GENETIC AND HORMONAL MECHANISMS CONTROLLING GRAIN DORMANCY AND PREHARVEST SPROUTING TOLERANCE IN WHITE WHEAT

By

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A dissertation submitted in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
Program in Molecular Plant Sciences

MAY 2018

To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of SHANTEL AMEALIA MARTINEZ find it satisfactory and recommend that it be accepted.

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ACKNOWLEDGMENTS

Research requires an army, not an individual and this work was no different. Dr. Camille Steber, my mentor, has very patiently molded me into a successful wheat geneticist and professional. Her lessons will ripple beyond me onto all subsequent mentees I encounter. Dr. Kimberly Garland Campbell, has provided me the foundation to become a successful wheat breeder and has also become, and will remain, a strong role model in my career. The lessons I've learned from both Kim and Camille have allowed me to become a basic scientist that will never forget her statistics and a wheat breeder who will always remember the little details that cannot be forgotten, no matter how large the experiment. My remaining two committee members, Dr. Arron Carter and Dr. Scot Hulbert, have been a driving force and positive influence toward obtaining my Ph.D. while pushing my knowledge and abilities beyond what I expected of myself.

Without the continuing support of my fellow lab mates, working day-to-day towards my Ph.D. would not have been as fulfilling and enlightening. Tracy Harris, without a doubt, has been the glue behind the scenes of the lab, organizing hourly help, ordering all the seed storage I require, and patiently allowing me to plant just one more greenhouse experiment, over and over. Thanks to Rehana Parveen for the unquestionable assistance throughout all of these projects and giving me the feeling of home and community while pursuing my Ph.D. Patricia DeMacon has given me the ability to independently manage all of my field projects from start to finish, provided this research with ample amounts of field labor, and has created both a work and home environment I too wish to be able to provide someone in the future. Many thanks are given to Dr. Sven Nelson for the valuable feedback on figure construction, R programming, and endless exciting research conversations, related and un-related to our projects. A special thank you goes to Stephanie Sjoberg for attentively learning everything about the preharvest sprouting project and continuing the PHS story after I leave. I would also like to sincerely thank the current and past Steber lab members for their support and collaboration: Dr. Elizabeth Schramm, Dr. Amber Hauvermale, Samantha Conselman, Dustan Cwick, Wenjing Ge, and Chang Liu. I would also like to thank the current and past Garland Campbell lab members for their support and collaboration: Dr.

Alison Thompson, Dr. Nuan, Wen, Scott Carle, Yvonne Manning-Thompson, and Nikayla Struass. Much gratitude goes to Dr. Lesley Murphy for beginning my career in wheat science and fostering my passion towards research. Special thanks go to both the Garland Campbell and Steber labs for helpful feedback on the research and manuscripts. With the Garland Campbell and Steber labs combined, I've become a better basic and applied scientist. Finally, I owe a debt of gratitude towards the members of the Garland Campbell, Carter, Dr. Michael Pumphrey, and Steber lab members for assistance with planting, sampling, and harvesting.

Special thanks go to the Oluwaseyi Shorinola and Cristobal Uauy for their fruitful and successful collaboration. I sincerely thank Dan Dreesman for constructing the greenhouse misting system and patiently assisting me with all greenhouse needs and management. Thanks are due to Emily Klarquist and Victor DeMacon for generous greenhouse assistance during population development and assistance in physiological maturity harvesting. I hope that at one point, I've provided the same amount of support and help to all of my colleagues that they've provided me.

I would like to thank my father Ronald, my sisters Sophia, Elouise, and Pamela, and my nephew Brissious who have kept my happiness and sanity intact throughout this journey. And finally, I also thank my partner, friends, and family who undoubtedly provide their love and support in all that I do.

GENETIC AND HORMONAL MECHANISMS CONTROLLING GRAIN DORMANCY AND PREHARVEST SPROUTING TOLERANCE IN WHITE WHEAT

Abstract

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May 2018

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Preharvest sprouting (PHS), germination of mature grain while still on the mother plant, occurs when conditions become cool and wet before harvest. The hydrolytic enzyme α-amylase, induced during germination, mobilizes starch into simple carbohydrates to fuel seedling growth. Because this enzyme activity in flour causes poor end-use quality, sprouted grain sells at a severe discount. The falling number (FN) test measures α-amylase activity in wheat meal or flour in the wheat industry. Seed dormancy, the inability to germinate even under favorable conditions, contributes about 60-80 % of genetic PHS tolerance. Red wheat varieties have higher seed dormancy and PHS tolerance than white. To improve white wheat, a genome-wide association study (GWAS) examined the genetic architecture of PHS tolerance in 469 soft white winter wheat accessions. Based on FN after natural or artificial rain, the GWAS identified 9 QTL (*QFN.wsu*), of which 4 co-localized with known PHS QTL and 3 with known FN/quality QTL. Based on visible sprout in spike-wetting tests, the GWAS identified 34 QTL (*QPHS.wsu*), of which 19 co-localized with known PHS loci and genes such as *MOTHER OF FLOWERING TIME* (*TaMFT*) and *mitogen-activated protein kinase kinase 3* (*TaMKK3-A*). PHS tolerance in white wheat can result from higher sensitivity to the seed dormancy-inducing hormone abscisic acid (ABA). *Enhanced Response to ABA* (*ERA8*) is a semi-dominant ABA hypersensitive mutant, resulting in increased seed dormancy and PHS tolerance in the soft white spring wheat 'Zak'. The *ERA8* locus was mapped to a large region of chromosome 4A relative to mutagen-induced SNPs in a

Zak/Zak*ERA8* backcross population using bulk segregant analysis (BSA) of exome sequence from $BC_3F_{2:3}$ wild-type and mutant DNA. Fine mapping using mutagen-induced SNPs in additional backcross lines localized *ERA8* to a 4.5 Mb region containing 70 predicted genes. The only mutagen-induced coding region mutation strongly linked to *ERA8* (LOD 16.51) resulted in a missense mutation in *MKK3-A*, a gene involved in Arabidopsis ABA signaling. Natural variation in wheat and barley *MKK3* was previously shown to control seed dormancy and PHS tolerance.

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DEDICATION

This dissertation is dedicated to my grandparents. Without your foundation and formation of who I am, I would not have achieved this unimaginable milestone.

CHAPTER ONE: Introduction

Preharvest Sprouting in Wheat

Preharvest sprouting (PHS) is the germination of mature seeds on the mother plant before harvest under rainy or humid conditions (reviewed by Gerjets *et al*., 2010; Rodriguez *et al*., 2015). PHS is associated with low seed dormancy in some cereal varieties such as wheat (*Triticum aestivum L.*), barley (*Hordeum vulgare* subsp. *vulgare*), and sorghum (*Sorghum bicolor*), likely due to selection for rapid and synchronous seedling emergence (Paterson and Sorrells, 1990; Gualano *et al*., 2007; Ullrich *et al*., 2009). Seed dormancy refers to the inability to germinate even under favorable environmental conditions (light, moisture, and temperature) (McCaig and DePauw, 1991; reviewed by Bewley and Black, 1994). Seeds are typically most dormant at maturity, and then lose dormancy through a period of dry storage called after-ripening, through imbibition in the cold (called cold stratification), or through scarification of the seed coat (Figure 1; Paterson *et al*., 1989; reviewed by Finkelstein *et al*., 2008). Seed coat-imposed seed dormancy is relieved by cutting or scarifying the seed coat, whereas embryo dormancy is not relieved by cutting the seed coat. A seed is considered germinated when the radical or any other part of the embryo emerges from the seed coat. When wheat seed germination is initiated, alpha-amylase is induced in order to mobilize stored reserves for use by the growing embryo (Clarke *et al*., 1984).

For wheat, even mild cases of PHS damage the quality of the grain because the induction of alpha-amylase enzyme leads to starch degradation, resulting in flour that produces poor quality bread and cakes (Figure 1). Sprouting damage is detected using the Hagberg-Perten Falling Number (FN) test for starch degradation. Briefly, a slurry of flour and water is heated to 100 °C with stirring to form a gravy-like mixture, and then FN machine measures the time in seconds that it takes for a stirrer to fall through the slurry. The higher the alpha-amylase activity, the thinner the slurry, the faster the stirrer falls, and the lower the FN. If the FN is below 300 -350 sec, then the farmer receives significantly less money for his grain. Chapter 2 determined loci contributing to PHS tolerance and high FN in PNW wheat. While efforts to improve synchronous germination benefit

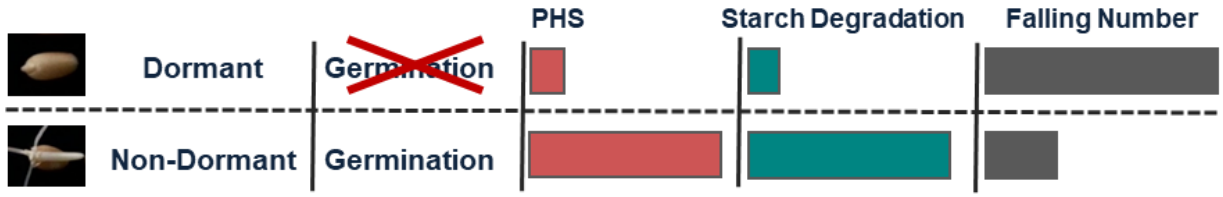

Figure 1 - Overall preharvest sprouting (PHS) effect on wheat. When a rain event occurs after maturity of a dormant wheat seed, germination does not occur. This, in turn, results in little to no PHS or starch degradation due to α-amylase. The Falling Numbers remain high in the absence of PHS and α-amylase activity. In contrast, a non-dormant wheat seed can germinate when rain events occur. This results in increased PHS, increased starch degradation due to α-amylase, and lower Falling Numbers due to the increased α-amylase activity.

agronomic production when grain is used as seed, a balance between rapid germination and dormancy is required to maintain grain quality in the grain trade.

The spike-wetting test is often used by breeders to examine PHS tolerance since it examines and controls for multiple variables affecting PHS in the field, such as maturity differences (Paterson *et al*., 1989). Intact spikes are harvested from the field at physiological maturity and allowed to dry after-ripen for the same number of days (Paterson and Sorrells, 1989). Spikes are then stored in a freezer in order to maintain the grain at an equivalent degree of after-ripening, thus allowing for control of differences in maturity date. In addition to seed dormancy, the artificial misting of intact spikes accounts for the effect of awns, erectness of the spike, gloom tightness, and head type (Hong, 1979; Pool and Patterson, 1958; Ibrahim, 1966; King and Richards, 1984). Spikes are assigned a sprouting score using a scale based on visible germination and postgerminative growth throughout the spike (Figure 2; Table 1; McMaster and Derera, 1976). Other methods for assessing PHS tolerance include plating assays to assess degree of seed dormancy, the Falling Numbers test, and variations on the spike-wetting test (Zhang *et al*. 2014; Jimenez *et al*., 2016; Zhou *et al*, 2017). The spike-wetting test method can be conducted using misting of intact spike, immersion of spikes, or the use of wet sand (McMaster and Derera, 1976; Paterson *et al*., 1989; Humphreys and Noll, 2002; Anderson *et al*., 1993; Rehman Arif *et al*., 2012). Within each method, the number of days of after-ripening, the duration of spikes wetting, and the day scored vary between studies. Numerous studies have used the misting system of Anderson *et al*. (1993) and the scoring system of McMaster and Derera (1976).

Seed Dormancy

Multiple genetic and environmental factors control the degree of seed dormancy and its loss. Loci controlling seed dormancy and PHS tolerance have been identified on practically every wheat chromosome (Jaiswal *et al*., 2012; Kulwal *et al*., 2012; reviewed in Martinez *et al*., 2018a). In wheat, seed dormancy increased when the environment is cool and dry and decreases when conditions are hot during embryo maturation (Nakamura *et al*., 2011). Red kernel color is generally associated with higher seed dormancy

Figure 2 – Visualization of preharvest sprouting (PHS) wheat spikes. **A**) Wheat widely varies in tolerance and susceptibility to PHS. **B**) McMaster and Derera's (1976) 1-10 scale measures visible root, coleoptile, and leaf emergence. PHS scale photo (**B**) by Kent Loeffler, Plant Pathology, Cornell University.

Table 1. Description of the preharvest sprouting scoring system used in screening sprouted wheat spikes (McMaster and Derera, 1976).

Score	Seminal roots		Coleoptiles	
	Number	Length	Number	Length
1	---------- No visible sprouting ----------			
2	1-2 perv spike	Just emerging	-	-
3	3-4 per spike	1-2 mm	-	-
4	1 or 2 on 65-75 % of the spikelets	3-4 mm	-	-
5	Uniform over the spike	4-6 mm	-	-
6	Uniform over the spike	6mm – 1cm	1 or 2 per spike	Just emerging
7	Uniform over the spike	1-2 cm	>2 per spikes	Just emerging
8	Uniform over the spike	2-4 cm	Uniformly emerging	0-0.5 cm
9	Uniform over the spike	>4 cm	Uniform over the spike	1-2 cm
10	Uniform over the spike	>4 cm	Uniform over the spike	3-4 cm

and PHS tolerance, whereas white kernel color is associated with lower seed dormancy and lower PHS tolerance (Paterson and Sorrells, 1990; Flintham, 2000; Warner *et al*., 2000; Himi *et al*., 2002; Torada and Amano, 2002). Red seed coat color is controlled by the three *Red-1* loci encoding the transcription factors *TaMyb10-A1*, *TaMyb10-B1*, and *TaMyb10-D1* (Himi *et al*., 2011). However, it is clear that other factors control seed dormancy and dormancy loss because red kernelled wheat can be PHS susceptible and white kernelled wheat can be PHS tolerant.

Abscisic Acid

Plant hormone signaling also controls seed dormancy and dormancy loss. The hormone abscisic acid (ABA) induces seed dormancy during embryo maturation, and the hormone gibberellin A (GA) stimulates seed germination (reviewed by Finkelstein *et al*., 2008). ABA establishes seed dormancy during embryo maturation, maintains dormancy in mature seeds, and inhibits the germination of mature seeds when exogenously applied. In addition to inducing seed dormancy and desiccation tolerance during embryo dormancy, ABA induces resistance to multiple environmental stresses including drought tolerance, cold tolerance, and salt tolerance (reviewed by Finkelstein, 2013). The ABA hormone is produced in response to dehydration stress. Freezing and salt stress can effectively reduce the availability of water inside the leaf leading to ABA production. ABA induction of osmotic protectants such as proline and dehydrin proteins can increase plant resistance to dehydration, freezing, and salt stress. ABA also controls plant development, causing decreased growth of lateral roots and increased growth of the primary root which facilitate deeper root growth towards deeper moisture. ABA signaling can be controlled both at the level of hormone accumulation and at the level of the ABA sensitivity.

The ABA signaling pathway was recently defined based on mutant studies in Arabidopsis (Figure 3; Park *et al*., 2009; Santiago *et al*., 2009; reviewed by Cutler *et al*., 2010). The ABA signal is perceived by a family of receptors referred to as PYR/PYL/RCAR1 (*Pyrabactin Resistance*/ *Pyrabactin Resistance 1-Like*/ *Regulatory Component of ABA Receptor1*). In the absence of ABA, a family of PP2Cs (Protein Phosphatases type 2Cs) negatively regulate ABA responses by dephosphorylating a protein kinase called

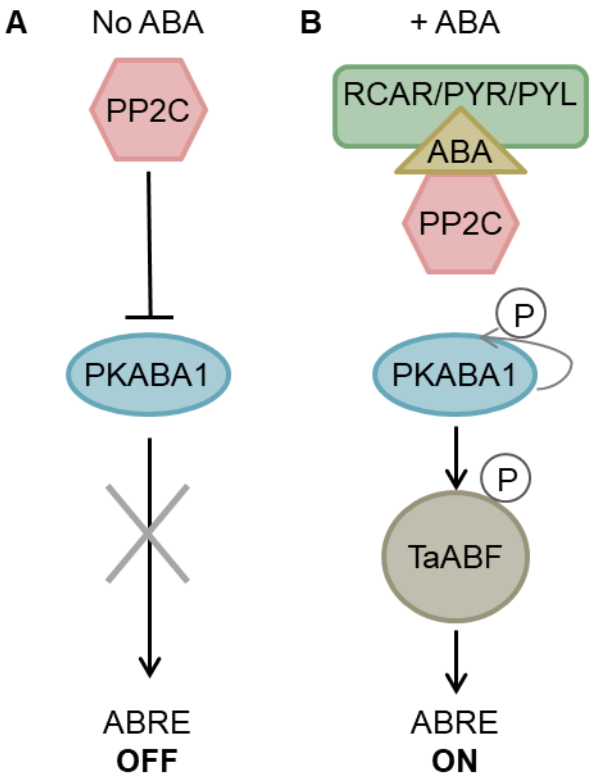

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Figure 3 - Model showing positive and negative regulators of ABA signaling from Martinez *et al*. (2016). **A**) Without ABA, the protein phosphatase type 2C (PP2C) represses ABA signaling and ABA-responsive gene expression by inactivating the PKABA1 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.

PKABA1 (Protein Kinase of ABA1) in wheat or the SnRKs (SNF1-related protein kinases) in Arabidopsis (Anderberg and Walker-Simmons, 1992; Umezawa *et al*., 2009; Vlad *et al*., 2009). Dephosphorylation of PKABA1 blocks its ability to induce ABA responses. In the presence of ABA, PYR1 binding of ABA results in a conformational change allowing PYR1 to bind the PP2C, thereby inhibiting its ability to dephosphorylate PKABA1 (Nakashima *et al*., 2009). This then, allows PKABA1 to activate itself through autophosphorylation, which in turn allows PKABA1 to phosphorylate a family of basic leucine zipper (bZIP) transcription factors called TaABF (Johnson *et al*., 2002, 2008). Activation of the transcription factor through phosphorylation promotes downstream changes in ABA-regulated gene expression. Gain-of-function mutations in the PP2C negative regulators resulted in ABA-insensitivity associated with lack of seed dormancy and a wilty phenotype (Finkelstein, 2013). Conversely, loss of the PP2C ABI1 was associated with a mild ABA hypersensitive phenotype likely due to the fact ABI1 is part of a multigene family (Leung *et al*., 1997). Loss of function mutations in positive regulators of ABA response, including the bZIP transcription factors and the farnesyl transferase *Era1* (Enhanced Response to ABA1), also resulted in an ABA hypersensitive phenotype (Cutler *et al*., 1996). The ABA hypersensitive *era1* mutants of Arabidopsis and of canola showed increased survival of imposed drought, suggesting that it may be possible to improve drought tolerance using increased ABA hormone sensitivity (Pei *et al*., 1998; Wang *et al*., 2005).

ABA Signaling Mutant

Consistent with previous work in Arabidopsis, ABA insensitive mutants of wheat show decreased seed dormancy, whereas ABA hypersensitive mutants show increased seed dormancy (Kawakami *et al*., 1997; Rikiishi and Maekawa, 2010; Schramm *et al*., 2010, 2012, 2013). The *EH47* and *RSD32* (Reduced Seed Dormancy32) mutants were isolated based on an assay for reduced seed dormancy at physiological maturity, and were subsequently shown to have decreased embryo ABA sensitivity. The *ScABI* (Scarlet ABA Insensitive) mutants were isolated based on decreased ABA sensitivity during seed germination (Schramm *et al*., 2012). Although these mutants had some seed dormancy at maturity, they were found to

lose dormancy more rapidly than the wild-type (WT) wheat cultivar 'Scarlet' through dry after-ripening. ABA hypersensitive mutants, *ScERA* (Scarlet Enhanced Response to ABA) and *ABA27*, of wheat were associated with increased seed dormancy and slower after-ripening (Kobayashi *et al*., 2008; Schramm *et al*., 2013).

Enhanced Response to ABA Mutant, *ERA8*

ERA8 (Enhanced Response to ABA8) is a semi-dominant ethyl methanesulfonate (EMS)-induced mutation in the soft white wheat cultivar called 'Zak' (Kidwell *et al*., 2002; Schramm *et al*., 2013). The *ERA* mutants, Zak*ERA8*, *ERA19A*, and *ERA19B* were identified by screening for increased sensitivity to 5µM ABA compared to Zak WT. Of the three mutants, the semi-dominant *ERA8* line showed strongest increase in ABA sensitivity and seed dormancy. *ERA8* was backcrossed three times to WT Zak to clean up unrelated EMS mutations (Schramm *et al*., 2013; Martinez *et al*., 2014, 2016). *ERA8* shows more PHS tolerance than WT and other spring wheat cultivars from the PNW (Figure 4). Since PHS often occurs under cool conditions, a cold-induced germination assay was conducted on *ERA8*. The PHS tolerant mutant will maintain dormancy in temperatures as low as 15 °C compared to WT, but not as well at 10 °C (Martinez *et al*., 2016). Wheat dormancy at low temperatures (5-15 °C) has also been reported in other wheat cultivars (Cao *et al*., 2016; Kashiwakura *et al*., 2016).

Increasing seed dormancy in order to prevent preharvest sprouting could potentially cause poor seedling emergence after planting. This would be a problem if the *ERA8* gene was introgressed into a winter wheat cultivar where the next crop was planted in the fall, approximately 8 weeks after harvest. Previous work showed that 70 % of hand threshed *ERA8* seeds will germinate after 8 weeks of after-ripening compared to 96.7 % germination of WT Zak (Martinez et. al., 2014). In practice, field material is harvested using a combine, resulting in scarification of the seed coat by the machinery which could efficiently break seed dormancy. In order to assay the combine harvested grain, percent germination was measured at 9 weeks of after-ripening. The germination of the *ERA8* mutant was 91.5 % compared to the

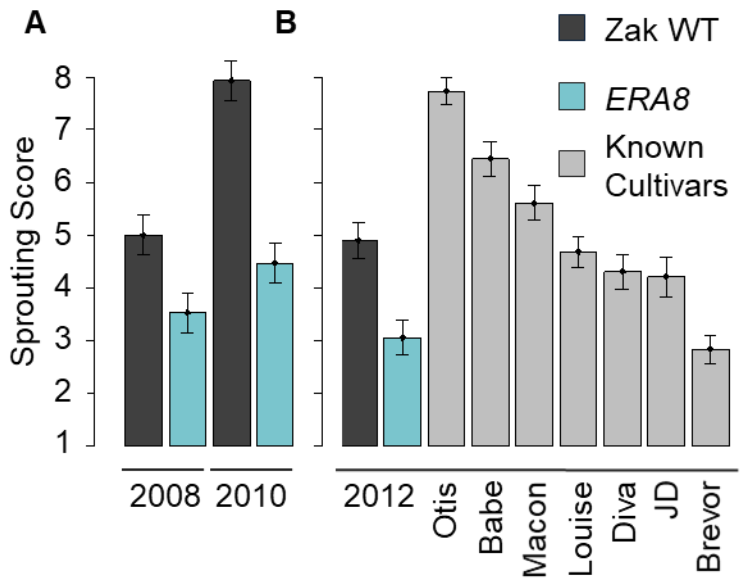

Figure 4 - The effect of *ERA8* on PHS tolerance based on sprouting score from Martinez *et al*. (2016). Spike wetting tests were performed on intact spikes of Zak (black), *ERA8* (blue), and spring wheat cultivars (grey) grown in the Pacific Northwest. Increasing sprouting scores on a 1-10 scale indicate increasing PHS susceptibility in the indicated year. The LS-mean sprouting score and SE bars are shown. **A**) Zak and *ERA8* were grown in 2008 and 2010 in New York State, whereas (**B**) Zak, *ERA8*, and indicated cultivars were grown in Pullman WA in 2012.

germination of Zak WT at 98.3 % germination. This suggested that combine harvested *ERA8* has sufficient germination potential to plant in the fall approximately two months after harvest. Germination potential is only one component contributing to seedling emergence, especially when sown deep into stored moisture. Deeply planted seed must rely on stored reserves to fuel seedling growth because they cannot do photosynthesis until the seedling clears the soil. Since α-amylase is GA-induced and ABA-repressed, it is possible that an ABA hypersensitive mutant would have problems inducing α-amylase. Thus, measurements of coleoptile length of dark-germinated seedlings are used to screen for problems with poor seedling emergence. *ERA8* coleoptiles were not significantly shorter than those of Zak WT or of other winter wheat breeding lines harvested at the same time (Figure 5). Further studies will examine whether *ERA8* introgression negatively impacts spring or winter wheat emergence in field trials.

Examining hormone accumulation and response of the seed gave insight into *ERA8* and its role in plant hormone signaling (Martinez *et al*., 2016). The *ERA8* mutant was more dormant and ABA sensitive even in later periods of after-ripening compared to WT in an after-ripening time course. Moreover, during initial after-ripening stages, *ERA8* was more GA insensitive than WT. Both *ERA8* and WT decreased endogenous ABA content during after-ripening in the embryo. Conversely, ABA content didn't change in the aleurone tissue with after-ripening. When WT and *ERA8* had the same germination profile (dormant or after-ripened), *ERA8* has equal or lower ABA content in the embryo compared to WT. Endogenous GA levels were too low to detect significance due to a small sample size but there still appeared to be an increasing trend in GA with after-ripening. Future work will need to increase the sample size for the GA hormone measurements and study the effects of endogenous GA levels in *ERA8* during after-ripening (Nelson, 2015). The plant hormone auxin, Indole Acetic Acid (IAA), had significantly higher levels in *ERA8* than in WT at the beginning of the after-ripening time course, but IAA levels dropped down to WT equivalent levels over time. Liu *et al*. (2013) also showed that IAA hormone levels in whole seeds decrease with after-ripening. Exogenously applied IAA precursors, such as L- and D- tryptophan, and IAA, also reduced germination (Morris *et al*., 1988; Ramaih *et al*., 2003). The ABA hypersensitive phenotype of *ERA8* appears to be largely seed-specific and does not show any vegetative

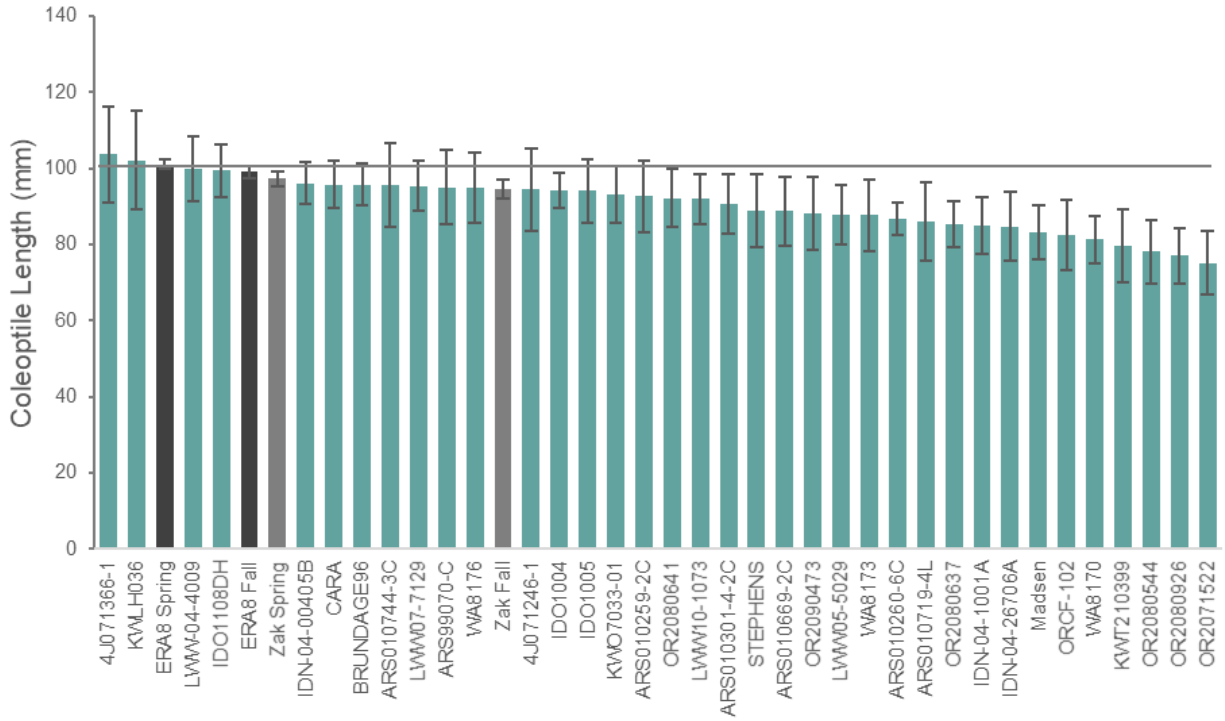

Figure 5 - *ERA8*, Zak, and western regional soft winter wheat nursery coleoptile emergence from Pullman 2013. Coleoptile lengths of 10 technical reps over 2 biological reps were measured after 4 days at 4 °C and 16 days at 15 °C. Seeds were imbibed in the dark on germination paper with sterile water. The average *ERA8* coleoptile length is indicated by the grey horizontal line.

ABA sensitivity. The *ERA8* allele could be explained as either a mutation in a positive regulator of the ABA signaling pathway (sensitivity) or a mutation in a gene controlling ABA metabolism resulting in the inability to degrade the hormone (accumulation). In chapter 3 we identify the *ERA8* region of the wheat genome for easier deployment of the PHS tolerance gene after introgression into breeding programs.

ABA and Drought

ABA triggers the rapid closure of stomates under dry conditions, thereby preventing water loss through transpiration. Closing stomates can have the undesirable effect of reduced photosynthesis by limiting carbon dioxide (CO_2) uptake through stomates. Many physiological traits have been considered as mechanisms to improve wheat yield under water limitation including water use efficiency (WUE), photosynthetic efficiency, and root depth. Higher WUE is correlated with higher grain yield in some environments, but not in others (Monneveux *et al*., 2006). Shrestha (2014) found little or no correlation between higher WUE as measured with carbon isotope discrimination in the flag leaf tissue, and grain yield in eastern Washington. The spring wheat cultivar 'Louise' has both high yield under dry conditions and high WUE. Thus, it is possible that WUE is useful in this semi-arid environment in some genetic backgrounds. Interestingly, the cultivar Louise appeared to have higher sensitivity to applied ABA when compared to the spring wheat cultivars 'Alpowa' and 'Avocet' (Murphy, Steber, and Campbell, unpublished). This was based on observing the effect of ABA application on photosynthetic assimilation and transpiration rates. Moreover, greenhouse gas exchange measurements using the LI-COR 6400XT (LI-COR, Lincoln, NE) showed that Alpowa had higher transpiration rates than Louise under drought conditions, over 5 days without additional water in a greenhouse environment (Murphy and Campbell unpublished; Figure 6). This observation was consistent with the observation that Louise has high WUE, whereas Alpowa has low (Shrestha, 2014). Thus, the creation of a Louise/Alpowa RIL population provided an opportunity to examine the variation at loci affecting WUE (and possibly ABA sensitivity). Louise and Alpowa appear to have different tolerance mechanisms. Whereas Louise has high WUE

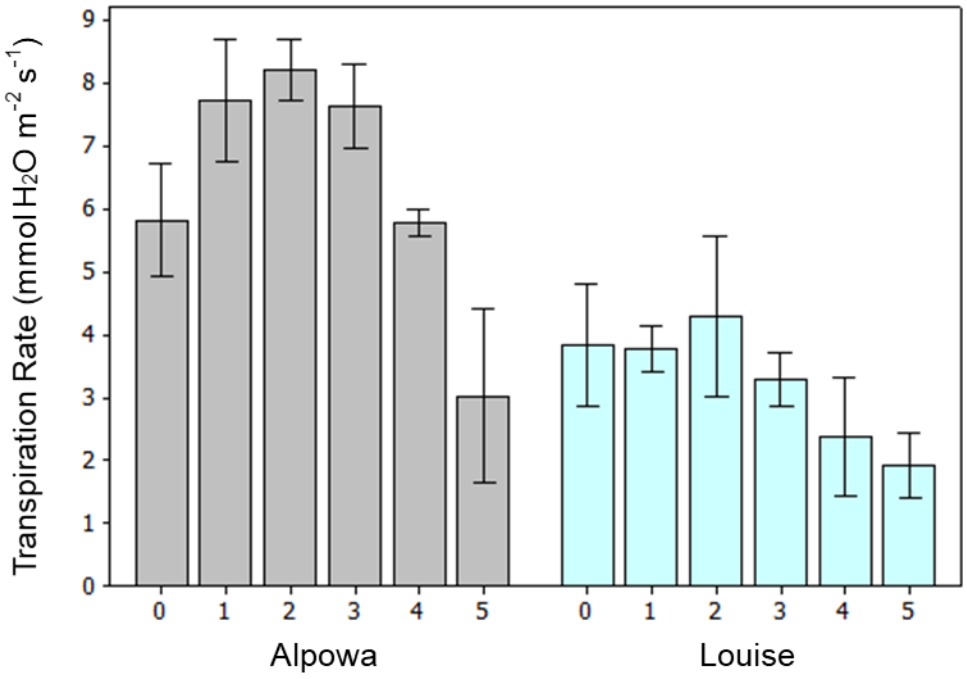

Figure 6 - Drought response of Alpowa and Louise after 5 days without water. Transpiration rate was measured using the LI-COR 6400XT. Analysis of variance was performed across genotypes and the bars represent LSMeans.

associated with high yield under drought, Alpowa has low WUE and high yield under drought. Drought tolerance in Alpowa seems to be due to a growth pattern that allows it to escape the effects of drought in the field. Alpowa has a slower growth rate in early growth that may leave stored moisture in the soil, but then shows rapid growth during flowering that may allow it to complete grain filling before soil moisture becomes critically low (Martinez *et al*., 2018b). In chapter 4 we examine the Louise/Alpowa drought tolerant mapping population genetics and variation for important agronomic traits.

Wheat Genome

Bread wheat (*Triticum aestivum* L.) has a large allohexaploid genome (17 billion bp) (Dubcovsky and Dvorak, 2007; Brenchley *et al*., 2012). The three diploid progenitors each contributed 7 homoeologous chromosomes referred to as the A, B, and D genomes (*2n* = 6x = 42, genomes AABBDD). The 21 chromosomes are numbered 1 through 7, followed by the genome identifying letter (chromosome 1A, 1B, 1D, etc.). The A genome was derived from *Triticum monococcum subsp. Uratu* while and the B genome from *Aegilops speltoides* (Gill and Jiang, 1994; El Baidouri *et al*., 2017). Current research has shown that the D genome may be due to multiple hybridization events and was closest to the subspecies L2, *strangulata*, of *Aegilops tauschii* (Jia *et al*. 2013; Wang *et al*. 2013). Sequencing of the wheat genome is currently in progress with 100 % of the survey sequence, physical map, and reference sequence completed (IWGSC, 2017; www.wheatgenome.org). A fairly complete sequence of the expressed portion of the wheat genome has been derived from cDNA sequencing (Brenchley *et al*., 2012; IWGSC, 2017; www.wheatgenome.org).

Mapping techniques in wheat are becoming higher throughput as technologies improve. Simple sequence repeat (SSR) markers for wheat were developed around 1993 and almost all have known chromosome location (Grist *et al*., 1993; www.graingenes.org). The current use of SSR markers with new marker technology would be to either 1) improve linkage groups that have limited known chromosome locations or 2) assay previously published SSR markers that flank loci of interest. Single nucleotide markers (SNP) were identified and verified for genome specificity and a 9k and 90k single

nucleotide polymorphic (SNP) assay was developed on the Illumina Infinium iSelect beadchip platform (Lai *et al*., 2012; Allen *et al*., 2013). For both the 9k and 90k SNP assay, 3,958 and 40,267 marker locations have been published, respectively (Cavanagh *et al*., 2013; Wang *et al*., 2014). High throughput marker technologies such as genotyping-by-sequencing are another resource for breeders to identify polymorphic SNPs (Poland *et al*., 2012). In GBS, genomic DNA is digested with two restriction enzymes and then ligated to DNA linkers. Sequencing is initiated with primers that anneal to the linker sequence. This method requires intensive bioinformatic analysis of the resulting whole genome sequence data, and markers are not always associated with previously defined important loci. Sequencing multiple lines such as a whole populations and parents will result in an optimal number of SNPs to map with. But it is not cost-efficient to whole-genome sequence an entire mapping population. Alternative approaches, such as bulk-segregant analysis (BSA), include sequencing only pooled tail-ends of a phenotype of interest.

Bulk-segregant analysis (BSA) consists of pooled-DNA samples that contrast in phenotype to identify polymorphic markers co-segregating in a specific genomic region for the trait of interest (Michelmore *et al*. 1991). BSA has been conducted in wheat in addition to numerous species such as *Arabidopsis thaliana*, *Zea maize* (corn), and *Oryza sativa* (rice) (Liu *et al*., 2001; Venuprasad *et al*., 2009; Becker *et al*., 2011; Liu *et al*., 2012). Wheat biotic stress resistance to Fusarium Head Blight and aphid resistance has been mapped using BSA (Liu *et al*., 2001; Shen *et al*., 2003). A combination of next generation resequencing and BSA has also been proven to fine map a known grain protein content, *GPC-B1*, gene (Trick *et al*., 2012). A major stripe rust (*Puccinia striiformis* f. sp. *tritici*) gene, *Yr15*, was also mapped to a 0.77cM interval using BSA RNA-Sequencing (Ramirez-Gonzalez *et al*., 2015). Other stripe rust and leaf rust (*Puccinia triticina*) genes have been identified in other BSA studies (Ren *et al*., 2012a, b; Wu *et al*., 2018). Mo *et al*. (2017) used the combination of BSA and exome re-sequencing in order to look for height genes in the Kronos TILLING population. One can reduce the sequencing cost by using exome capture on only bulk-phenotyped lines instead of a whole mapping population. Another advantage would be sequencing only coding regions of the genome instead of sequencing the entire 16 Gb genome.

In chapter 3, we used BSA-exome sequencing to identify *ERA8*-linked SNPs in addition to traditional linkage analysis.

Linkage analysis or association mapping (AM) can be used to locate regions of a chromosome influencing a specific trait through identification of areas of the genome that are in linkage disequilibrium, that are in association with the variation in the trait. Association mapping localizes quantitative trait loci (QTL) with higher resolution than linkage analysis (10-20 cM versus > 10 cM intervals) because it exploits the historical and evolutionary recombination events (reviewed in Zhu *et al*., 2008). Linkage analysis in bi-parental population generates pertinent information about traits that can be applied to other genetically related populations whereas significant markers resulting from AM may be applied to a much wider germplasm base, depending on the germplasm used in the AM panel. The results of AM are usually termed genome-wide association studies (GWAS) and because of the higher resolution, GWAS studies using whole genome molecular markers can identify loci associated with complex traits (Risch and Merikangas, 1996). The methods in linkage analysis and association mapping have been previously compared in wheat through examination of numerous phenotypes (Breseghello and Sorrells, 2006). Conducting both linkage analysis with bi-parental populations and GWAS is often useful in wheat for given traits because of the complexity of the wheat genome and the ability to reduce the likelihood of false positives for QTLs.

PHS and Dormancy QTL

QTL for PHS, dormancy, and ABA sensitivity have been identified throughout the wheat genome. Major QTLs for PHS tolerance have been found on chromosome 2B, 3A, 3D, 4A, 6D, and 7B (Zanetti *et al*., 2000; Ogbonnaya *et al*., 2008; Munkvold *et al*., 2009; Jaiswal *et al*., 2012; Kulwal *et al*., 2012; Zhou *et al*., 2017). Moreover, an embryo seed dormancy QTL called *MOTHER OF FT AND TFL1* (*TaMFT*) was found on chromosome 3A using gene expression and was also cloned (Nakamura *et al*., 2011). *TaMFT* was independently discovered and previously named *TaPHS1* before candidate gene confirmation (Liu *et al*., 2008, 2014). In diploid wheat, QTLs for ABA sensitivity and seed dormancy were found on

chromosomes 3A, 4A, and 5A (Nakamura *et al*., 2007). Another group found homoeologous QTL on chromosome 2A, 2B, and 2D of three ABA-insensitive Five-binding Protein (*TaAFP*) that interacts with the wheat bZIP transcription factor (*ABI5*) of the ABA signaling pathway (Ohnishi *et al*., 2008). A preharvest sprouting QTL, *QPhs.ccsu-2A.5*, found in the dormant accession 'SPR8198' (Mohan *et al*., 2009). Another dormancy QTL, *QPhs.cnl-2B.1*, in the cultivar 'Cayuga' was identified in the 2B chromosome which was also near a QTL found from a European winter wheat GWAS (Somyong *et al*., 2014; Albrecht *et al*., 2015). The club wheat gene, *C* locus, has been mapped near the centromere on chromosome 2D (Johnson *et al*., 2008a). In previous studies, the *C* locus was associated with having more water uptake and preharvest sprouting (King and Richards, 1986; Hong, 1979). Future research should elucidate if the *C* locus is the cause of PHS susceptibility in previous work, or just two tightly linked genes co-segregation with the *C* locus.

The *Phs-A1* locus, found on chromosome 4A, was identified by at least 16 other studies (Martinez *et al*., 2018a). Previous work proposed that the candidate genes tandem duplicate *Plasma Membrane 19* (*PM19-A1* and *PM19-A2*) and the *mitogen-activated protein kinase kinase 3* (*TaMKK3*) located within the *Phs-A1* locus contribute to PHS tolerance and increased seed dormancy (Barrero *et al*., 2015; Shorinola *et al*., 2016; Torada *et al*., 2016). Haplotype analysis of multiple mapping populations and accession suggested that *TaMKK3*, and not *PM19*, contributed to PHS tolerance (Shorinola *et al*., 2017). Homologous *TaMKK3* genes are found on 4AL, 5B, and 5D (Table 2). Chromosome 4AL is known to have historical translocations events during evolution that include the 4AS, 5AL, and 7BS chromosome (Devos *et al*., 1995). *TaMKK*3 is homologous to map kinase kinases from multiple species, such as Arabidopsis (*AtMKK3*), barley (*HvMKK3*), rice (*OsMKK3*), and tobacco (*Nicotiana tabacum* L.; *NtNPK2*) (Shibata *et al*., 1995; Hamel *et al*., 2006; Nakamura *et al*., 2016; Torada *et al*., 2016). Sequencing of the wheat *MKK3* across 58 accessions identified 6 different SNPs in *TaMKK3-A*, one in the fourth exon and five within introns (Shorinola *et al*., 2017). *TaMKK3-A-A660*^{s} is the non-dormant allele whereas *TaMKK3-A- C660*^{R} is the dormant PHS tolerant allele. Progenitor analysis of *TaMKK3*

Table 2 – *TaMKK3-A* BLAST results against the Chinese Spring RefSeqv1.0 reference genome.

Gene [a]	Chrm	Score	Identities	%	Expect	Start	End
TaMKK3-A	Unknown	663	367/367	100	0	244924670	244924304
	3A	639	494/587	84	6.00E-180	32155167	32154581
	3B	607	486/586	83	4.00E-170	40251504	40250919
TaMKK3-D	5D	581	351/370	95	1.00E-162	556549977	556549608
TaMKK3-B	5B	581	351/370	95	1.00E-162	710233713	710234082
	7D	223	203/253	80	2.00E-54	285681130	285680888
	7A	214	201/253	79	8.00E-52	317144054	317144296
	7B	208	184/226	81	3.00E-50	251133495	251133271
	5A	203	177/220	80	1.00E-48	267941276	26794149

[a] Homologous gene names were classified in Torada *et al*. (2016).

suggested that the non-dormant allele, *TaMKK3-A-A660*^{s}, was derived from the dormant C allele, *TaMKK3-A- C660*^{R}, but the mutation occurred before the hybridization of bread wheat. Moreover, an N260T substitution in the kinase domain of the homologous barley *MKK3* gene resulted in a loss-of-function allele that improved PHS tolerance (Nakamura *et al*., 2016). *TaMKK3-A* gene expression can be identified in seeds, leaves, cotyledon, and root tissues (Torada *et al*., 2016). *AtMKK3* has been known to be involved in ABA, JA, and ethylene signaling pathways (Takahashi *et al*., 2007; Liu, 2012; Enders *et al*., 2017; Li *et al*., 2018). Based on our results in chapter 3, the *ERA8* gene may co-localize near one of these loci or it may be a homologue of the positive regulators in the ABA signaling pathway.

The purpose of this dissertation was to elucidate genes contributing to PHS, Falling Numbers, and increased ABA sensitivity. This was achieved through association mapping, QTL linkage mapping, and BSA-exome-sequencing. A mapping population was also created for future QTL analysis on drought tolerance and other agronomic traits. All the QTL identified and populations created in this dissertation are useful tools and information that contribute to a broader understanding of wheat molecular genetics.

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Individual Contributions to this Research

In addition to work I've done, many other individuals have collaborated their research towards this thesis:

Chapter Two

Shantel A. Martinez (SAM), Jayfred Godoy (JG), Meng Huang (MH), Zhiwu Zhang (ZZ), Arron H. Carter (AC), Kimberly A. Garland Campbell (KGC), Camille M. Steber (CMS):

SAM, KGC, and CMS designed the experiments. SAM conducted the experiments, analyzed the phenotypic data, performed the statistical analysis and GWAS, and constructed the visualizations. JG curated and filtered the genotypic data and performed the LD analysis. MH calculated the phenotypic variation. MH and ZZ provided expert guidance on performing the GWAS. KGC and AC provided field resources. CMS, KGC, AC, and ZZ obtained funding. SAM and CMS wrote the manuscript.

Chapter Three

Shantel A. Martinez (SAM), Oluwayesi Shorinola (OS), Samantha Conselman (SC), Deven See (DS), Dan Skinner (DS), Cristobal Uauy (CU), and Camille M. Steber (CMS):

SAM, OS, CMS, and CU designed the experiments. SAM and CMS developed the population. SAM and SC conducted the LZ8 ABA sensitivity, linkage map, and QTL analysis. DS and DS conducted GBS and analyzed the raw sequence data. SAM conducted the ZZ8 ABA sensitivity, *ERA8* fine mapping, and introgression *ERA8* into breeding lines. OS performed the exome capture, BSA, and developed KASP markers. SAM and OS conducted the RNA sequencing and *TaMKK3* alignment. CMS and CU provided resources. SAM and CMS wrote the manuscript.

Chapter Four

Shantel A. Martinez (SAM), Alison L. Thompson (AT), Nuan Wen (NW), Lesley Murphy (LM), Karen A. Sanguinet (KAS), Camille M. Steber (CMS), and Kimberly A. Garland Campbell (KGC):

SAM, KGC, and CMS designed the experiments. LM and AT extracted the DNA. AT and NW ran the SSR and KASP marker. SAM called the 9k SNP, created the linkage map, and constructed the visualizations. KS, KGC, and CMS provided resources. SAM, KGC, and CMS wrote the manuscript.

CHAPTER TWO: Genome-wide Association Mapping for Tolerance to Preharvest Sprouting and Low Falling Numbers in Wheat.

(Published as a research paper in Frontiers in Plant Science)

Abstract

Preharvest sprouting (PHS), the germination of grain on the mother plant under cool and wet conditions, is a recurring problem for wheat farmers worldwide. α-amylase enzyme produced during PHS degrades starch resulting in baked good with poor end-use quality. The Hagberg-Perten Falling Number (FN) test is used to measure this problem in the wheat industry, and determines how much a farmer's wheat is discounted for PHS damage. PHS tolerance is associated with higher grain dormancy. Thus, breeding programs use germination-based assays such as the spike-wetting test to measure PHS susceptibility. Association mapping identified loci associated with PHS tolerance in U.S. Pacific Northwest germplasm based both on FN and on spike-wetting test data. The study was performed using a panel of 469 white winter wheat cultivars and elite breeding lines grown in six Washington state environments, and genotyped for 15,229 polymorphic markers using the 90k SNP Illumina iSelect array. Marker-trait associations were identified using the FarmCPU R package. Principal component analysis was directly and a kinship matrix was indirectly used to account for population structure. Nine loci were associated with FN and 34 loci associated with PHS based on sprouting scores. None of the *QFN.wsu* loci were detected in multiple environments, whereas six of the 34 *QPHS.wsu* loci were detected in two of the five environments. There was no overlap between the QTN detected based on FN and PHS, and there was little correlation between the two traits. However, both traits appear to be PHS-related since 19 of the 34 *QPHS.wsu* loci and four of the nine *QFN.wsu* loci co-localized with previously published dormancy and PHS QTL. Identification of these loci will lead to a better understanding of the genetic architecture of PHS and will help with the future development of genomic selection models.

Keywords: *preharvest sprouting, Falling Number, seed dormancy, wheat, association mappin*g

Introduction

Rainy conditions before harvest can cause mature grain to initiate germination while still on the mother plant (Rodriguez *et al*., 2015). This problem, called preharvest sprouting (PHS), occurs in many cereal crops such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and sorghum (*Sorghum bicolor*) (Paterson and Sorrells, 1990; Gualano *et al*., 2007; Ullrich *et al*., 2009). Germination is associated with α-amylase enzyme induction in order to mobilize starch reserves for use by the growing embryo (Clarke *et al*., 1984). This α-amylase induction during PHS in wheat grain leads to problems with poor end-use quality due to starch degradation. Thus, sprouted wheat grain is discounted in the marketplace.

The degree of PHS tolerance is associated with multiple environmental and genetic factors. Grain dormancy can account for up to 60 % of variation in PHS tolerance, although spike morphology and epicuticular waxes are also associated with PHS response (King, 1984; King and Richards, 1984; King and Wettstein-Knowles, 2000; DePauw and McCaig, 1991). Dormant seeds cannot germinate under favorable environmental conditions (light, moisture, and temperature) (Bewley and Black, 1994). Seeds are most dormant at physiological maturity, and then lose dormancy through a period of dry storage called after-ripening (Finkelstein *et al*., 2008). Dormancy can also be broken by moist chilling (called cold stratification) or seed coat scarification (Paterson *et al*., 1989; Finkelstein *et al*., 2008). The degree of wheat grain dormancy and PHS tolerance depends on environmental conditions both before and after the grain reaches physiological maturity. Grain dormancy is higher when the mother plant is exposed to cooler conditions during the maturation phase of grain development (Nakamura *et al*., 2011). Conversely, cold stratification of mature grain breaks dormancy. If rain and cold temperatures occur after physiological maturity, seed dormancy can be broken through cold stratification and grain is more likely to sprout. Thus, cold temperatures have opposite effects depending on whether they occur prior to or after the maturation date. Because seed dormancy is also broken through dry after-ripening, wheat also becomes more likely to sprout the longer unharvested mature grain stands dry in the field before it rains

(Gerjets *et al*., 2010). Thus, variation in PHS tolerance also depends on when the rain occurred relative to maturation date. Higher PHS tolerance is associated with genetic loci that increase grain dormancy including red kernel color and the synthesis or response to the dormancy-inducing hormone ABA (abscisic acid) (Walker-Simmons, 1987; Flintham, 2000; Warner *et al*., 2000; Schramm *et al*., 2010; Himi *et al*., 2011; Jaiswal *et al*., 2012; Kulwal *et al*., 2012; Martinez *et al*., 2016). Since lack of red kernel color reduces dormancy, other genetic mechanisms supporting PHS tolerance must be identified and selected in wheat with white kernels.

The spike-wetting test is often used to assess PHS tolerance in breeding programs because it examines multiple variables affecting PHS (Paterson *et al*., 1989). In order to control for variation due to maturity date, intact spikes are harvested from the field at physiological maturity and allowed to dry after-ripen for the same number of days (5-14 days) before placing them under a greenhouse misting system. The use of intact spikes takes into account the effect of awns, erectness of the spike, gloom tightness, and head type (Hong, 1979; Pool and Patterson, 1958; Ibrahim, 1966; King and Richards, 1984). Spikes are assigned a sprouting score using a scale based on visible germination and post-germinative growth throughout the spike (McMaster and Derera, 1976). The sprouting scores of McMaster and Derera (1976) actually reflect three biological stages: 1) initial germination or the first appearance of roots (scores 1-5), 2) coleoptile emergence (scores 6-8), and 3) seedling growth (scores 9-10). Other methods for assessing PHS tolerance include plating assays to assess degree of seed dormancy, the Falling Numbers test (see below), and variations on the spike-wetting test (Paterson *et al*., 1989; Kumar *et al*., 2009; Zhang *et al*., 2014; Jimenez *et al*., 2016; Zhou *et al*, 2017).

Damage due to α-amylase induction from PHS is measured in the wheat industry using the Hagberg-Perten Falling Numbers (FN) test (Perten, 1964). During the FN test, a slurry of wheat meal and water is mixed while being heated to 100 °C. Then the FN machine measures the time in seconds (sec) that it takes for a stirrer to fall through the slurry. The higher the α-amylase level, the thinner the slurry, allowing the stirrer to fall faster resulting in a lower FN. If the FN is below 300 sec, then the farmer receives significantly less money for his/her grain. Because some studies have shown a significant

correlation between FN and sprouting scores, one might expect spike-wetting tests and FN to map similar PHS tolerance loci (reviewed by DePauw *et al*., 2012). However, this has not been directly tested.

Here we present a genome-wide association mapping study (GWAS) for PHS tolerance in white wheat based on sprouting scores and FN. A large panel of 469 white wheat lines representing 6 northwestern U.S. breeding programs was examined over multiple environments in order to characterize the genetic architecture of PHS tolerance/susceptibility. The goal was to identify quantitative trait nucleotides (QTN) associated with PHS tolerance in existing breeding programs, while examining the phenotypic connection between FN and spike-wetting test scores.

Materials and Methods

Plant Materials

This study used a mapping panel of 469 winter wheat accessions, consisting of advanced soft white breeding lines and cultivars from US Pacific Northwest breeding programs (Supplemental Table 1; Supplemental Figure 1). The accessions included 36 % club (*T. aestivum* ssp *compactum*) genotypes with compact spike morphology and 64 % soft white genotypes with lax spike morphology. The same panel was recently analyzed for soil acidity, aluminum tolerance, Cephalosporium stripe resistance, and stripe rust resistance (Froese and Carter, 2016; Froese *et al*., 2016; Liu *et al*., 2017).

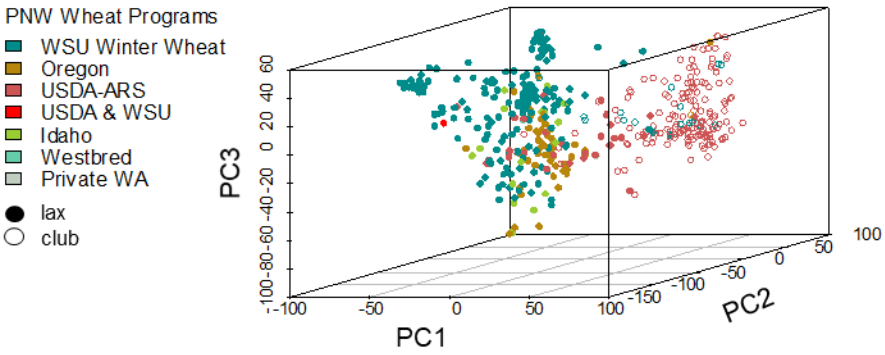
Field Research Environments

The mapping panel was grown as 1.5 m long headrows at Central Ferry, WA in 2014, 2015, and 2016 (C14, C15, C16), or as 8 m^2 plots at the Washington State University Spillman Agronomy Farm in Pullman, WA in 2014, 2015, and 2016 (P14, P15, P16) using recommended agronomic practices for those locations. The panel was also grown as headrows in Pullman, WA in 2013 (P13). Heading dates from the Pullman 2014 environment were determined after 50 % of the plot reached full spike emergence from the boot. Table 1 lists the planting dates and harvest dates for each environment. Spikes were harvested

Supplemental Table 1 - Mapping panel accession names and the breeding program each accession was developed in. Head type is also indicated.

Dissertation Attachment: SMartinez_10857505_Chapter2_TableS1

Online Resource found at:
https://www.frontiersin.org/articles/10.3389/fpls.2018.00141/full#supplementary-material



Supplemental Figure 1 - Principle components. The mapping panel was derived from at least six soft white winter wheat breeding programs and was comprised of three principle genetic components. Note that most of the club wheat (open circle) came from the USDA-ARS program.

Table 1 - Environments tested for preharvest sprouting traits. For the (A) Falling Numbers test (seconds) and the (B) spike-wetting test (sprouting score), planting dates, harvest dates, and general statistics are reported.

Location [a]	Year	Planting Date	Harvest Date [b]		Rain Event Precipitation	Rain Event Temperature	n [c]	t rep [d]	Mean ± SD [e]	Range (min/max)
(**A**)			PM	HM	cm	°C				
Pullman	2013	- [f]	-	Aug 15, 2013	0.43	22 ± 3	459	1	379 ± 55	88/504
Central Ferry	**2014**	Oct 2, 2013	-	Aug 6, 2014	1.91	26 ± 5	458	2	331 ± 39	202/538
Pullman	**2015**	Oct 8, 2014	-	Aug 1, 2015	0.00	-	464	2	326 ± 31	187/410
Central Ferry	**2015**	Oct 1, 2014	-	Jul 31, 2015	3.80	31 ± 2	397	2	389 ± 55	111/537
Central Ferry	2016	Oct 13, 2015	-	Jul 14, 2016	1.80	17 ± 2	426	2	347 ± 54	154/538
(**B**)										
Pullman	2014	Oct 10, 2013	Jul 11-18, 2014	-	-	-	427	5	3.9 ± 1.9	1/10
Central Ferry	**2014**	Oct 2, 2013	Jun 30- Jul 8, 2014	-	-	-	230	5	4.2 ± 1.7	1/9
Pullman	**2015**	Oct 8, 2014	Jul 1-10, 2015	-	-	-	416	5	4.05 ± 2.2	1/10
Central Ferry	**2015**	Oct 1, 2014	Jun 15-25, 2015	-	-	-	275	5	5.8 ± 2.3	1/10
Pullman	2016	Oct 12, 2015	Jul 15-22, 2016	-	-	-	437	5	6.5 ± 1.9	1/10

[a] Pullman, WA and Central Ferry, WA. **Bold** environments had both the FN and spike-wetting test conducted whereas the other environments had either the FN or spike-wetting test conducted.

[b] FN samples were harvested at harvest maturity (HM) whereas spike-wetting test samples were harvested at physiological maturity (PM).

[c] Number (n) of accessions harvested and conducted in the FN and sprouting tests.

[d] Technical (t) replicates used for each test and environment.

[e] Mean and standard deviation (SD) were calculated; Sprouting scores from Day 5 are reported.

[f] Planting date was not recorded.

at physiological maturity, right after the peduncle turned yellow, for the spike-wetting test (see below) only in Pullman 2014, 2015, and 2016 and Central 2014 and 2015.

Reduced FN was examined in field-grown material after PHS induced either by natural or artificial rain events. PHS-inducing natural rain events occurred after physiological maturity in Pullman 2013 and Central Ferry 2016. Therefore, these two environments were not used for spike-wetting tests, since the material was already sprouted. In Pullman 2013, rain occurred over 3 consecutive days with precipitation amounts of 0.38, 0.025, and 0.025 cm, and high temperatures of 22.8, 18.2, and 23.8 °C respectively (AgWeatherNet 2016, weather.wsu.edu). Central Ferry 2016 received 1.8 cm of precipitation over four days with an average maximum temperature of 17 °C. Artificial rain was used to induce PHS in Central Ferry 2014 and 2015 using overhead sprinkler irrigation at 2 weeks past the average physiological maturity date of the trial (precipitation = 1.91, 3.8 cm, average maximum temperature = 26 °C and 31 °C respectively). Approximately, 0.64 cm was applied daily over 3 days in order to induce mild sprouting. Pullman 2015 was included in the analysis as a "no event" control because there was no natural or artificial rain event after physiological maturity. Grain was harvested from plots 2-3 weeks after physiological maturity for FN tests (see below) when grain moisture was less than 12 %. In Pullman, single plot replicates were harvested using a Wintersteiger Classic small plot combine (Wintersteiger Ag, Ried im Innkreis, Austria). In Central Ferry, one headrow was hand-harvested with a sickle per accession and machine threshed using the Vogel headrow thresher (Bill's Welding, Pullman, WA). All harvested grain was cleaned of chaff using a gravity cleaner.

Preharvest Sprouting Evaluation

Spike-wetting tests were used to evaluate preharvest sprouting tolerance of field samples (Anderson *et al*., 1993). Intact spikes were hand-harvested from the field at physiological maturity, and allowed to dry after-ripen for the five days before storing at -15 °C to maintain dormancy until tests were conducted (within 2 to 4 months). A representative set of spikes were tested for moisture content at physiological maturity, following 5 d of after-ripening, and after storage at -15 °C, and found to be an average of 31 %,

14 %, and 10 %, respectively. Spike-wetting tests were conducted in a greenhouse with a 16 h day/8 h night photoperiod and 22-25 °C day and 16 °C night temperature. Supplemental lighting was used to maintain the photoperiod with a light intensity of 300-400 μmol/m^2/sec. Spikes were misted for six seconds every minute. The rare moldy spikes were thrown out of the experiment. Sprouting scores based on the McMaster and Derera (1976) 1-10 scale were determined every 24 hours for seven days, except that the "11" value was not used. Note that no sprouting was observed until day 3 of each experiment. Since the greenhouse misting system could test a maximum of 194 genotypes at a time, each environment had to be tested over multiple weeks. Two PHS tolerant controls, 'Brevor' and 'Clark's Cream', and two PHS susceptible controls, 'Greer' and 'Bruneau', were included in every experiment as a check for consistency (Walk-Simmons, 1987; Tuttle *et al*., 2015). Spikes were arranged in a randomized order, including five technical replicates (*i.e.*, five spikes) for each genotype.

Analyses of the spike-wetting tests were performed using sprouting scores and a sprouting index designed to give more weight to earlier than later sprouting. Sprouting index (SI) was calculated as (7 x s_{day1} + 6 x s_{day2} … + 1 x s_{day7}) / (7 x n) where s is the sprouting score on each day and n is the maximum sprouting score. SI ranged from 0 to 1, where an SI of 1 indicated that the spike reached 100 % highly sprouted by day 1 of misting. For the day 3, 4, 5, 6, and 7 sprouting scores and for the SI of each accession, best linear unbiased predictors (BLUPs) were calculated within each environment over the five technical replicates using the MIXED procedure in SAS/STAT v9.4 (Piepho *et al*., 2008). Furthermore, the week the accession was tested and the tray location in the misting system were used as covariates in the model and accessions were treated as random effects.

Falling Number Evaluation

The Hagberg-Perten FN test was conducted using cleaned machine-threshed grain from Central Ferry, WA in 2014, 2015, and 2016 and from Pullman, WA in 2013 and 2015 (Table 1). FN can gradually increase during storage at higher temperatures (Ji and Baik, 2016). Grain was stored in sealed containers at -15 °C to reduce problems with increasing FN. The FN test was conducted according to the ICC

standard No. 107/1 (1995) and the AACC Method 56-81.03A (1999) expect that a 25 g sample was used to represent a plot or headrow rather than a 250 g sample used to represent a field. 25 grams of grain was ground to meal using a Udy Cyclone Sample Mill with a 0.5 mm screen, and stored in air-tight 2 oz jars. Meal moisture content was averaged over four random samples, and applied to a subset of 48 samples. The sample weight used for the test was adjusted for moisture in order to be equivalent to 7 g of meal at 14 % moisture. After 25 mL of distilled water was added to a sample, it was placed in a shaker for 5 sec, then placed in a Perten Falling Number machine (Model 1600 or 1700). The FN machine determines the time needed for a stirrer to fall to the bottom of the tube after stirring and heating the samples for 60 sec (minimum FN is 60 sec). A lower FN is indicative of more α-amylase digestion, leading to lower gelling capacity. The FN was corrected for an altitude of 2500 ft (762 m) using FGIS Directive 9180.3 (2009). The material was examined using two technical replicates per accession with the exception of Pullman 2013, which only had one technical replicate. Each technical replicate was run on different days in 2014, within five minutes of one another in 2015, and side-by-side in 2016.

BLUPs for FN were also calculated over the artificial rain environments, the natural rain environments, and the no-rain event environment. Due to each year and environment having a different rain or no-rain event, we did not analyze BLUPs over all years or all environments (Supplemental Figure 2). An analysis of variance between accessions was performed using the MIXED procedure in SAS/STAT v9.4. Covariates were added to the analysis when relevant and included the individual who ran the test, the FN Machine used, and the seed-moisture sample subset (sets of 48 milled and tested together).

Spearman's significant rank correlations between the FN and the sprouting scores were conducted using the CORR procedure in SAS/STAT v9.4 (Supplemental Table 2). Since this is an association panel, the genotypic repeatability (R^2), rather than the heritability (H^2), was calculated using the lme4 package v1.1-13 in R (Campbell and Lipps, 1998; Bates, 2010). For the repeatability calculations, genotypes and covariates were considered to be random effects, whereas FN, sprouting score, or SI was used as the dependent variable.

Genotyping

DNA was harvested and extracted as described in Froese and Carter, (2016). Extracted DNA was genotyped using the Illumina Infinium iSelect 90K SNP array, and polymorphic markers were identified and curated using GenomeStudio v2011.1 (Illumina) (Wang *et al*., 2014). Monomorphic markers were filtered out based on the criteria of only having 0 or 1 accession with an alternate allele (out of 469). Markers with a 20 % or more missing data and minor allele frequency (MAF) less than 5 % were excluded from the analysis. A consensus map consisting of SNP markers were used to align chromosome locations of polymorphic markers (Wang *et al*., 2014). Genetic locations of unmapped (unk) markers were cross referenced with the GrainGenes database and are reported in Table 4, without reference to a cM position on a chromosome (www.graingenes.org). Missing values for markers with published locations were imputed using default parameters in BEAGLE v3.3.2 (Browning and Browning, 2016). This resulted in a total of 15,229 polymorphic markers, of which 12,681 had known locations covering all chromosomes.

Genome-wide Association Study

The GAPIT R package panel identified three principal component sub-groups associated with market class and breeding program of origin in the mapping panel (Tang *et al*., 2016). Variances captured by the first three principal components (PCs) accounted for 29.6 % of the total variance among the genotypes (Liu *et al*., 2017). In order to account for the presence of population structure, the top three PCs were fitted into the model as fixed effects (Supplemental Figure 1).

A portion of the data were analyzed using multiple statistical models, and the best statistical method selected based on how the observed *p*-values exceeded the null expectation on the Q-Q plot from GAPIT and FarmCPU (Lipka *et al*., 2012; Tang *et al*., 2016; Liu *et al*., 2016). Using the Pullman 2014 FN and sprouting score phenotypic data, a general linear model (GLM), mixed linear model (MLM), compressed mixed linear model (CMLM), SUPER model, and FarmCPU model were compared (Supplemental Figure 3; Zhang *et al*., 2010; Wang *et al*., 2014; Liu *et al*., 2016). The results indicated

that the FarmCPU model performed better than the other models. All subsequent genome-wide association analyses used only the FarmCPU model.

FarmCPU default parameters were used except that the 'optimum' bin method with default range and interval parameters was used instead of the 'static' method. The FN trait least squares means (LSMeans) and the sprouting score BLUPs were used as dependent variables in this GWAS (Supplemental Figure 3). Markers were identified as significantly associated with the trait after a 1 % Bonferroni multiple test correction ($p < 2.85E\text{-}07$; $-\log_{10}(p) > 6.55$).

The proportion of explained phenotypic variance was calculated as follows:

$$r^2 = \sum_{i=1}^{n}(\hat{y}_i - \bar{\hat{y}})^2 \Big/ \sum_{i=1}^{n}(y_i - \bar{y})^2$$

where y_i is observed phenotype value, $\hat{y}_i$ is the estimated phenotype value from a multiple linear regression model that was fitted to all significant SNPs as an independent variable with fixed effect.

Linkage disequilibrium (LD) was calculated using JMP software v6.0 (SAS, Cary, NC). LD of significant markers were used to estimate boundaries of potential quantitative trait loci (QTL) using the criteria of LD ($R^2 > 0.2$), chromosome location (cM) based on Wang *et al*. (2014), correlation between markers, and marker-trait information among the significant markers. For each designated QTL, the marker with the strongest association with either FN or sprouting scores was reported. Criteria for a strong association include: a) phenotypic variation explained by the marker (r^2); b) allelic effect; and c) marker *p* value.

Tolerant nucleotides of each significant marker were used to determine the pyramiding effect of PHS tolerant loci. A linear model regression was applied to the phenotypic estimates and number of favorable loci per accession. 'Favorable' loci nucleotides were those that lowered the sprouting score or increased the FN. Pearson's correlation coefficients were calculated between the trait and number of favorable loci using the 'cor' function in R v.3.2.5.

Comparison of QTN Locations with Previously Reported PHS Genes and QTL

A comparison of identified QTN with previous studies was performed using the integrated map of Maccaferri *et al*. (2015) that includes SSR markers, 9k SNP markers, 90k SNP markers, Synthetic x Opata DH GBS markers, and the Diversity Array Technology markers (Supplemental Table 3; Akbari *et al*., 2006; Cavanagh *et al*., 2013; Saintenac *et al*. 2013; Wang *et al*. 2014). Maccaferri *et al*. (2015) converted distances in cM into relative % length distances by dividing them by total chromosome length. The approximate relative positions of QTN were estimated based on known marker positions. Note that the LD of QTL from other studies were not recalculated.

Results

Environmental Response of PHS-Related Traits in Soft White Wheat

A panel of 469 soft white wheat accessions was evaluated for PHS tolerance based on spike-wetting tests and FN following rain in the field. FN was examined in five environments over four years and at two locations. The mean FN ranged from 326 to 389 sec for all environments (Table 1). α-amylase activity tends to correlate with FN below 325 sec (Perten, 1964; Yu *et al*., 2015). All environments had multiple accessions below the 325 sec threshold (ranging from 36 to 234 accessions) (Figure 1; Supplemental Figure 2). Natural sprout-inducing rain events occurred in Pullman 2013 and Central Ferry 2016. Artificial rain was applied to induce sprouting in Central Ferry in 2014 and 2015. Pullman 2013, and Central Ferry 2015 and 2016 had wide variation for FN but Central Ferry 2015 had very few accessions below 325 sec. The Pullman 2015 data was included in the analysis in an attempt to differentiate PHS-induced differences in FN from differences in FN due to starch or protein composition because no rain event occurred in Pullman 2015 so PHS did not occur (AgWeatherNet 2016, weather.wsu.edu). Pullman 2015 had 234 accessions below 325 sec but had lower variation than other environments. This is likely due to poor grain filling since that season had unusually high summer temperatures. QTN mapped based on FN data will be referred to as *QFN.wsu*.

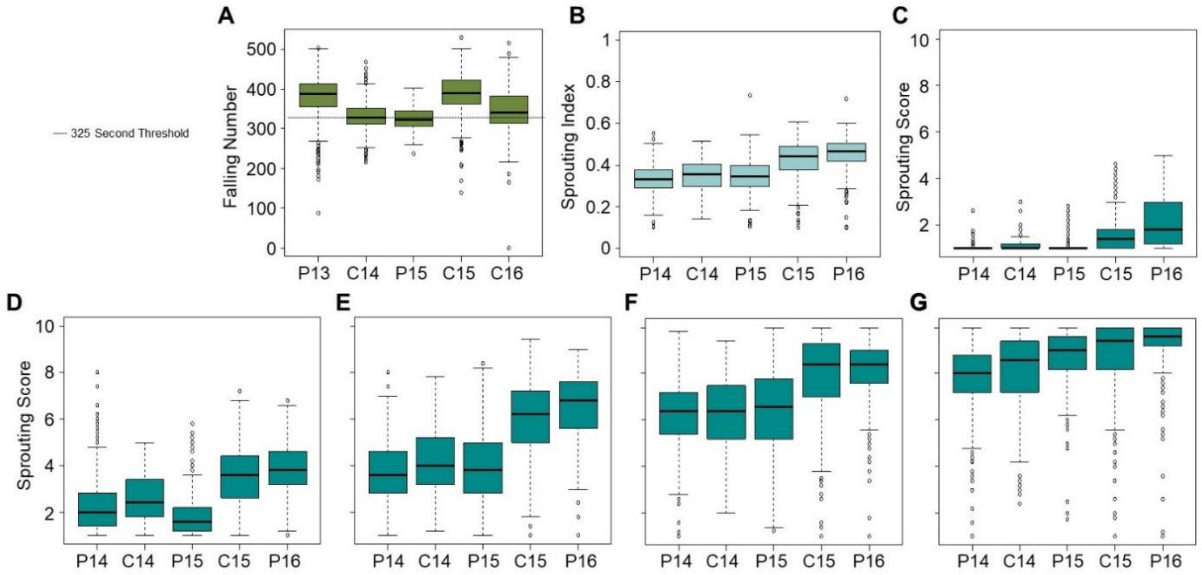
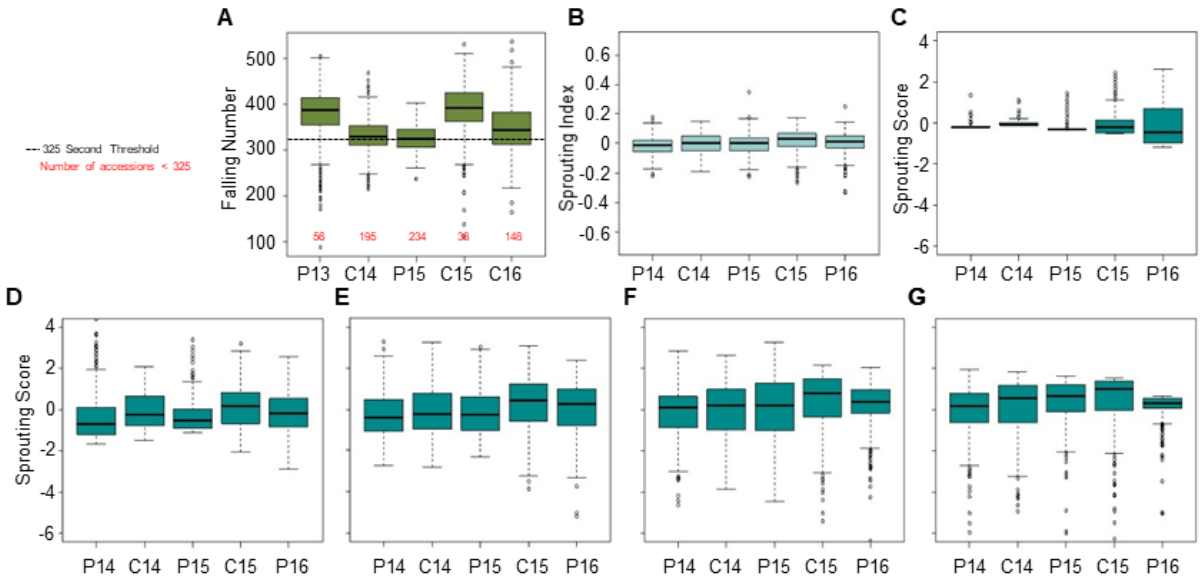


Figure 1 - FN and PHS distributions. (**A**) FN distributions across environments including the natural rain events in Pullman 2013 (P13) and Central Ferry 2016 (C16), an artificial rain event in Central Ferry 2014 (C14) and 2015 (C15), and an environment without rain in Pullman 2015 (P15). Spike-wetting tests were performed for Pullman 2014 (P14) as well as C14, P15, C15, and Pullman 2016 (P16). (**B**) Sprouting index was calculated over all 7 days of misting. Sprouting scores after (**C**) 3, (**D**) 4, (**E**) 5, (**F**) 6, and (**G**) 7 days of misting were based on a 1-10 scale.



Supplemental Figure 2 - FN and sprouting score distributions. GWAS was performed using the FN least square means and the spike-wetting test BLUPs. (**A**) FN distributions across environments including the natural rain events in Pullman 2013 (P13) and Central Ferry 2016 (C16), an artificial rain event in Central Ferry 2014 (C14) and 2015 (C15), and an environment without rain in Pullman 2015 (P15). Spike-wetting tests were performed for Pullman 2014 (P14) as well as C14, P15, C15, and C16. (**B**) Sprouting index was calculated over all 7 days of misting. Sprouting scores after (**C**) 3, (**D**) 4, (**E**) 5, (**F**) 6, and (**G**) 7 days of misting

In spike-wetting tests, environments had similar effects on results regardless of whether we examined sprouting scores or the sprouting index. For example, Central Ferry 2015 and Pullman 2016 consistently showed the highest level of sprouting (Figure 1; Table 1). BLUP analysis was performed in order to reduce environmental variation in sprouting scores prior to mapping (Supplemental Figure 2). BLUPs calculated over all environments were highly correlated over days of sprouting and with the sprouting index (SI) (Supplemental Table 2A). Based on the range of values, we expected to have enough variation to map QTN associated with PHS tolerance due to reduced germination or slower post-germinative growth. For ease of communication, QTN identified based on sprouting scores or SI will be referred to collectively as PHS loci or *QPHS.wsu*.

PHS Trait Correlations

Correlations were used to examine whether genotypic differences in FN or sprouting scores were consistent between environments, and to examine whether FN and sprouting scores were related. The FN values showed a weak but significant positive correlation between environments, ranging from 0.23 to 0.46 ($p<0.001$; Table 2). Sprouting scores were positively correlated between environments, but not always significantly (Supplemental Table 2B-G). Day 3 sprouting scores showed the least significant correlation between environments, whereas day 5 and day 6 sprouting scores showed significant positive correlations between environments. SI had the strongest and most significant correlation between environments. The genotypic repeatability of FN ($R^2 = 0.50$) was greater than sprouting scores ($R^2 < 0.31$), especially when covariates like operator, machine, subset, and replicate, were taken into account (Table 3). When repeatability was calculated on a line mean basis, both traits had similar repeatability in our experiments (FN $R^2 = 0.67$; SI $R^2 = 0.70$). The genotypic repeatability of sprouting was highest when spikes were misted longer or when all measurements were integrated in the SI (Table 3). Because seed germination induces α-amylase, which in turn lowers FN, we expected higher sprouting scores to negatively correlate with lower FN, but this was not the case (Table 2). BLUPs for SI generated over all days scored showed the strongest and most significant negative correlation with FN in years when natural

Table 2- Rank correlation coefficients for (A) FN LSMeans across environments and (B) PHS score BLUPs compared to FN environments.

	(**A**)	FN:	P13[a]	C14	P15	C15		
FN	C14		0.29**					
	P15		0.23**	0.42**				
	C15		0.23**	0.29**	0.29**			
	C16		0.33**	0.46**	0.30**	0.34**		
	(**B**)	PHS:	3 days	4 days	5 days	6 days	7 days	SI
FN	P13		-0.16**	-0.24**	-0.17**	-0.18**	-0.20**	-0.21**
	C14		-0.07	-0.09*	-0.06	-0.09	-0.10*	-0.10*
	P15		-0.07	-0.13*	-0.12*	-0.12*	-0.17**	-0.15**
	C15		-0.09	-0.04	0.00	0.01	0.00	-0.01
	C16		-0.17**	-0.19**	-0.18**	-0.17**	-0.17**	-0.19**

* represents a *p-value* $\leq$ 0.05 and ** represents a *p-value* $\leq$ 0.001

[a] Environments Pullman (P) and Central Ferry (C)

Supplemental Table 2 – Correlations between spike-wetting test environments over (G,M) SI, (B,H) 3, (C,I) 4, (D,J) 5, (E,K) 6, and (F,L) 7 days of misting.

BLUPs

A

All Environ	3 days	4 days	5 days	6 days	7 days
4 days	0.70**				
5 days	0.59**	0.87**			
6 days	0.49**	0.83**	0.94**		
7 days	0.45**	0.77**	0.87**	0.91**	
SI	0.63**	0.89**	0.96**	0.95**	0.93**

* represents a *p-value* ≤ 0.05 and ** represents a *p-value* ≤ 0.001

LSMeans

B

Day3	C14	P16	P14	C15	P15
C14	-				
p					
P16	0.19284	-			
p	0.0017				
P14	0.0126	0.03292	-		
p	0.841	0.4827			
C15	0.27428	0.06171	0.05252	-	
p	<.0001	0.2811	0.3655		
P15	0.07793	-0.02443	-0.03125	0.20677	-
p	0.2186	0.6068	0.5137	0.0003	

C

Day4	C14	P16	P14	C15	P15
C14	-				
p					
P16	0.42648	-			
p	<.0001				
P14	0.2883	0.21289	-		
p	<.0001	<.0001			
C15	0.47904	0.23633	0.35428	-	
p	<.0001	<.0001	<.0001		
P15	0.20391	0.07889	0.28951	0.32272	-
p	0.0012	0.0961	<.0001	<.0001	

D

Day5	C14	P16	P14	C15	P15
C14	-				
p					
P16	0.35426	-			
p	<.0001				
P14	0.43199	0.29901	-		
p	<.0001	<.0001			
C15	0.37238	0.20597	0.46418	-	
p	<.0001	0.0003	<.0001		
P15	0.31392	0.10822	0.49301	0.36943	-
p	<.0001	0.0223	<.0001	<.0001	

E

Day6	C14	P16	P14	C15	P15
C14	-				
p					
P16	0.38753	-			
p	<.0001				
P14	0.39478	0.39473	-		
p	<.0001	<.0001			
C15	0.38216	0.30193	0.39522	-	
p	<.0001	<.0001	<.0001		
P15	0.34186	0.16453	0.46462	0.25505	-
p	<.0001	0.0005	<.0001	<.0001	

F

Day7	C14	P16	P14	C15	P15
C14	-				
p					
P16	0.34795	-			
p	<.0001				
P14	0.36042	0.29515	-		
p	<.0001	<.0001			
C15	0.35687	0.29019	0.30197	-	
p	<.0001	<.0001	<.0001		
P15	0.34323	0.25626	0.40361	0.25413	-
p	<.0001	<.0001	<.0001	<.0001	

G

SI	C14	P16	P14	C15	P15
C14	-				
p					
P16	0.45936	-			
p	<.0001				
P14	0.42941	0.34628	-		
p	<.0001	<.0001			
C15	0.45929	0.27471	0.50059	-	
p	<.0001	<.0001	<.0001		
P15	0.34361	0.16683	0.51395	0.40306	-
p	<.0001	0.0004	<.0001	<.0001	

H

Day3	C14	P16	P14	C15	P15
C14	-				
p					
P16	0.19899	-			
p	0.0011				
P14	0.00932	0.06165	-		
p	0.8809	0.184			
C15	0.2516	0.06749	0.26826	-	
p	<.0001	0.2338	<.0001		
P15	0.15545	0.04608	0.125	0.28988	-
p	0.0128	0.3268	0.008	<.0001	

I

Day4	C14	P16	P14	C15	P15
C14	-				
p					
P16	0.43882	-			
p	<.0001				
P14	0.2785	0.20057	-		
p	<.0001	<.0001			
C15	0.47717	0.24577	0.35641	-	
p	<.0001	<.0001	<.0001		
P15	0.23332	0.1105	0.28477	0.34774	-
p	0.0002	0.0196	<.0001	<.0001	

J

Day5	C14	P16	P14	C15	P15
C14	-				
p					
P16	0.36218	-			
p	<.0001				
P14	0.42715	0.29676	-		
p	<.0001	<.0001			
C15	0.36992	0.20841	0.45788	-	
p	<.0001	0.0002	<.0001		
P15	0.3195	0.128	0.48985	0.36207	-
p	<.0001	0.0063	<.0001	<.0001	

K

Day6	C14	P16	P14	C15	P15
C14	-				
p					
P16	0.39539	-			
p	<.0001				
P14	0.38998	0.39859	-		
p	<.0001	<.0001			
C15	0.39189	0.30214	0.39322	-	
p	<.0001	<.0001	<.0001		
P15	0.34003	0.17945	0.45821	0.26322	-
p	<.0001	0.0001	<.0001	<.0001	

L

Day7	C14	P16	P14	C15	P15
C14	-				
p					
P16	0.34458	-			
p	<.0001				
P14	0.3618	0.29856	-		
p	<.0001	<.0001			
C15	0.37829	0.3051	0.30369	-	
p	<.0001	<.0001	<.0001		
P15	0.3603	0.27544	0.41131	0.27298	-
p	<.0001	<.0001	<.0001	<.0001	

M

SI	C14	P16	P14	C15	P15
C14	-				
p					
P16	0.4639	-			
p	<.0001				
P14	0.42073	0.34492	-		
p	<.0001	<.0001			
C15	0.46004	0.2757	0.49625	-	
p	<.0001	<.0001	<.0001		
P15	0.35712	0.17915	0.50527	0.40873	-
p	<.0001	0.0001	<.0001	<.0001	

Table 3 - Genotypic repeatability (R^2) of FN, spouting scores, and SI across all environments.

Trait	Simple $R^{2\,a}$	Covariate $R^{2\,b}$	Line mean basis $R^{2\,c}$
FN	0.197	0.500	0.667
PHS d3 [d]	0.109	0.s145	0.459
PHS d4	0.163	0.240	0.612
PHS d5	0.151	0.214	0.577
PHS d6	0.229	0.276	0.656
PHS d7	0.218	0.230	0.599
SI	0.228	0.315	0.697

[a] Genetic and environmental variances were calculated using a simple y ~ x model with x (genotypes) as a fixed effect and repeatability $R^2 = V_g / (V_g + V_e)$ was calculated.

[b] Genetic and environmental variances were calculated using a simple y ~ x + covariates model with x (genotypes) and covariates (time, machine, operator, etc.) as a fixed effect.

[c] Repeatability $R^2 = V_g / (V_g + (V_e / n))$ was expressed by a line mean basis by dividing the environmental variance (V_e, residuals) with the number of technical reps (n; FN = 2 and spike-wetting test = 5).

[d] Sprouting score (PHS) on days (d) 3 through 7

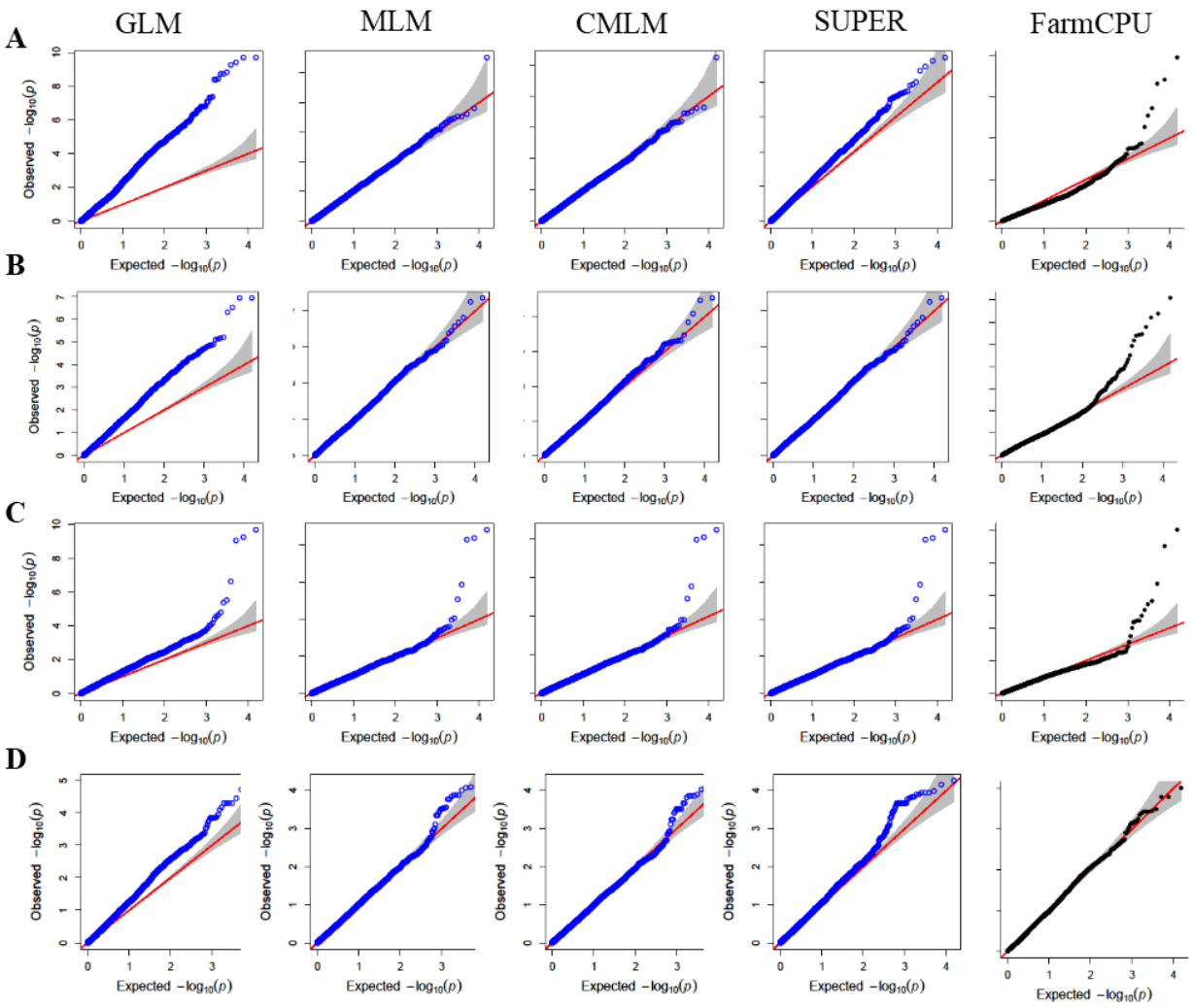
sprouting events occurred; Table 2B). In Central Ferry 2015, the FN did not correlate to the sprouting scores and SI. Overall, 4 days of misting had the highest correlation across all FN environments.

Association Analysis for Falling Numbers

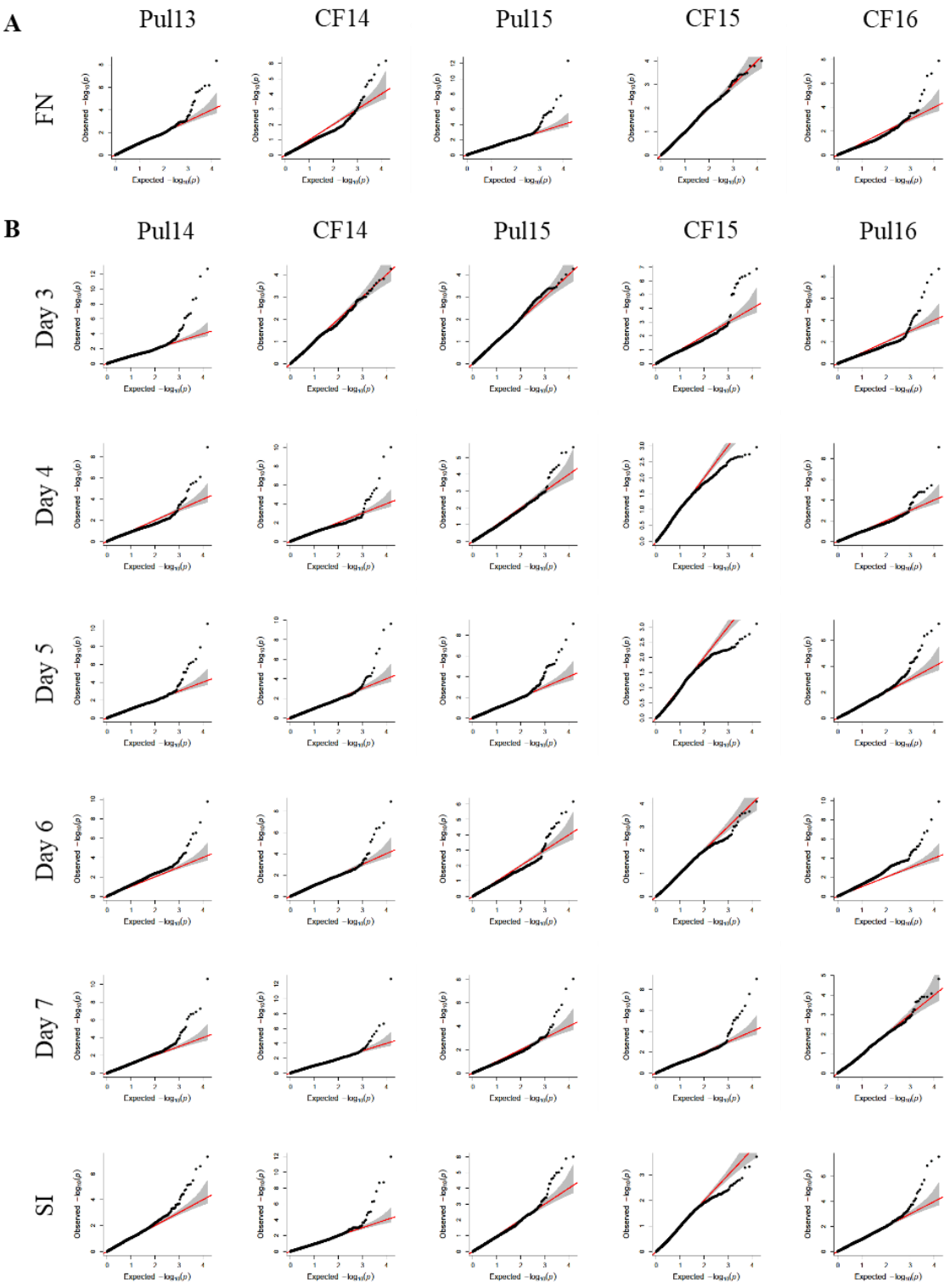
GWAS for FN was performed using FarmCPU within each environment because environments varied drastically. All environments fit the FarmCPU association mapping model (Supplemental Figure 4). However, Central Ferry 2015 showed no significant marker-trait associations, regardless of which model was used for GWAS (Supplemental Figure 3D). Nine significant QTN associated with the FN were mapped on chromosomes 4A, 5A, 5D, 7A, and 7B (Table 4). The three QTN identified without rain in Pullman 2015 likely represent grain characteristics that are independent of PHS. The remaining QTN were identified in natural rain events. The QTN detected on chromosomes 6D ($-\log_{10}(p) = 5.88$) and 7A ($-\log_{10}(p) = 6.17$) in Central Ferry 2014 were just below the stringent threshold for significance ($-\log_{10}(p) = 6.55$). *QFN.wsu-7A.1* and *QFN.wsu-7B.2* had the largest effects, increasing FN by 26 and 27 sec, respectively. *QFN.wsu-7A.2* had the highest significance ($-\log_{10}(p) = 12.36$) and had an 8 sec effect. In order to reduce the effect of loci unrelated to PHS tolerance on the GWAS, the data were re-analyzed with 400 sec set as the maximum possible FN. When this was done, none of the nine *QFN.wsu* were detected and two unique significant QTN, *QFN.wsu-6A* and *QFN.wsu-7A.3* were identified in Central Ferry 2016 (Supplemental Table 4). These two QTN had large effects, increasing FN by 17 and 13 sec, respectively, but only explained 1 % of the phenotypic variation. A GWAS was also conducted for heading date from Pullman 2014 and there were no heading date QTN discovered that overlap with QTNs for either FN or PHS (Supplemental Table 5).

Association Analysis for Sprouting Score and Sprouting Index

For the spike-wetting tests, 34 significant *QPHS.wsu* were detected based on sprouting scores or on SI (Table 4). There were 12 *QPHS.wsu* associated with early germination and root emergence (3-4 days of misting) (Figure 1; Table 4; McMaster and Derera, 1976). Seven *QPHS.wsu* were identified after 5 days



Supplemental Figure 3 - Comparison of association mapping models. Quantile-Quantile plots from GLM, MLM, CMLM, SUPER, and FarmCPU models are compared for environments: (**A**) Central Ferry 2016 Falling Numbers, (**B**) Pullman 2015 sprouting scores, (**C**) Central Ferry 2014 sprouting scores, and (**D**) Central Ferry 2015 sprouting scores. The grey region shows the 95 % confidence interval for the QQ-plot under the null hypothesis of no association between the SNP marker (black or blue dot) and the trait. The FarmCPU model showed an excellent fit of observed and expected -$\log_{10}(p)$ values except for the extreme markers (upper right) that were used to make marker-trait associations.



Supplemental Figure 4 - Quantile-Quantile plots of the observed versus the expected -$\log_{10}(p)$ using the FarmCPU model. QQ-plots of (**A**) Falling Number environments and (**B**) sprouting scores across different days of misting and SI environments. The grey region shows the 95 % confidence interval for the QQ-plot under the null hypothesis of no association between the SNP marker (black dot) and the trait.

Table 4. Loci significantly associated with Falling Numbers (FN), early preharvest sprouting (PHS) scores (days 3-4), 5 days of misting, late PHS scores (days 6-7), and PHS sprouting index (SI).

QTL[a]	Marker	Chr[b]	cM[b]	-log10(*p*)	maf	Effect[c]	r^2	Environment	Favorable Allele[d]
*QFN.wsu-4A**	IWB1884	4A	152	6.63	0.48	10.28	0.00	C16 FN	**A**/C
*QFN.wsu-5A.1**	IWB60191	5A	23	7.27	0.27	7.53	0.00	P15 FN	**A**/G
QFN.wsu-5A.2	IWB9800	5A	141	7.77	0.20	7.43	0.00	P15 FN	**A**/G
QFN.wsu-5D	IWB36060	5D	202	6.11	0.35	11.70	0.08	P13 FN	A/**C**
QFN.wsu-7A.1	IWB22966	7A	35	8.34	0.06	26.09	000	P13 FN	A/**G**
QFN.wsu-7A.2	IWA334	7A	126	12.36	0.41	7.99	0.01	P15 FN	A/**C**
QFN.wsu-7B.1	IWB39063	7B	162	7.91	0.48	10.88	0.01	C16 FN	A/**G**
QFN.wsu-7B.2	IWB75387	7B	-	6.15	0.09	27.35	0.00	P13 FN	A/**C**
QFN.wsu-unk	IWB37658	unk	-	6.82	0.09	15.49	0.00	C16 FN	T/**C**
QPHS.wsu-1A.1	IWB2320	1A	82	6.73	0.15	-0.04	0.00	P14 d3	**T**/C
QPHS.wsu-1A.2	IWB6759	1A	155	11.70	0.47	-0.31	0.15	P14 d3	**A**/G
	IWB77968	1A	155	12.72	0.47	-0.02	0.10	P14 d3	**A**/G
QPHS.wsu-1B.2	IWB64868	1B	135	9.00	0.15	-0.40	0.17	C14 d4	**A**/G
	IWB31676	1B	137	8.18	0.08	-0.37	0.00	P16 d3	**A**/G
QPHS.wsu-2A.1	IWB42693	2A	25	6.87	0.22	-0.16	0.02	C15 d3	**T**/G
QPHS.wsu-2D	IWB7652	2D	52	10.03	0.37	-0.46	0.00	C14 d4	T/**C**
	IWA8544	2D	50	8.73	0.46	-0.28	0.01	P16 d3	**A**/G
	IWA8544	2D	50	9.12	0.46	-0.32	0.07	P16 d4	**A**/G
QPHS.wsu-3A.2	IWB50719	3A	68	6.71	0.14	-0.29	0.04	C14 d4	A/**G**
QPHS.wsu-4A.1	IWA7535	4A	58	8.57	0.05	-0.07	0.03	P14 d3	A/**G**
QPHS.wsu-4B.2	IWB21707	4B	75	8.93	0.10	-0.42	0.07	P14 d4	A/**G**
*QPHS.wsu-4B.3**	IWB22055	4B	101	6.57	0.08	-0.37	0.00	P16 d3	A/**G**
QPHS.wsu-5B.1	IWB31067	5B	26	8.75	0.08	-0.06	0.01	P14 d3	T/**G**
QPHS.wsu-7B.1	IWB54418	7B	3	7.46	0.03	-0.26	0.01	P16 d3	A/**G**
*QPHS.wsu-1B.1**	IWB22868	1B	31	7.88	0.18	-0.30	0.01	P14 d5	**T**/C
QPHS.wsu-2D	IWB46396	2D	54	9.63	0.39	-0.49	0.02	C14 d5	**A**/G
QPHS.wsu-3B.2	IWA6185	3B	62	6.55	0.44	-0.23	0.01	P14 d5	A/**G**
QPHS.wsu-4A.2	IWB54609	4A	66	7.30	0.17	-0.35	0.01	P16 d5	**A**/G
*QPHS.wsu-5A.2**	IWB10250	5A	70	9.13	0.32	-0.44	0.03	P15 d5	**T**/C
QPHS.wsu-5B.3	IWB73511	5B	129	6.73	0.30	-0.28	0.01	P16 d5	A/**G**
*QPHS.wsu-6B**	IWA1838	6B	65	10.53	0.07	-0.29	0.05	P14 d5	**A**/G
*QPHS.wsu-7B.2**	IWB7099	7B	133	9.00	0.00	-0.34	0.00	C14 d5	A/**G**
*QPHS.wsu-1D**	IWB71680	1D	163	7.61	0.06	-0.60	0.09	P14, P16 d6	**A**/G
*QPHS.wsu-2A.2**	IWB17580	2A	53	9.02	0.07	-0.69	0.02	C15 d7	T/**C**
QPHS.wsu-2A.3	IWB79387	2A	-	6.88	0.01	-0.31	0.00	C14 d6	A/**G**
QPHS.wsu-2D	IWB7652	2D	52	12.69	0.37	-0.85	0.12	C14 d6, d7	T/**C**
QPHS.wsu-3A.1	IWB32631	3A	15	6.63	0.26	-0.31	0.02	C14 d7	A/**G**
*QPHS.wsu-3B.1**	IWB6430	3B	11	8.92	0.08	-0.38	0.01	P14 d7	T/**C**
QPHS.wsu-3B.3	IWB9902	3B	-	7.76	0.07	-0.59	0.06	P14 d7	**T**/C

QTL[a]	Marker	Chr[b]	cM[b]	-log10(*p*)	maf	Effect [c]	r^2	Environment	Favorable Allele [d]
QPHS.wsu-4A.2	IWB46089	4A	73	6.83	0.16	-0.33	0.04	P16 d6	A/**G**
QPHS.wsu-4A.3	IWB1389	4A	151	7.51	0.23	-0.45	0.02	C14 d7	**T**/G
QPHS.wsu-4B.1	IWB72936	4B	60	7.92	0.25	-0.46	0.02	C14 d7	**A**/G
QPHS.wsu-4B.2	IWA1382	4B	73	8.04	0.06	-0.53	0.00	P15 d7	A/**G**
QPHS.wsu-5A.2	IWB60303	5A	70	7.56	0.34	-0.39	0.01	C15 d7	A/**G**
*QPHS.wsu-5A.3******	IWB6049	5A	84	9.92	0.19	-0.31	0.00	P16 d6	**A**/G
QPHS.wsu-6A	IWB6726	6A	77	7.52	0.07	-0.48	0.03	P14 d7	**T**/G
QPHS.wsu-6B	IWB76583	6B	65	9.76	0.05	-0.33	0.03	P14 d6	**A**/G
*QPHS.wsu-6D******	IWB49280	6D	153	7.17	0.03	-0.39	0.00	P15 d7	**A**/G
*QPHS.wsu-7A******	IWB51129	7A	152	6.64	0.00	-0.27	0.06	P16 d6	A/**G**
QPHS.wsu-7B.3	IWB10815	7B	171	10.62	0.05	-0.41	0.00	P14 d7	T/**C**
QPHS.wsu-1D*	IWB71680	1D	163	7.22	0.06	-0.03	0.10	P16 SI	**A**/G
QPHS.wsu-2B	IWB30853	2B	87	7.59	0.21	-0.02	0.00	C14 SI	A/**G**
QPHS.wsu-2D	IWB46396	2D	54	11.97	0.39	-0.03	0.07	C14 SI	**A**/G
QPHS.wsu-3B.3	IWB9902	3B	-	7.31	0.07	-0.03	0.00	P14 SI	**T**/C
QPHS.wsu-5A.1	IWB10998	5A	53	8.70	0.41	-0.02	0.25	C14 SI	T/**C**
QPHS.wsu-6B	IWB57747	6B	64	6.85	0.07	-0.02	0.07	P16 SI	**A**/G
	IWB76583	6B	65	6.57	0.02	-0.01	0.00	P14 SI	**A**/G
*QPHS.wsu-7B.2**	IWB7099	7B	133	8.63	0.00	-0.02	0.01	C14 SI	A/**G**
	IWB7099	7B	133	7.58	0.01	-0.02	0.00	P16 SI	A/**G**

[a] QTL in bold explained 10 % (r^2 >0.1) or more of the phenotypic variation. QTL underlined were significant in 2 environments. Loci considered to be novel are indicated with ***.**

[b] Chromosome and position according to Wang *et al*. (2014). Positions are not reported if the location was identified on the GrainGenes database.

[c] The allelic effect is shown in FN seconds or sprouting score.

[d] The significant allele is favorable (in bold) if it decreases sprouting scores in the spike-wetting tests or increases Falling Numbers.

Supplemental Table 4 - Loci significantly associated with low FN when the maximum FN is 400 sec (FN >400 = 400) and early sprouting with the maximum sprouting score set to 5 (scores 1-5).

QTL[a]	Marker	Chr[b]	cM[b]	-log10(*p*)	maf	Effect[c]	r^2	Enviro-nment	Favorable Allele[d]
QFN.wsu-6A	IWB12127	6A	37	6.24	0.05	17.1	0.013	C16 FN	**A**/G
QFN.wsu-7A.3	IWB73683	7A	61.8	6.61	0.11	13.3	0.015	C16 FN	**A**/G
QPHSg.wsu-1A.2	IWB44485	1A	76	7.05	0.49	-0.06	0.20	P15 d3	**T**/G
QPHSg.wsu-1D.1	IWB5944	1D	67	7.32	0.07	-0.04	0.00	P14 d3	T/**C**
QPHSg.wsu-2B.1	IWB75872	2B	88	8.87	0.07	-0.04	0.00	P14 d3	A/**G**
QPHSg.wsu-2D	IWB81540	2D	50	9.84	0.46	-0.31	0.00	P16 d3	**A**/G
	IWB81540	2D	50	9.44	0.46	-0.23	0.01	P16 d4	**A**/G
	IWB7652	2D	52	27.42	0.37	-0.72	0.02	C14 d4	T/**C**
QPHSg.wsu-3B.2	IWB48693	3B	62	6.71	0.47	-0.20	0.00	C14 d4	**A**/G
	IWB54142	3B	62	8.82	0.49	-0.17	0.00	C15 d3	A/**G**
QPHSg.wsu-4A.1	IWB80864	4A	58	10.84	0.05	-0.08	0.00	P14 d3	A/**G**
QPHSg.wsu-4A.2	IWB61756	4A	109	7.04	0.49	-0.06	0.00	P15 d3	**A**/G
QPHSg.wsu-5B.1	IWB33287	5B	19	10.40	0.38	-0.19	0.04	P15 d4	A/**G**
QPHSg.wsu-5B.2	IWB22696	5B	40	7.01	0.25	-0.05	0.02	P15 d3	A/**G**
QPHSg.wsu-5B.3	IWB33771	5B	117	7.10	0.43	-0.25	0.01	P14 d4	**A**/G
QPHSg.wsu-6A.3	IWB40151	6A	85	11.40	0.48	-0.07	0.00	P15 d3	A/**G**
QPHSg.wsu-7A.2	IWB34499	7A	97	7.61	0.39	-0.29	0.00	C15 d4	A/**C**
	IWB59295	7A	97	8.82	0.39	-0.20	0.01	P15 d4	A/**G**
QPHSg.wsu-7A.5	IWB79354	7A	203	8.00	0.24	-0.07	0.20	P15 d3	A/**G**
QPHSg.wsu-7A.6	IWB26780	7A	216	7.25	0.34	-0.20	0.19	C14 d4	**T**/C
QPHSg.wsu-1B	IWB63380	1B	90	7.29	0.11	-0.22	0.03	P15 d5	A/**G**
QPHSg.wsu-1D.2	IWB71680	1D	163	9.35	0.06	-0.24	0.00	P16 d5	**A**/G
QPHSg.wsu-2D	IWB46396	2D	54	29.29	0.39	-0.54	0.10	C14 d5	**A**/G
QPHSg.wsu-3A.4	IWB8288	3A	151	7.57	0.12	-0.16	0.00	P16 d5	A/**C**
QPHSg.wsu-3B.3	IWB8629	3B	67	7.11	0.08	-0.15	0.00	P16 d5	A/**G**
QPHSg.wsu-4D	IWB10053	4D	79	7.20	0.09	-0.29	0.05	P15 d5	T/**C**
QPHSg.wsu-5A.1	IWB10250	5A	70	8.07	0.32	-0.27	0.01	P15 d5	**T**/C
QPHSg.wsu-6A.2	IWB51108	6A	77	6.97	0.34	-0.15	0.09	P14 d5	**A**/G
QPHSg.wsu-7B.2	IWB7099	7B	133	6.57	0.17	-0.21	0.00	C14 d5	A/**G**
QPHSg.wsu-unk.2	IWB12564	unk	-	8.64	0.06	-0.39	0.00	P15 d5	T/**C**
QPHSg.wsu-unk.3	IWB74624	unk	-	6.74	0.18	-0.10	0.03	P16 d5	A/**G**
QPHSg.wsu-1A.1	IWB9223	1A	16	7.58	0.18	-0.09	0.02	P15 d7	T/**C**
QPHSg.wsu-1D.2	IWB71680	1D	163	8.29	0.06	-0.14	0.00	P14 d7	**A**/G
	IWB71680	1D	163	13.58	0.06	-0.19	0.02	P16 d6	**A**/G
QPHSg.wsu-2A.1	IWB17580	2A	53	11.58	0.07	-0.29	0.01	C15 d6	T/**C**
	IWB17580	2A	53	14.21	0.07	-0.28	0.01	C15 d7	T/**C**
QPHSg.wsu-2A.2	IWB64379	2A	128	6.96	0.08	-0.17	0.00	P14 d6	**A**/G
QPHSg.wsu-2B.1	IWB27957	2B	92	11.90	0.06	-0.13	0.02	P16 d6	A/**G**
QPHSg.wsu-2B.3	IWB64082	2B	141	9.54	0.10	-0.14	0.07	C14 d7	**A**/G
QPHSg.wsu-2D	IWB7652	2D	52	18.90	0.37	-0.39	0.11	C14 d6	T/**C**
	IWB7652	2D	52	15.04	0.37	-0.21	0.07	C14 d7	T/**C**

QTL[a]	Marker	Chr[b]	cM[b]	-log10(*p*)	maf	Effect[c]	r^2	Environment	Favorable Allele[d]
QPHSg.wsu-3A.1	IWB50475	3A	15	6.65	0.33	-0.11	0.01	C14 d6	**A**/G
QPHSg.wsu-3A.2	IWB32758	3A	113	7.53	0.07	-0.15	0.02	P16 d6	**T**/G
QPHSg.wsu-3B.1	IWB7629	3B	5	8.85	0.07	-0.14	0.00	P14 d7	T/**G**
QPHSg.wsu-3B.3	IWB45058	3B	67	7.62	0.10	-0.11	0.00	P16 d6	A/**G**
QPHSg.wsu-3B.4	IWB70167	3B	137	7.86	0.05	-0.15	0.04	P16 d6	A/**G**
QPHSg.wsu-5A.2	IWB6049	5A	84	7.20	0.19	-0.07	0.00	P16 d6	**A**/G
QPHSg.wsu-5B.4	IWB71749	5B	144	9.99	0.11	-0.28	0.01	C15 d6	**A**/G
	IWB71749	5B	144	17.54	0.11	-0.36	0.00	C15 d7	**A**/G
QPHSg.wsu-6D	IWB49280	6D	153	7.10	0.09	-0.22	0.00	P15 d6	**A**/G
QPHSg.wsu-7A.1	IWB47598	7A	45	7.58	0.45	-0.07	0.04	P16 d6	A/**G**
QPHSg.wsu-7A.3	IWB54820	7A	178	6.65	0.46	-0.07	0.00	P14 d7	**A**/G
QPHSg.wsu-7B.1	IWB78079	7B	77	8.29	0.07	-0.04	0.00	P14 d7	**A**/G
	IWB81211	7B	77	7.87	0.06	-0.35	0.02	P14 d7	A/**G**
	IWB59498	7B	77	7.87	0.06	-0.35	0.00	P14 d7	**A**/G
	IWB25969	7B	78	8.11	0.06	-0.37	0.00	P14 d7	**A**/G
	IWB26968	7B	78	8.11	0.06	-0.37	0.00	P14 d7	**A**/G
	IWB26969	7B	78	8.11	0.06	-0.37	0.01	P14 d7	**A**/G
QPHSg.wsu-7D	IWB7177	7D	149	7.75	0.45	-0.09	0.01	C14 d7	**A**/G
QPHSg.wsu-unk.1	IWB12177	unk	-	10.02	0.32	-0.21	0.01	C15 d6	A/**G**
QPHSg.wsu-unk.4	IWB50340	unk	-	8.12	0.09	-0.18	0.00	P16 d6	**A**/G
QPHSg.wsu-2B.2	IWB63900	2B	104	7.77	0.22	-0.02	0.00	C14 SI	A/**G**
QPHSg.wsu-2D	IWB81540	2D	50	7.64	0.46	-0.02	0.03	P16 SI	**A**/G
	IWB7652	2D	52	37.13	0.37	-0.06	0.00	C14 SI	T/**C**
QPHSg.wsu-3A.3	IWB46039	3A	138	8.17	0.08	-0.03	0.00	C14 SI	A/**G**
QPHSg.wsu-5A.1	IWB10250	5A	70	9.33	0.32	-0.02	0.16	P15 SI	**T**/C
QPHSg.wsu-5A.3	IWB66227	5A	96	6.55	0.43	-0.02	0.03	C15 SI	T/**C**
QPHSg.wsu-6A.1	IWB80412	6A	5	8.38	0.22	-0.02	0.00	C14 SI	**A**/G
QPHSg.wsu-7A.2	IWB59295	7A	97	10.04	0.39	-0.03	0.02	C15 SI	A/**G**
QPHSg.wsu-7A.4	IWB29877	7A	181	7.23	0.10	-0.02	0.01	C14 SI	**T**/C

[a] QTL in bold are not found in the original GWAS analysis (Table 4) assuming QTN within 10cM are the same QTL.

[b] Chromosome and position according to Wang *et al*. (2014).

[c] The allelic effect is shown in FN seconds or sprouting score BLUPs.

[d] The significant allele is favorable (in bold) if it decreases sprouting scores in the spike wetting tests or increases Falling Numbers.

Supplemental Table 5 - Loci significantly associated with heading date (HD) from Pullman 2014.

QTL[a]	Marker	Chr[b]	cM[b]	-log10(*p*)	maf	Effect[c]	r^2	Envir-onment	Later Allele[d]
QHD.wsu-1B.1	IWB64963	1B	60	12.83	0.05	0.99	0.09	CF16 FN	A/**G**
QHD.wsu-1B.2	IWB68096	1B	160	9.39	0.33	0.46	0.00	PUL15 FN	**A**/G
QHD.wsu-2B	IWB37419	2B	91	7.26	0.34	0.44	0.00	PUL13	**T**/C
QHD.wsu-4A	IWB1396	4A	41	11.29	0.15	0.56	0.01	PUL15 FN	**T**/G

[a] QTL in bold explained 10 % (R^2 >0.1) or more of the phenotypic variation.
[b] Chromosome and position according to Wang *et al*. (2014).
[c] The allelic effect is in days.
[d] The significant allele which causes later heading date is highlighted in bold.

of misting, only one of which was also seen with 3-4 days of misting. There were 16 significant *QPHS.wsu* associated with coleoptile emergence and elongation at 6-7 days of misting, 13 of which were unique to 6-7 days of misting. There were 3 additional *QPHS.wsu* uniquely detected by SI (Figure 2B). An association at the *QPHS.wsu-2D* locus was detected over all days of scoring, suggesting that it is not unique to any one sprouting stage (Figure 2A). The *QPHS.wsu-1B.2* and *QPHS.wsu-6B* were detected on two of the scoring days. Of the 34 total QTN found in the spike-wetting tests, only 6 *QPHS.wsu* on chromosomes 1B, 1D, 2D, 5A, 6B, and 7B were significant in two environments (Figure 2C). No *QPHS.wsu* were detected in more than 2 environments. Five *QPHS.wsu* on chromosomes 1A, 1B, 1D, 2D, and 5A explained more than 10 % of the phenotypic variation. In Central Ferry 2015, only sprouting scores on days 3 and 7 fit the expected -log(*p*), whereas days 4, 5, 6, and SI did not (Supplemental Figure 4). Therefore, very few significant *QPHS.wsu* were identified in this environment.

In order to reduce the effect of post-germinative growth versus germination *per se*, the GWAS was repeated with the maximum sprouting score set at 5 and referred to as *QPHSg.wsu* (Supplemental Table 4). Out of 46 QTN detected, 32 QTN were unique to this analysis and 21 were no longer significant (Supplemental Figure 5). The major QTN, *QPHS.wsu-1D*, *QPHS.wsu-2D*, *QPHS.wsu-5A.2*, and *QPHS.wsu-7B.2* were also detected in this germination-based analysis. The *QPHSg.wsu-2D* locus was highly significant in this GWAS ($7.64 < -\log_{10}(p) < 37.13$), and had strong effects (0.02 to 0.72).

Association Analysis without Using the Club Wheat Breeding Program as a Covariate

Based on the fact that the strong *QPHS.wsu-2D* locus was near the *C* locus region for the compact spike morphology, we examined whether the club wheat breeding program contributed more PHS tolerance to the GWAS than the lax wheat breeding programs (Figure 3). In Central Ferry 2014, the tolerant and susceptible loci from the strongest marker within *QPHS.wsu-2D* were compared and 98 % of the tolerant loci were club (Figure 3A). When the BLUPs were compared across all environments, the same trend was observed (Figure 3B). In fact, the club wheat breeding program generally contributed more PHS tolerance in the GWAS than the other programs (Figure 3C). Thus, it is possible that using the principle

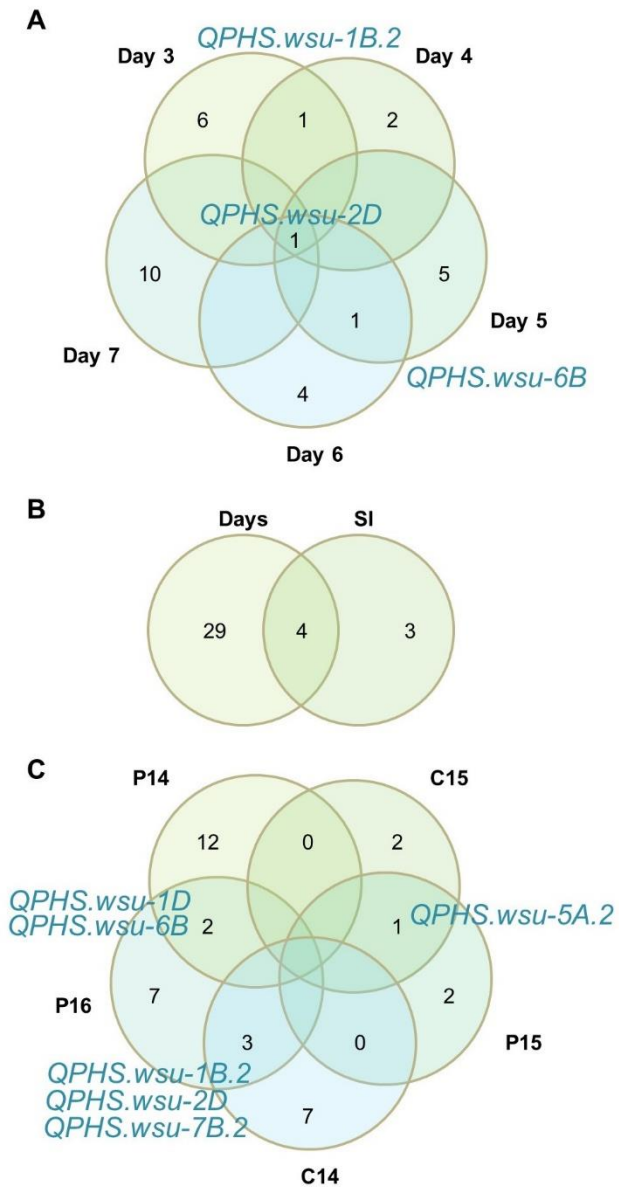
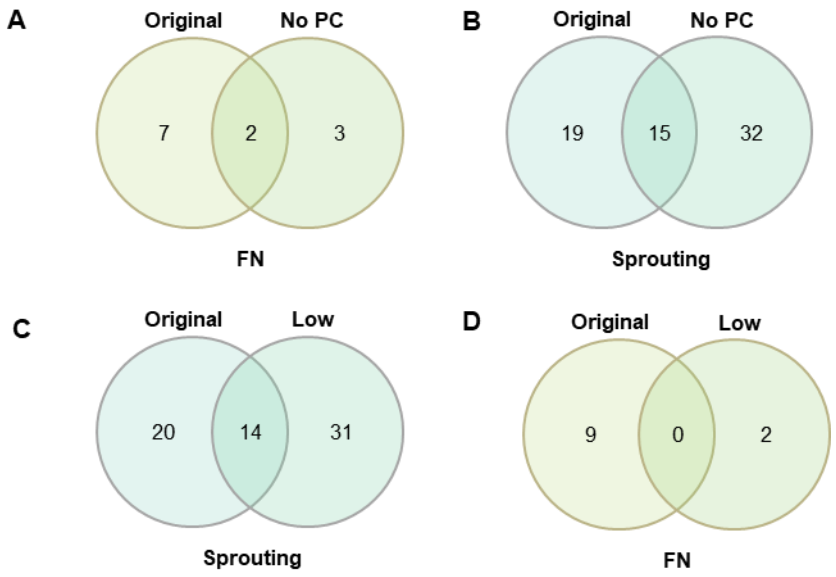


Figure 2 - Venn Diagrams comparing sprouting score QTN across (**A**) days misted, (**B**) all days misted and sprouting index, and (**C**) the five environments. Shared QTN are printed in blue.



Supplemental Figure 5 - Venn Diagrams comparing significant QTN between (**A**) the original GWAS *QFN.wsu* loci versus the GWAS without using principal components for *QFNnPC.wsu* loci, (**B**) the original GWAS *QPHS.wsu* loci versus the GWAS without using principal components for *QPHSnPC.wsu* loci, (**C**) the original sprouting GWAS *QPHS.wsu* loci versus the low sprouting score GWAS *QPHSg.wsu* loci (score range only 1-5), and (**D**) the *QFN.wsu* loci from the original GWAS versus the GWAS in which the maximum FN was set to 400 sec. QTN within 10 cM region of each other based on the Wang *et al*. (2014) consensus map were considered to be the same QTL.

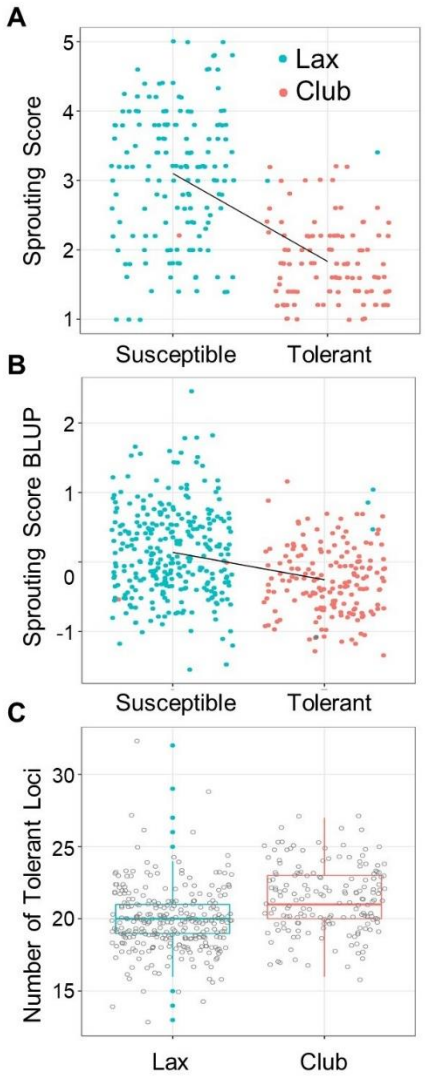


Figure 3 - *QPHS.wsu-2D* in club versus lax mapping lines. Scatter plot of sprouting score (**A**) raw means over the environment, Central Ferry 2014, that *QPHS.wsu-2D* had the strongest effect and (**B**) Sprouting score BLUPs across all five environments after 4 days of misting versus the presence of the *QPHS.wsu-2D* susceptible (S) or tolerant (T) allele. Club genotypes are in red and lax in blue. (**C**) Total number of favorable loci are compared to club versus lax spike morphology.

Supplemental Table 6 - Loci significantly associated with FN, sprouting (PHS) scores across days (d) 3-7, and sprouting index (SI) with no principal components (nPC) incorporated into the association mapping model.

QTL[a]	Marker	Chr[b]	cM[b]	-log10(*p*)	maf	Effect[c]	r^2	Enviro-nment	Favorable Allele[d]
QFNnPC.wsu-1B	IWB24969	1B	75	8.62	0.12	10.07	0.00	P15	**T**/C
QFNnPC.wsu-4A.1	IWB59982	4A	54	8.57	0.12	16.11	0.00	C16	A/**G**
QFNnPC.wsu-4A.2	IWB59774	4A	164	7.01	0.07	21.46	0.00	C15	**A**/G
QFNnPC.wsu-7A	IWB14901	7A	124	6.69	0.18	15.85	0.02	C15	**T**/C
	IWB75568	7A	126	13.88	0.41	8.19	0.03	P15	A/**C**
QPHSnPC.wsu-1A.1	IWB34707	1A	16	6.81	0.41	-0.36	0.15	P14 d4	A/**C**
QPHSnPC.wsu-1A.2	IWB44485	1A	76	7.06	0.49	-0.06	0.00	P15 d3	**T**/G
QPHSnPC.wsu-1A.3	IWB77080	1A	87	7.25	0.35	-0.02	0.00	C15 SI	A/**G**
QPHSnPC.wsu-1B.1	IWB22868	1B	31	8.04	0.18	-0.34	0.14	P14 d5	**T**/C
QPHSnPC.wsu-1B.2	IWB47637	1B	67	6.63	0.21	-0.28	0.06	C15 d4	A/**G**
	IWB12334	1B	70	6.85	0.14	-0.33	0.06	P14 d5	**A**/G
QPHSnPC.wsu-1B.3	IWB49534	1B	81	7.13	0.46	-0.14	0.02	C15 d3	A/**G**
QPHSnPC.wsu-1B.4	IWB63380	1B	90	10.18	0.11	-0.34	0.01	P15 d5	A/**G**
	IWB77753	1B	90	6.84	0.10	-1.05	0.00	P15 d5	**A**/G
QPHSnPC.wsu-1D.1	IWB5944	1D	67	7.32	0.07	-0.04	0.00	P14 d3	T/**C**
QPHSnPC.wsu-1D.2	IWB71680	1D	163	12.65	0.06	-0.64	0.01	P16 d6	**A**/G
	IWB71680	1D	163	15.54	0.06	-0.61	0.00	P16 d7	**A**/G
QPHSnPC.wsu-2A.1	IWB26001	2A	150	7.19	0.16	-0.02	0.31	C14 SI	**T**/C
QPHSnPC.wsu-2B.1	IWB75872	2B	88	8.87	0.07	-0.04	0.01	P14 d3	A/**G**
	IWB33642	2B	90	7.01	0.10	-0.37	0.08	C14 d5	**A**/G
QPHSnPC.wsu-2B.2	IWB63970	2B	114	6.62	0.36	-0.27	0.08	C14 d6	A/**G**
	IWB63970	2B	114	7.29	0.36	-0.02	0.00	C14 SI	A/**G**
QPHSnPC.wsu-2D	IWB81540	2D	50	9.84	0.46	-0.31	0.01	P16 d3	**A**/G
	IWB81540	2D	50	7.05	0.46	-0.26	0.07	P16 d4	**A**/G
	IWB7652	2D	52	23.74	0.37	-0.69	0.00	C14 d4	T/**C**
	IWB7652	2D	52	25.31	0.37	-1.08	0.10	C14 d6	T/**C**
	IWB46396	2D	54	30.03	0.39	-0.82	0.24	C14 d5	**A**/G
	IWB46396	2D	54	11.44	0.39	-0.61	0.08	C14 d7	**A**/G
	IWB46396	2D	54	23.14	0.39	-0.04	0.17	C14 SI	**A**/G
QPHSnPC.wsu-3A.1	IWB60440	3A	15	7.52	0.47	-0.30	0.01	P14 d7	A/**G**
QPHSnPC.wsu-3A.2	IWB78290	3A	89	6.80	0.41	-0.29	0.08	P15 d5	A/**G**
QPHSnPC.wsu-3A.3	IWB78959	3A	105	9.81	0.19	-0.44	0.01	C14 d6	A/**G**
QPHSnPC.wsu-3A.4	IWB8288	3A	151	6.56	0.12	-0.36	0.00	P16 d6	A/**C**
	IWB8288	3A	151	7.64	0.12	-0.18	0.03	P16 d7	A/**C**
QPHSnPC.wsu-3B.1	IWB54142	3B	62	8.40	0.49	-0.16	0.02	C15 d3	A/**G**
	IWB36421	3B	62	7.00	0.46	-0.26	0.00	P14 d6	**A**/G
	IWB36421	3B	62	8.10	0.46	-0.01	0.00	P14 SI	**A**/G
	IWB79762	3B	62	8.92	0.47	-0.29	0.00	P16 d6	A/**G**
QPHSnPC.wsu-3B.2	IWB8629	3B	67	7.46	0.08	-0.52	0.01	P16 d6	A/**G**

QTL[a]	Marker	Chr[b]	cM[b]	-log10(*p*)	maf	Effect[c]	r^2	Environment	Favorable Allele[d]
QPHSnPC.wsu-3B.3	IWB10839	3B	139	8.95	0.40	-0.33	0.00	P16 d6	**T**/G
QPHSnPC.wsu-3D	IWB27554	3D	155	6.77	0.05	-0.51	0.07	P15 d5	**A**/G
QPHSnPC.wsu-4A.1	IWB80864	4A	58	10.85	0.05	-0.08	0.01	P14 d3	A/**G**
QPHSnPC.wsu-4A.2	IWB61756	4A	109	7.04	0.49	-0.06	0.00	P15 d3	**A**/G
QPHSnPC.wsu-4B.1	IWB8210	4B	61	9.86	0.09	-0.57	0.01	P14 d7	A/**G**
QPHSnPC.wsu-4B.2	IWB76214	4B	73	7.60	0.06	-0.48	0.00	P15 d7	A/**G**
QPHSnPC.wsu-4D	IWB10053	4D	79	9.21	0.09	-0.53	0.02	P15 d5	T/**C**
QPHSnPC.wsu-5A.1	IWB10998	5A	53	8.74	0.40	-0.02	0.26	C14 SI	T/**C**
QPHSnPC.wsu-5A.2	IWB10250	5A	70	8.61	0.32	-0.40	0.01	P15 d5	**T**/C
	IWB76988	5A	73	6.95	0.33	-0.26	0.00	P14 d5	A/**G**
	IWB76988	5A	73	6.69	0.33	-0.01	0.01	P14 SI	A/**G**
QPHSnPC.wsu-5A.3	IWB6049	5A	84	8.42	0.19	-0.29	0.01	P16 d6	**A**/G
	IWB40144	5A	86	7.27	0.41	-0.37	0.00	P15 d6	**A**/G
QPHSnPC.wsu-5A.4	IWB66227	5A	96	7.46	0.43	-0.29	0.01	C15 d4	T/**C**
QPHSnPC.wsu-5B.1	IWB22696	5B	40	7.02	0.25	-0.05	0.01	P15 d3	A/**G**
QPHSnPC.wsu-5B.2	IWB56759	5B	60	7.27	0.05	-0.31	0.00	C15 d3	**A**/G
QPHSnPC.wsu-5B.3	IWB71749	5B	144	10.33	0.11	-0.83	0.00	C15 d7	**A**/G
QPHSnPC.wsu-5D	IWB81433	5D	60	8.70	0.47	-0.34	0.00	P14 d7	**A**/G
QPHSnPC.wsu-6A.1	IWB40151	6A	85	11.40	0.48	-0.07	0.00	P15 d3	A/**G**
QPHSnPC.wsu-6A.2	IWB3818	6A	130	6.96	0.21	-0.24	0.02	C14 d4	**T**/C
QPHSnPC.wsu-6D	IWB49280	6D	153	7.27	0.09	-0.36	0.03	P15 d7	**A**/G
QPHSnPC.wsu-7A.1	IWB36251	7A	33	6.70	0.28	-0.14	0.03	P15 d5	**A**/C
QPHSnPC.wsu-7A.2	IWB34499	7A	97	6.77	0.39	-0.30	0.04	C15 d4	A/**C**
	IWB59295	7A	97	9.58	0.39	-0.27	0.00	P15 d4	A/**G**
	IWB22776	7A	97	7.43	0.40	-0.36	0.01	P15 d5	**T**/C
QPHSnPC.wsu-7A.3	IWB79354	7A	203	8.00	0.24	-0.08	0.00	P15 d3	A/**G**
QPHSnPC.wsu-7A.4	IWB25497	7A	230	8.58	0.12	-0.46	0.00	P15 d5	**A**/G
QPHSnPC.wsu-7B.1	IWB25434	7B	24	10.26	0.08	-0.52	0.04	P16 d7	T/**G**
QPHSnPC.wsu-7B.2	IWB7099	7B	133	9.92	0.17	-0.57	0.03	C14 d7	A/**G**
QPHSnPC.wsu-unk	IWB71903	unk	-	7.91	0.28	-0.37	0.02	P15 d5	A/**G**
QPHSnPC.wsu-unk	IWB71903	unk	-	6.79	0.28	-0.02	0.01	P15 SI	A/**G**
QPHSnPC.wsu-unk	IWB7559	unk	-	7.31	0.40	-0.34	0.01	P16 d5	T/**C**
QPHSnPC.wsu-unk	IWB65338	unk	-	9.17	0.05	-0.39	0.04	P16 d7	A/**G**
QPHSnPC.wsu-unk	IWB35677	unk	-	7.22	0.13	-0.64	0.03	P16 d7	A/**C**

[a] QTL in bold are not found in the original GWAS analysis (Table 4) assuming QTN within 10cM are the same QTL.

[b] Chromosome and position according to Wang *et al*. (2014).

[c] The allelic effect is shown in FN seconds or sprouting score BLUPs.

[d] The significant allele is favorable (in bold) if it decreases sprouting scores in the spike wetting tests or increases Falling Numbers.

components as a covariate in the GWAS may artifactually remove some of the PHS loci contributed by the club wheat breeding program. To test this hypothesis, the GWAS was repeated without incorporating the principle components into the model (Supplemental Table 6). The *QPHS.wsu-2D* QTN became stronger in this analysis, increasing to a $-\log_{10}(p)$ of up to 30.03 and an effect of 1.08. An additional 32 *QPHSnPC.wsu* and 2 *QFNnPC.wsu* were detected, whereas 15 *QPHS.wsu* and 2 *QFN.wsu* were found in common (Supplemental Figure 5).

Pyramiding Effects of FN and PHS QTN

Next, we examined whether an increasing number of QTN were associated with increasing FN or PHS tolerance. The number of favorable *QFN.wsu* loci within accessions ranged from 2 to 9 (Figure 4A-C). An increasing number of *QFN.wsu* loci was only weakly correlated to higher FN. The correlation was actually stronger when there was no rain event than when there was a natural or artificial rain event (r = 0.23, 0.19, 0.09, respectively). The number of *QPHS.wsu* loci varied more widely within the accessions (8 to 26), making it easier to assess the effects of pyramiding multiple tolerance loci (Figure 4D-I). An increasing number of tolerance loci was negatively correlated with sprouting scores ranging from r = -0.47 to r = -0.55 (Supplemental Figure 6). Thus, having more *QPHS.wsu* loci was associated with more PHS tolerance. Tolerant FN loci only slightly correlated with the increasing sprouting scores, and vice versa ($r < 0.20$) (Supplemental Figure 6).

Within the mapping panel, there was only one accession, 'A00154', with all 9 *QFN.wsu* that had an average FN of 461 sec across natural rain events. The accession '6J020288-1' had the highest number of favorable alleles, including 26 out of 34 *QPHS.wsu* and 6 of 9 *QFN.wsu*. This was reflected in the phenotype since 6J020288-1 had an average FN of 380 sec across natural rain events and a sprouting score of 1 after 5 days of misting from all environments. In contrast, the accession 'J950409-10-2' had only 9 out of 34 *QPHS.wsu* and 5 of 9 *QFN.wsu*, associated with an average FN of 272 sec and a high sprouting score of 7 after 5 days of misting. Interestingly, 'Lewjain' had only 8 of the 34 *QPHS.wsu* and 5 of the 9 *QFN.wsu* but had an average FN of 401 sec and a sprouting score of 4 after 5 days. Thus, these

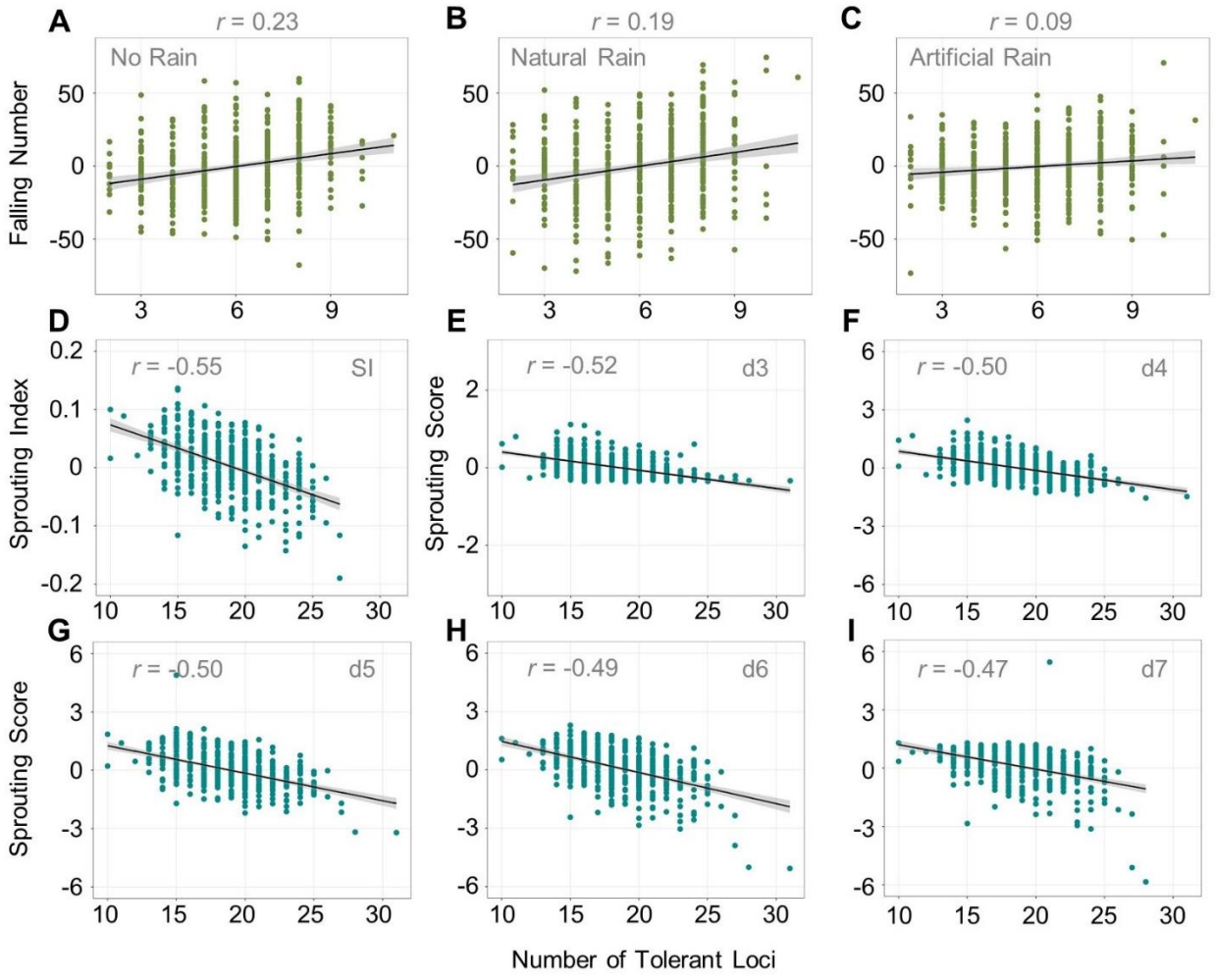
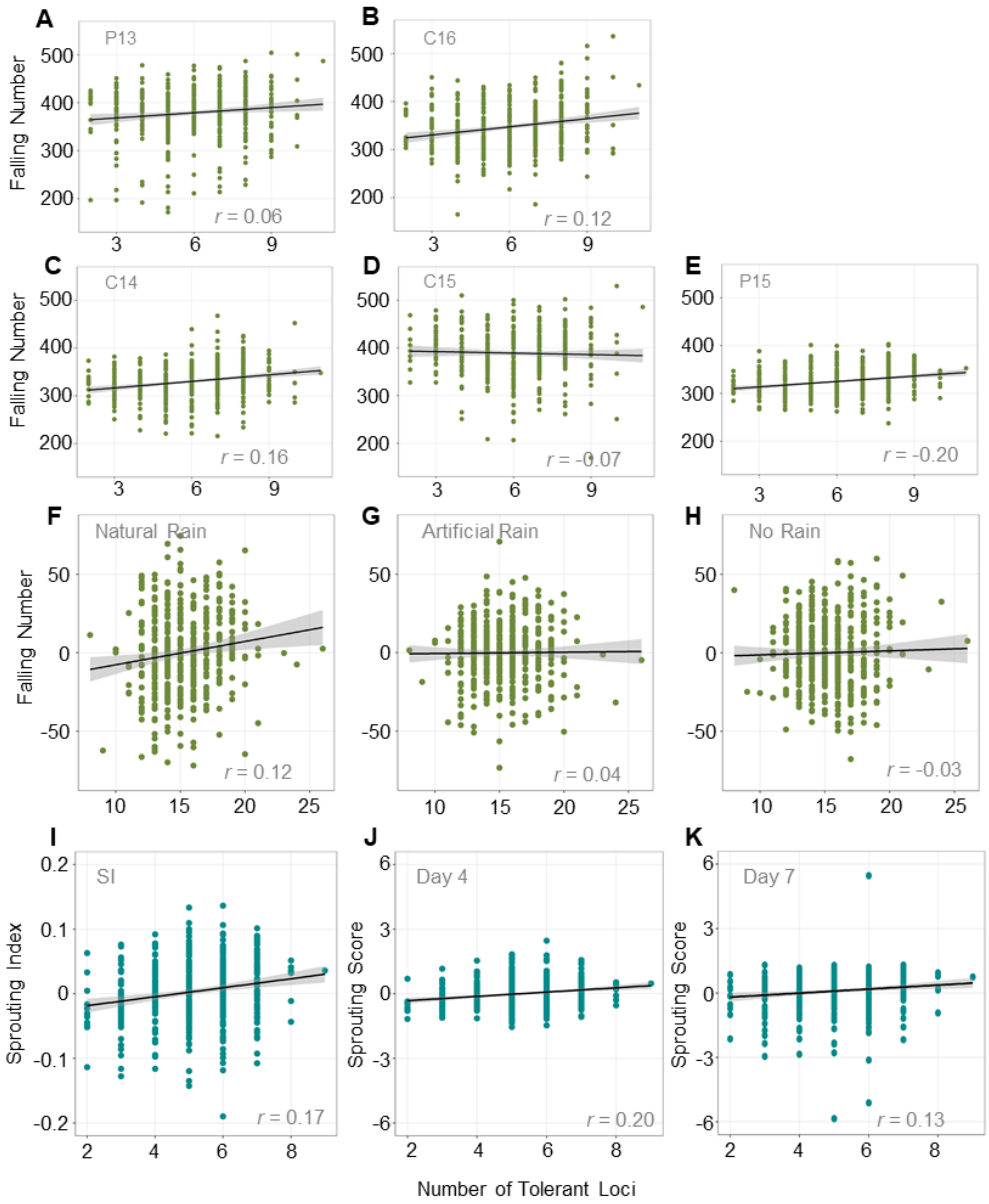


Figure 4 - The effect of pyramiding multiple *QFN.wsu* and *QPHS.wsu* loci. Scatter plots of the number of favorable *QFN.wsu* loci versus FN BLUPs across: (**A**) in the absence of rain, (**B**) both natural rain environments combined, and (**C**) both artificial rain environments combined. Scatter plots of the number of favorable *QPHS.wsu* loci versus BLUPs calculated across all environments for (**D**) sprouting index, and sprouting scores on days (**E**) 3, (**F**) 4, (**G**) 5, (**H**) 6, and (**I**) 7 of misting. *r* is the Pearson correlation coefficient between the trait and number of tolerant loci.



Supplemental Figure 6 - The pyramiding effect of significant *QPHS.wsu* and *QFN.wsu* loci against FN and sprouting scores. Scatter plots of the number of tolerant *QFN.wsu* loci and the FN least squared means across (**A**) Pullman 2013, (**B**) Central Ferry 2016, (**C**) Central Ferry 2014, (**D**) Central Ferry 2015, and (**E**) Pullman 2015. Tolerant *QPHS.wsu* loci were also compared to FN BLUPs across (**F**) the natural rain environments, (**G**) the artificial rain environments, and (**H**) in the absence of rain. Scatter plots of the number of tolerant *QFN.wsu* loci against (**I**) sprouting index BLUPs, (**J**) sprouting score BLUPs after 3 and (**K**) 7 days of misting. *r* is the Pearson correlation coefficient between the trait and number of tolerant loci.

QTN do not always behave in an additive fashion, suggesting that there are epistasis effects. For example, some of the few favorable QTN in Lewjain may have a stronger effect on the phenotype than the unfavorable alleles.

Comparative Mapping for PHS

The location of QTN for FN and sprouting scores were compared to locations of PHS-related loci identified in 54 previous studies (Figure 5). This was done using the comparative map of commonly used wheat markers created by Maccaferri *et al*., (2015). The studies used in the comparison are shown in Supplemental Table 3. Mapped traits that are related to FN and end-use quality included FN, starch content, protein content, and α-amylase activity. Mapped traits related to sprouting and dormancy included germination assays, kernel color, and sprouting scores from spike-wetting tests. There is currently no experimental standard for the spike-wetting test, and methods vary from misting of intact spike to spike immersion to the use of wet sand (McMaster and Derera, 1976; Paterson *et al*., 1989; Humphreys and Noll, 2002; Anderson *et al*., 1993; Rehman Arif *et al*., 2012). Even within each method, the number of days of after-ripening, the duration of spikes wetting, and the day scored vary between studies. Only a few studies score PHS after 4-6 days of misting (Anderson *et al*., 1993; Munkvold *et al*., 2009; Kulwal *et al*., 2012; Somyong *et al*., 2014). This inconsistency across studies led us to ask whether or not our assay could map previously published cloned genes and QTL. Nineteen of the 34 sprouting QTN detected in this study co-localized with known major PHS QTL and cloned genes such as *TaMFT* on chromosome 3A (Figure 5; Nakamura *et al*., 2011). Interestingly, 14 of the 34 sprouting QTN were identified near known FN or quality QTL. Three of 9 FN QTN were identified near known FN and quality QTL, whereas 4 of 9 FN QTN were identified near dormancy or PHS QTL. Of these FN QTN, 2 co-localized with both PHS and FN/quality QTL. A total of 2 QTN for FN and 10 QTN for sprouting score appear to be unique to this study (marked with a star in Table 4).

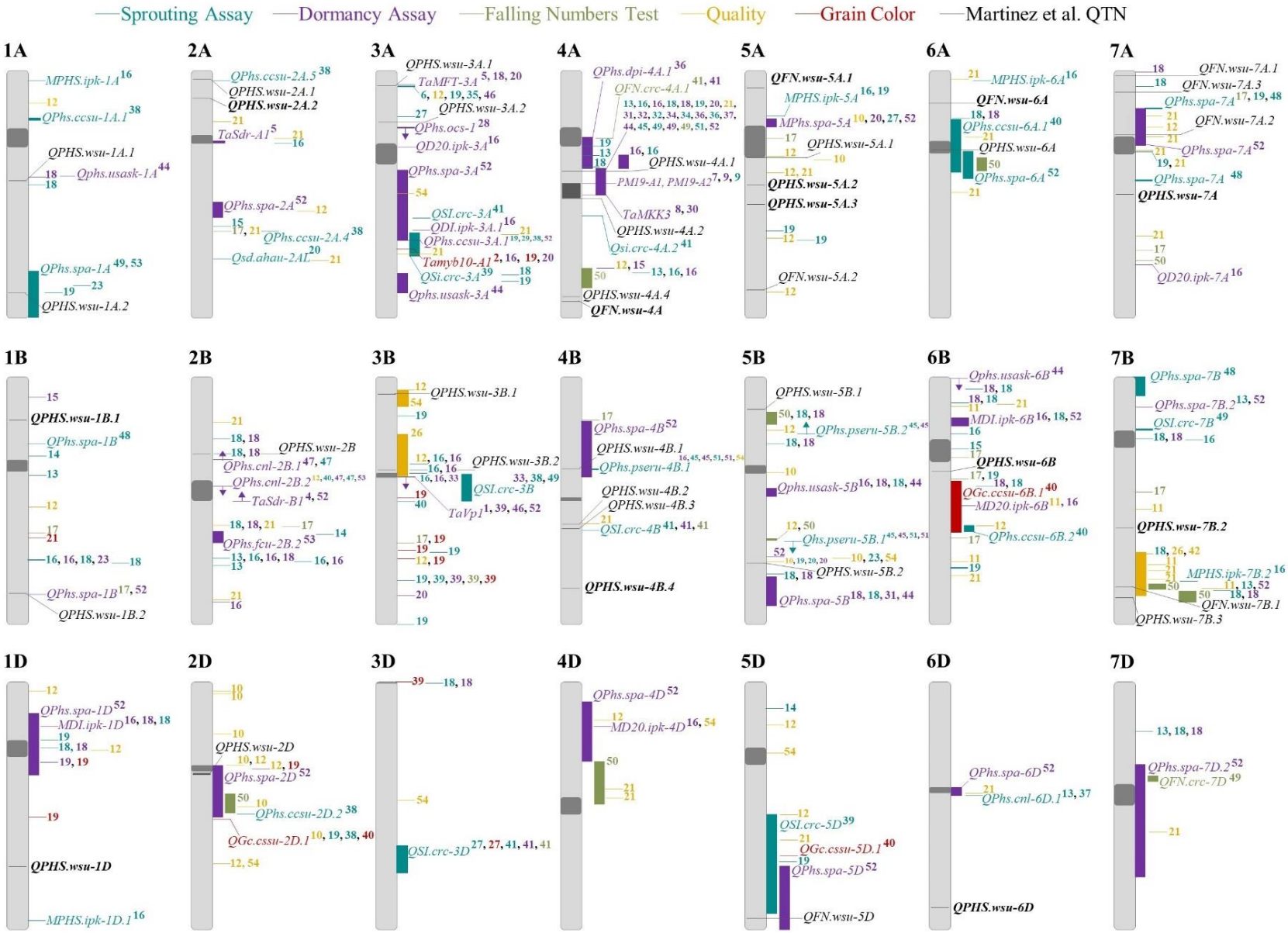

Sprouting Assay
Dormancy Assay
Falling Numbers Test
Quality
Grain Color
Martinez et al. QTN
1A
2A
3A
4A
5A
6A
7A
1B
2B
3B
4B
5B
6B
7B
1D
2D
3D
4D
5D
6D
7D

Figure 5 - Chromosome positions of PHS-related loci. Comparative mapping of quantitative trait nucleotides identified in this study (black), and previously published quantitative trait loci (QTL) for PHS tolerance (blue), dormancy (purple), Falling Number (green), grain color (red), and quality (orange). Potentially novel *QPHS.wsu* or *QFN.wsu* QTN are in bold. Published QTL were aligned to the Maccaferri *et al*. (2015) comparative map using the flanking markers or significant associated markers. Chromosomes are presented as a standardized relative length. An arrow along a QTL indicates the QTL direction when only one flanking marker was found in the comparative map. The centromeric region is a dark grey oval. ID numbers to the right of the QTL correspond to the references found in Supplemental Table 3. Note: Illumina 9k or 90k SNP markers were not reported on chromosome 3D, resulting in an incomplete map.

Supplemental Table 3 - Previously published PHS-related QTL or genes aligned with Martinez *et al*. FN and sprouting QTN on a comparative map created by Maccaferri *et al*. (2015). (Bold parents have increased dormancy (D), PHS tolerance, or increased quality)

ID Number	Gene/QTL/Loci	Name(s)	Trait/Phenotype	Chrm(s)	Parents	Size	Population type	Marker Type	Grain Color	Reference
1	1 (Vp1)	*TaVp1*	embryo D	-	**Minamino**, Tozan	-	-	-	-	Nakamura et al. 2001
2	1 (Myb10)	*yb10-A1, Tamyb10-B1, Tamyb10-*	grain color	3A, 3B, 3D	CS	-	-	-	red/white	Himi et al. 2011
3	1 (MFT)	*TaMFT*	Temp D	3A	SK **N61 CS(Zen3A)** BW **Zen**	-	-	microarray	red/white	Nakamura et al. 2011
4	1 (sdr)	*TaSdr-B1*	D	2B	**Yangxiaomai** x Zhongyou9507	201	RILs	CAPS	white	Zhang et al. 2014
5	1 (sdr)	*TaSdr-A1*	D	2A	**Yangxiaomai** x Zhongyou9507	200	RILs	CAPS	white	Zhang et al. 2017
6	1 (PHS1)	*TaPHS1 (Qphs.pseru-3AS)*	PHS	3A	**Rio Blanco** x NW97S186(S078)	1874	NILs	SSR	red/white	Liu et al. 2014
7	2 (PM19)	*PM19-A1, PM19-A2*	D	4A	Baxter/Chara/Westonia/**Yitpi**	1579	MAGIC	SNP, DaRT	white	Barrero et al. 2015
8	1 (MKK3)	*TaMKK3-A*	D	4A	**Leader**, Haruyokoi	3535	NILs	EST, CAPS, dCAPS	red	Torada et al. 2016
9	2 (PM19)	*PM19-A1, PM19-A2*	D, PHS	4A	**ption** × Claire , **Alchemy** × Robigı	48, 122	DH	SNP, SSR	white?	Shorinola et al. 2017
10	6, 4, 4 markers	-	Kernel Morph, Milling	2D, 5A, 5B	white winter wheat	95	AM	SSR	white?	Breseghello and Sorrells, 2006
11	7, 4 markers	-	LMA	6B, 7B	synthetic wheat	91	AM	DArT	-	Emebiri et al 2010
12	6, 4, 5, 12, 3 markers	-	Quality (TKW, PC, Sed, TW, Sta	all but 2B, 4B, 6A, 6D, 7	european soft winter wheat	207	AM	SSR	white?	Reif et al 2011
13	13 markers	-	PHS	1B, 2B, 2D, 4A, 6D, 7B,	white winter wheat	208	AM	SSR, DArT	white	Kulwal et al. 2012
14	3FDR+7env (20 other	-	PHS	1B, 2A, 2B, 3D, 5B, 5D,	Indian/Exotic common wheat	242	AM	SSR	-	Jaiswal et al. 2012
15	8 sig markers (34 total)	-	D, PHS	1B, 4A, 5D, unk	winter wheat	96	AM	DArT	-	Rehman Arif et al. 2012
16	19	*MTrait.ipk-CH*	PHS, D	14/21	**CS** x Synthetic6x, D Genome lines	85	AM	DArT	-	Lohwasser et al., 2013
17	13 markers	-	FN	1B, 2A, 2B, 3B, 4B, 5A,	hard white wheat	110	AM	SNP	white	Zhang et al 2014
18	22 QTL (40 markers)	-	D, PHS	1A, 1B, 1D, 2B, 3A, 3D,	European winter wheat	124	AM	SSR, DArT	red	Albrecht et al. 2015
19	16	-	PHS, grain color	3A, 3B, 3D, 4A, 7A	US Elite & cultivars	185	AM	SNP	red/white	Lin et al 2016
20	5 (47 loci)	*Qsd.ahau-2AL*	D, PHS	2A	411 × **Wanxianbaimaizi**, Jimai 20	260	AM, RIL	SSR	mix, white	Zhu et al. 2016
21	23 loci	-	Starch (a- b-)	15/21	Chinese wheat	166	AM	SNP	-	Li et al 2017
22	5	*Phst-qtl-gbs###*	D	3B, 3A	US Great Pl. hard winter	1118	AM	GBS	red/white	Moore et al. 2017
23	2	-	D	1A, 1B	Chinese founder parents	80	AM	SSR, SNP, DArT	-	Lin et al. 2017
24	3 (all), 32 (red)	-	D	5D, 3A, 3D	Chinese landraces wheat	717	AM	DaRT, SNP	red/white	Zhou et al 2017
25	3	*cs-4A.1, QPhs.ocs-4B.2, QPhs.oc*	D	4A, 4B, 4D	**AC Domai**n x Haruyutaka	119	DH	SSR	red	Kato et al. 2001
26	3	-	LMA		Cranbrook x **Halberd**	-	DH	-	-	Mares and Mrva 2001
27	7	-	PHS, grain color	3A, 3B, 3D, 5A, 5B, 1B,	**Renan** x Récital	194	RIL	SSR, RFLP, AFLP	red/white	Groos et al.2002
28	2	*QPhs.ocs-1, QPhs.ocs-2*	D	3A	**Zen** x CS	125	RIL	SSR	red	Osa et al. 2003
54				4A					red/white	Mares et al. 2005
29	1	*QPhs.ccsu-3A.1*	PHS	3A	**SPR8198** x HD2329	100	RIL	SSR, AFLP, SAPML	red/white	Kulwal et al. 2005
30	1	-	D	4A	**leader** x haruyokoi	282	BC	SSR	red	Torada et al. 2005
31	5	-	D	4A, 5B	Cascades x **AUS1408**	83	DH	DaRT	white	Tan et al.2006
32	7	*Phs.dpiv-CH.#*	PHS, D, grain color	1D, 2D, 3D, 4A, 6D	**Syn37/2** x Janz	271	BC1	SSR	red/white	Imtiaz et al. 2008
33	1	-	D	3B	QT7475 x **SUN325**	96	DH	SSR	red/white	Rathjen et al. 2008
34	1	-	PHS, D	4A	**Totoumai A** x Siyang 936	162	RIL	SSR	white	Chen et al. 2008
35	3	*ru-3AS, QPhs.pseru-2B.1, QPhs.ps*	PHS	3A, 2B	**RioBlanco** x NW97S186	171	RIL	SSR	white	Liu et al. 2008
36	2	*)Phs.dpivic.4A.1, QPhs.dpivic.4A.*	PHS, D	4A	**CN19055** x Annuello	169	RIL	SSR	white	Ogbonnaya et al.2008
37	9	*QPhs.cnl-CH.#*	PHS, D, heading	2B, 2D, 3D, 6D	**Cayuga** x Caledonia	209	DH	SSR, DArT	white	Munkvold et al. 2009
38	16	*QPhs.ccsu-CH.#*	PHS	1A, 2A, 2D, 3A, 3B	**SPR8198** x HD2329	90	RIL	SSR, AFLP, SAPML	red/white	Mohan et al. 2009
39	5	*QTrait.crc-CH*	PHS, D, FN, Grain color	3A, 3B, 3D, 5D,	**AC Domain** x RL4137	417	DH	SSR	red/white	Fofana et al. 2009
40	6	*QGc.ccsu-CH.#, QPhs.ccsu-CH.#*	PHS, grain color	1A, 2A, 2B, 2D, 3B, 5D,	**PH132** x WL711	100	RIL	SSR, ISSR, RAPD	red/amber	Kumar et al. 2009
41	6	*QTrait.crc-CH*	PHS, D, FN	3A, 3D, 4A, 4B, 7D	RL4452 x **AC Domain**	185	DH	SSR, EST	red/white	Rasul et al. 2009
42	-	-	LMA	7B, 3B	Australian Mexican wheat	39	-	SSR	-	McNeil et al. 2009
43	1	*Vp-1B*	D	3B	Wanxianbaimaizi* x Jing411	157	RILs	-	white (4 red)	Chang et al. 2010
44	7	*Qphs.usask-1CH*	D	1A, 2B, 3A, 4A, 5B, 6B,	**Argent** x W98616	151	DH	SSR, AFLP, DArT	white	Singh et al. 2010
54	10	-	LMA		WW1842 x whistler	188	DH	SSR, DArT	-	Tan et al.2010
45	4	*QPhs.pseru-CH.#*	PHS, D	4A, 4B, 5B	**Totoumai A** x Siyang 936	162	RIL	SSR	white	Liu et al. 2011
46	1, 1+1	*Qsd.ahau-3A, Qsd.ahau-3B*	D	3A, 3B	**izi** x Jing411 , **Wanxianbaimaizi** x	157, 221	RIL	SSR	white	Zhang et al. 2011
16	2 (wheat)	*QTrait.ipk-CH*	PHS, D	3A, 4A	**Opata85** x W7984 , **CS** x Synthetic	114	RIL	DArT	-	Lohwasser et al., 2013
47	2	*QPhs.cnl-2B.1*	PHS, D	2B	**Cauyuga** x Caledonia	1847	NIL	SSR, EST	white	Somyong et al. 2014
48	5	*QPhs.spa-CH*	PHS	1A, 1B, 5B, 7A, 7B	DT707 x **DT696**	122	DH	DArT	-	Singh et al 2014
49	4	*QTrait.crc-CH*	PHS, D, FN	3B, 4A, 7B, 7D	RL4452 x **'AC Domain'**	183	DH	SNP, DArT, EST	red	Cabral et al. 2014
50	16	-	FN	9/21	nx, Bussar x **W332-84**, BAUB4695	143, 68, 82	RIL, DH, DH	SSR	-	Mohler et al. 2014
51	4	*QPhs.pseru-CH*	PHS, D	4A, 4B, 5A, 5B	**Tutoumai A** x Siyang 936	155	RILs	SNP (GBS)	white	Lin et al. 2015
52	23	*QPhs.spa-CH*	D	all	**SC8021-V2** x AC Karma	91	DH	SSR	white	Kumar et al. 2015
53	1	*QPhs.fcu-2B.1, QPhs.fcu-2B.2,*	D	2B	Langdon (Durum) x **PI481521**	150	RIL	SNP, SSR	white	Chao et al. 2015

Discussion

This genome-wide association study was, to our knowledge, the first to map preharvest sprouting loci based both on sprouting scores from the spike-wetting test (*QPHS.wsu*) and FN (*QFN.wsu*). FN is an important and complex trait that determines the value of the grain in the wheat industry. While there were 34 significant *QPHS.wsu* loci across the five different sprouting time points and sprouting index, only nine significant *QFN.wsu* loci were identified. We expected FN and sprouting scores to identify some of the same QTN because the α-amylase expression that lowers FN is a consequence of germination. However, none of the identified significant FN and PHS loci were linked (Table 4). While *QFN.wsu-4A* and *QPHS.wsu-4A.3* appeared to be close to one another (1 cM apart), they were not in the same linkage group (*p* between 0.05-0.01), and had only a -0.13 correlation to one another. Moreover, we failed to find a strong correlation between FN and sprouting scores. This result contrasts with previous studies showing a correlation of up to -0.8 between spike-wetting tests and FN in Canadian and Chilean breeding lines (Rasul *et al*., 2009; Jimenez *et al*., 2016).

The lack of a strong correlation between FN and sprouting score in our study has multiple likely causes: our association panel was larger and sampled more variability for FN related traits; our environments were more variable; or the causes of low FN were not solely due to PHS. Eastern Washington is a semi-arid environment where grain is planted deeply to reach moisture and selection for emergence may have led to early and strong induction of α-amylase to fuel seedling growth. This, in turn, may have resulted in lower FN than would be expected for a given sprouting score. Future work may investigate this by determining if a propensity for low FN is associated with earlier expression of α-amylase during germination. The *QFN.wsu* loci identified, however, did appear to be related to preharvest sprouting because they co-localized with PHS-related loci identified in other studies. Thus, while sprouting scores were not predictive of FN in this study, both traits appeared to be useful for identifying PHS-related loci.

Comparison to Previously Published PHS QTL and Genes

Based on comparative mapping, 12 potentially novel PHS tolerance loci were identified (Figure 3). With the exception of *QPHS.wsu-4B.3*, the QTN identified during early sprouting (3-4 days misted) were near published QTL (Table 4). In contrast, six novel loci were identified during late sprouting (6-7 days misted). Previous PHS mapping studies mostly used spike-wetting test data collected after 4 to 6 days of misting (Anderson *et al*., 1993; Munkvold *et al*., 2009; Kulwal *et al*., 2012; Somyong *et al*., 2014).

Multiple FN QTN were located near published QTL or cloned genes governing PHS-related traits (Figure 5; Supplemental Table 3). Three of the six FN QTN identified in the presence of natural rain were near known preharvest sprouting and dormancy QTL (Fofana *et al*., 2009; Kumar *et al*., 2009; Kulwal *et al*., 2012; Albrecht *et al*., 2015; Kumar *et al*., 2015). Previous work has shown FN samples after a rainfall negatively correlate with dormancy which may be another explanation as to why we see FN QTN near dormancy QTL (Biddulph *et al*., 2008). *QFN.wsu-7A.1* had the largest effect on FN, and was near PHS and dormancy QTN found in a European winter wheat GWAS (Albrecht *et al*., 2015). *QFN.wsu-7B.1* localized in a region containing known QTL mapped based on spike-wetting tests, spike immersion, dormancy, and FN (Kulwal *et al*., 2012; Mohler *et al*., 2014; Kumar *et al*., 2015; Albrecht *et al*., 2015). Given that these QTN were detected after a natural rainfall event, it is curious that they were not detected in any of our spike-wetting test environments. This suggests that either these *QFN.wsu* loci resulted in higher FN due to PHS tolerance, or that the co-localization of these FN QTN with sprouting QTLs was a coincidence. FN is also controlled by grain starch characteristics. Consistent with this, the *QFN.wsu-5A.2* and *QFN.wsu-7A.2* loci identified in the absence of rain were located near starch content and starch granule size QTL (Reif *et al*., 2011; Li *et al*., 2017).

Many *QPHS.wsu* were found near published QTL or cloned genes associated with seed dormancy and PHS tolerance (Figure 5). *QPHS.wsu-2A.1* was located near a preharvest sprouting QTL, *QPhs.ccsu-2A.5*, found in the dormant accession 'SPR8198' (Mohan *et al*., 2009). *QPHS.wsu-2B* was located near the locus providing dormancy and PHS tolerance in the soft white wheat cultivar 'Cayuga' and in a European winter wheat QTL (Somyong *et al*., 2014; Albrecht *et al*., 2015). The *QPHS.wsu-2D* QTN had

the strongest effect, and co-localized with the *QPhs.spa-2D* locus identified in Canadian wheat (Kumar *et al*., 2015). *QPHS.wsu-3A.1* was within 1 cM of the major dormancy and PHS tolerance gene *MOTHER OF FT AND TFL1* (*TaMFT*), identified by map-based cloning in both Japanese and U.S. wheat (Nakamura *et al*., 2011; Liu *et al*., 2013). *QPHS.wsu-4A.1* and *QPHS.wsu-4A.2* are within the *Phs-A1* region associated with dormancy and PHS tolerance in mapping studies world-wide (Mares and Mrva, 2014; Barrero *et al*., 2015; Shorinola *et al*., 2016; Torada *et al*., 2016; Shorinola *et al*., 2017). Polymorphisms in the *MITOGEN-ACTIVATED PROTEIN KINASE KINASE 3* (*TaMKK3-A*) gene likely account for the seed dormancy providing PHS tolerance. Future work will need to examine if the PHS tolerance loci mapped in the current study are associated with the known dormancy-associated polymorphisms in *TaMFT* and *TaMKK3*. If so, then these perfect markers can be used for selecting PHS tolerance within the breeding programs represented in this GWAS. Identifying QTL near regions of known PHS QTL validates the GWAS and suggests that breeding programs in the northwestern U.S. have historically used multiple sources of PHS tolerance.

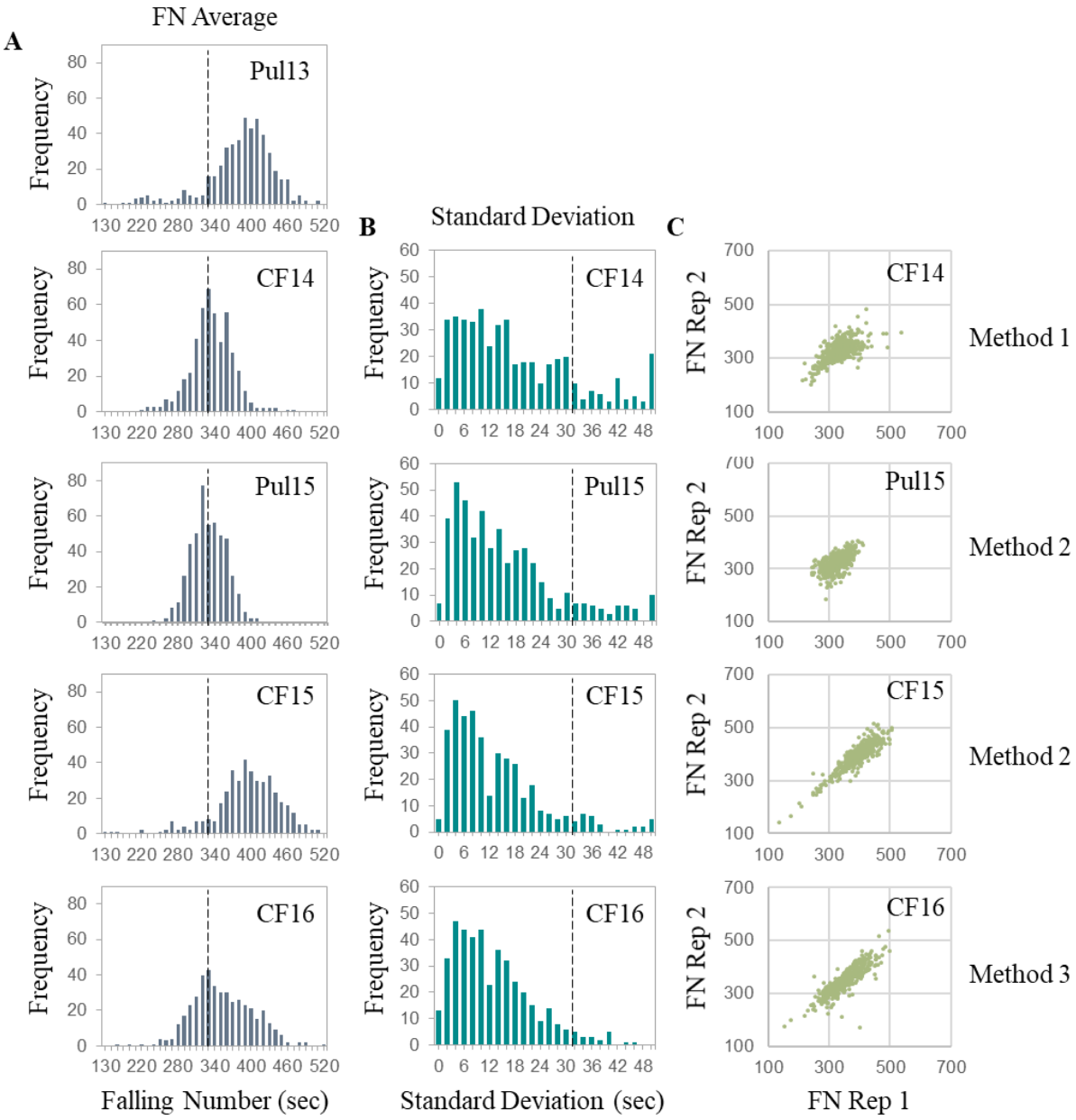
The strong *QPHS.wsu-2D* locus co-localized both with a known PHS locus and with the *C* locus that determines club head type (Supplemental Table 3; Johnson *et al*., 2008). The strong *QPHS.wsu-2D* locus associated with the *C* locus may partly be an artifact because the club wheat breeding program was the dominant source of this PHS tolerance locus in the mapping panel. Since *QPHS.wsu-2D* is less than 1 cM from the *C* locus flanking marker wmc144, this invited the question as to whether the *C* locus itself provided PHS tolerance or whether there was another PHS-tolerance locus in tight linkage with the *C* locus. The latter seemed more likely because previous studies found that the club head type took up more water during rain events and was sometimes associated with higher preharvest sprouting in near-isogenic lines (Hong, 1979; King and Richards, 1984; R.E. Allan, personal comm.). Interestingly, there were three PHS tolerant lax wheat lines that carried the *QPHS.wsu-2D* locus (J950409-10-4, J950409-10-5, and ID581), and there were two PHS susceptible club wheat lines (J970057-5 and ARS00226) that did not carry the *QPHS.wsu-2D* locus. While these counter-examples suggest that there was a PHS QTL strongly linked to the *C* locus, they are not proof because these PHS phenotypes may have resulted from variation

at other loci. Future work will need to examine this question using near-isogenic lines that differ only for the *C* locus and for the *QPHS.wsu-2D* locus.

Breeding for PHS Tolerance Based on Spike-Wetting Tests and FN

Sprouting scores for this population did not correlate strongly with FN (Table 2). While increasing number of *QPHS.wsu* loci correlated to increasing sprouting index and sprouting scores, we observed little or no correlation to increasing FN in natural rain, artificial rain, or no-rain environments (Figure 4; Supplemental Figure 6). Moreover, there was no strong association between increasing *QFN.wsu* loci and sprouting scores. In fact, sprouting scores and FN provided complementary information. It is possible that the tendency towards low FN is dependent more on the timing and strength of α-amylase induction during seed imbibition, then on the timing of visible sprout/germination *per se*. For example, there may be varieties that induce α-amylase earlier in the germination program, prior to germination *per se*. Such varieties would be prone to higher α-amylase/lower FN than expected based on the timing of visible sprout.

Breeding for FN is complicated by the fact that it is a complex trait governed by multiple factors. Although the intention was to map preharvest sprouting QTL, our FN field environments may have also experienced conditions that induced late maturity α-amylase (LMA). During LMA, α-amylase is induced in response to large temperature fluctuations during late grain filling (Farrell and Kettlewell, 2008; Mares and Mrva, 2014). While four of the five environments experienced either natural or artificial rain events, we cannot rule out the possibility that the wheat also experienced LMA. Indeed, the *QFN.wsu-7B.1* locus co-localized with a large LMA QTL (Figure 5; Mrva and Mares, 2001; McNeil *et al*., 2009; Emebiri *et al*., 2010). In the environment without rain, differences in FN likely resulted from differences in properties of grain starch and protein (Ross *et al*., 2012; Graybosch *et al*., 2000; Guo *et al*., 2003). Such properties likely also impact FN over 300 sec when there is a rain event. The FN test has a fairly high standard deviation within technical replicates (Supplemental Figure 7). The genotypic repeatability (R^2) of FN increased when we took experimental covariates (such as machine and operator) and technical



Supplemental Figure 7 - FN technical replicate reproducibility. (**A**) Histograms of FN across environments. The dotted line indicates 330 seconds, approximately the FN when we see α–amylase activity. (**B**) Histograms of the standard deviation per technical replicate across environments. The dotted line indicates 30 seconds, the expected standard deviation from the Hagberg-Perten Falling Numbers test. (**C**) Technical replicate 1 versus replicate 2 across the environments. Replicates 1 and 2 were tested on different days (method 1), within 5 minutes of each other (method 2), or side-by-side (method 3). Pullman 2013 had only 1 technical replicate.

replicates into account (Table 3). The FN test has other limitations for breeding such as the need for an expensive instrument, and the fact that it is more time-consuming to run FN than spike-wetting tests. Future research should examine whether α-amylase enzyme assays (Phadebas™ or Megazyme) or ELISA assays may serve as a faster, cheaper, or less variable proxy to FN (Mares and Mrva, 2008; Barrero *et al*., 2013). Environmental factors also caused variation in FN, resulting in only moderate correlations between environments in the current study (Table 2A). While the Zhang *et al*. (2014) study had higher correlations between FN in different environments ($0.43 > r > 0.80$), it had fewer samples below 300 sec suggesting that less sprouting occurred in their environments. Breeders cannot rely on natural rain occurring when they want to screen for low FN due to PHS, making the use of artificial rain necessary. Other differences in the environment, such as temperature during maturation or temperature during the sprout-inducing rainfall, can impact grain dormancy, PHS susceptibility, and FN. Future artificial rain experiments may be improved by applying the artificial rain during lower evening temperatures or letting the wheat after-ripen longer in the field prior to misting.

The spike-wetting test has long been favored for selecting preharvest sprouting tolerance because the experimental design takes into account after-ripening time, spike morphology, and grain dormancy/germinability (Paterson *et al*., 1989). A limitation of FN testing of field-harvested grain is that differences in maturation date can be a major covariate, since early maturing varieties may have lost more dormancy through after-ripening than late maturing varieties. The spike-wetting test reduces this problem by harvesting spikes at physiological maturity and then after-ripening for the same number of days before conducting the test. When screening large numbers of breeding lines, it would be convenient to avoid scoring daily over 3 to 7 days of misting. Based on the correlations between spike-wetting test and all FN environments (with and without rain), scoring after 4 days of misting should provide breeders with both adequate variation and higher correlation to FN (Table 2B). Scoring after 4 days misting also provides good insight into initial germination capacity (scores 1-5) rather than speed of seedling growth (scores 6-10) (Figure 1). However, it should be noted that the day 6 sprouting score had the highest genotypic repeatability in this study. One drawback of selecting for PHS tolerance based on seed dormancy, is that

too much dormancy may result in poor seedling emergence of winter wheat when grain is planted approximately eight weeks after harvest (Rodríguez *et al*., 2015). Future work will need to develop a genomic selection model for breeding wheat with sufficient seed dormancy to prevent preharvest sprouting without compromising seedling emergence.

Seed dormancy and PHS tolerance are stronger if temperatures are cool during grain maturation (Nakamura *et al*., 2011). One could remove temperature during grain development as a variable in spike-wetting tests by growing plants in a controlled environment instead of in the field. We might have also seen better correlations if spike-wetting tests had been performed for the entire trial in the two years with natural rain events. However, when spike-wetting tests were performed on 162 accessions in 2013, only a -0.26 correlation was observed. A more likely explanation is that FN in these environments was impacted by multiple factors in addition to preharvest sprouting, including LMA, starch, and protein characteristics. Thus, within this study, FN and spike-wetting tests were not two ways to measure the same trait.

PHS tolerance is profoundly impacted by environmental conditions during grain maturation and during the sprout-inducing rain event (Cao *et al*., 2016; Martinez *et al*., 2016; Kashiwakura *et al*., 2016). Thus, it is not uncommon to find lack of agreement between environments in PHS tolerance association studies based on spike-wetting tests (Kulwal *et al*., 2012; Jaiswal *et al*., 2012; Ogbonnaya *et al*., 2008; Zhou *et al*., 2017). A total of 6 out of 34 *QPHS.wsu* were identified in at least two of the five environments. In fact, the correlations between our environments were as good as those in other spike-wetting test studies (Kulwal *et al*. 2012; Jaiswal *et al*., 2012). The fact that this study identified some QTN in multiple environments and that many of the QTN identified agreed with previous studies, suggests that this association study will lay a strong foundation for future efforts to develop genomic selection for PHS tolerance in northwestern U.S. wheat.

Author Contributions

SM, KGC, and CMS designed the experiments. SM conducted the experiments, analyzed the phenotypic data, performed the statistical analysis and GWAS, and constructed the visualizations. JG curated and

filtered the genotypic data and performed the LD analysis. MH calculated the phenotypic variation. MH and ZZ provided expert guidance on performing the GWAS. KGC and AC provided field resources. CMS, KGC, AC, and ZZ obtained funding. SM and CMS wrote the manuscript.

Funding

This work was funded by the NIFA projects 2015-05798 and 2016-68004-24770, the USDA-ARS project 424575, and the WA Grain Commission projects 7599, 5389, and 126593.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

The authors wish to thank Rehana Parveen, Tracy Harris, Patricia DeMacon, Gary Shelton, and Xavian Thompson for expert technical assistance. The authors sincerely thank Dan Dreesman for constructing the greenhouse misting system. Thanks are due to members of the Garland Campbell, Carter, and Steber labs for assistance with planting, sampling, and harvesting. Special thanks go to the Garland Campbell and Steber labs for helpful feedback on the research and manuscript.

Supplemental Materials

Supplemental Table 1 - Germplasm names and information

Supplemental Table 2 – Spike-wetting test environment correlations

Supplemental Table 3 - Comparative map that includes PHS-related QTL or genes

Supplemental Table 4 - Significant loci associated with low FN and low sprouting

Supplemental Table 5 - Significant loci associated with Heading Date

Supplemental Table 6 - Significant loci associated with PHS without principal components

Supplemental Figure 1 - Mapping panel principal components

Supplemental Figure 2 - Phenotypic distributions of FN and spike-wetting test scores used in association mapping

Supplemental Figure 3 - Association mapping model comparisons

Supplemental Figure 4 - Q-Q plots of all PHS traits and environments

Supplemental Figure 5 - Venn diagrams of alternate GWAS versus the original GWAS

Supplemental Figure 6 - Favorable loci compared to FN and spike-wetting test

Supplemental Figure 7 - Falling Number technical replicate reproducibility

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CHAPTER THREE: Mapping the EMS-Induced ABA Hypersensitive *ERA8* Mutant using QTL Analysis and Bulk-Segregant Analysis-Exome-sequencing (BSA-Exome-seq)

(To be submitted as a research paper)

Abstract

Preharvest sprouting (PHS) is the germination of mature wheat grain on the mother plant when cool and wet conditions occur before harvest. PHS causes severe losses for wheat growers. Lack of seed dormancy accounts for 60-80 % of PHS susceptibility. *ERA8* is an EMS mutation that was selected for increased ABA hormone sensitivity, resulting in increased seed dormancy and PHS tolerance. This allele is a new source of wheat grain dormancy sufficient to protect white wheat from preharvest sprouting. The *ERA8* gene was mapped in a backcross population, Zak/Zak*ERA8*, relative to mutagen-induced sequence polymorphisms in coding regions of the wheat genome. This is the first example of mapping a gene in hexaploid wheat using bulked-segregant analysis of wild-type-like and mutant-like genomic DNA that was enriched for the coding region by exome-capture before genome resequencing. This approach localized *ERA8* to a large region of chromosome 4A spanning the centromere. Fine-mapping was performed by QTL analysis of a larger backcross population genotyped for mutagen-induced SNPs. The *ERA8* phenotype was strongly associated with *TaMKK3-A*, a gene previously identified as a source of natural variation for PHS tolerance in barley and wheat on the long arm of chromosome 4A. The *ERA8*-linked polymorphism, *TaMKK3-A-G1093A*, was not strongly linked to the ABA hypersensitive phenotype in a recombinant inbred line population, Louise/Zak*ERA8*. This is interesting because, while Louise showed less ABA sensitivity than Zak*ERA8*, it did carry that natural dormant/PHS tolerant allele *TaMKK3-A-A660*^{R}. It is possible *ERA8* could not be mapped in this population because both parents carried dormant alleles of *MKK3*, such that the only variation in ABA sensitivity resulted from segregation at other genes. This classic QTL analysis of an RIL population points out the main weakness in mapping a gene in a RIL population; researchers are blind to the exact sequence polymorphisms causing the phenotype until map-based cloning is nearly complete. The *ERA8* gene was predicted to be

within a 5 million base pair region containing 70 genes. None of these genes shows the differential regulation that would be expected of a promoter mutation, and *MKK3* is the only gene in the region that carries a mutagen-induced sequence polymorphism. Thus, the current model is that the *ERA8* phenotype results from the *TaMKK3-A-G1093A* mutation

Keywords: *Abscisic Acid, After-ripening, Seed Dormancy, Wheat, Bulk-Segregant Analysis, 4A*

Key Message: **Bulk segregant analysis exome sequencing of a backcross population resulted in a rapid method for fine mapping an EMS induced ABA hypersensitive mutant, *ERA8*.**

Figures - 23

Table - 15

Abstract Word Count - 367

Body Word Count - 10,900

Introduction

Preharvest sprouting (PHS) is the germination of mature seeds on the mother plant before harvest under rainy or humid conditions (reviewed by Rodriguez *et al*., 2015). The wheat seed or grain is, strictly speaking, a caryopsis or berry due to the presence of a pericarp outside of the seed coat. Lack of grain dormancy accounts for 60-80 % of the variation for preharvest sprouting susceptibility in wheat (*Triticum aestivum* L.) (McCaig and DePauw, 1991). Grain dormancy refers to the inability to germinate even under favorable environmental conditions (reviewed in Bewley *et al*., 2013). Grain is most dormant at physiological maturity and then loses dormancy through: 1) a period of dry storage called after-ripening, 2) imbibition in the cold (called cold stratification), or 3) scarification of the seed coat (Paterson *et al*., 1989; Gerjets *et al*., 2010). Seed coat-imposed dormancy is relieved by cutting or scarifying the seed coat, whereas embryo dormancy is not relieved by cutting the seed coat. Wheat can have both forms of seed dormancy (Schramm *et al*., 2010). A seed is considered germinated when the radical or any other part of the embryo emerges from the seed coat (reviewed in Bewley *et al*., 2013; Mares and Mrva, 2014). When seed germination is initiated, hydrolytic enzymes are induced to mobilize stored reserves for use by the growing seedling. Induction of hydrolytic enzymes such as alpha-amylase cause starch degradation when flour is made into dough, even in mild cases of PHS. This results in flour that produces poor quality end-use products such as bread, cakes, and noodles. While higher seed dormancy is beneficial for preventing preharvest sprouting, it has the drawback that too much dormancy at the time of planting causes problems with uneven or poor seedling emergence (reviewed by Rodriguez *et al*., 2015). Thus, it is important to understand the genetic control of wheat seed dormancy and germination so that we can develop varieties that have sufficient seed dormancy to prevent PHS, but not so much as to interfere with synchronous emergence.

Multiple environmental and genetic factors control the degree of seed dormancy. In wheat, seed dormancy is higher if the environment is cool and dry during embryo maturation, and lower if conditions are hot during embryo maturation (Nakamura *et al*., 2011; Mares and Mrva, 2014). Plant hormone

signaling controls seed dormancy and germination (reviewed in Finkelstein *et al*., 2008). The hormone abscisic acid (ABA), induces seed dormancy during embryo maturation, and the hormone gibberellin (GA) stimulates seed germination. ABA establishes seed dormancy during embryo maturation, maintains dormancy in mature seeds, and inhibits the germination of mature seeds when exogenously applied. Thus, increased ABA signaling or decreased GA signaling is associated with higher seed dormancy. Dormancy is also higher in wheat varieties with red grain coat color than white (Himi *et al*., 2002, 2011). While red kernel color is associated with higher ABA sensitivity, ABA and grain coat color can act independently and in concert to increase wheat grain dormancy (Himi *et al*., 2002). Mutant studies have shown that loss of ABA sensitivity can decrease seed dormancy in red wheat and that increased ABA sensitivity can increase seed dormancy in both red and white wheat varieties (Flintham 2000; Torada and Amano, 2002; Schramm *et al*., 2010, 2012, 2013). Since white wheat varieties have lower seed dormancy and PHS tolerance, we have identified ethyl methanesulfonate (EMS)-induced genetic variants with increased ABA sensitivity as a method to improve preharvest sprouting tolerance in white wheat.

The EMS-induced semi-dominant *ERA8* (Enhanced Response to ABA8) mutation in a soft white wheat called 'Zak' (Kidwell *et al*., 2002; Schramm *et al*., 2013). The ERA mutants, Zak*ERA8*, *ERA19A*, and *ERA19B* were identified by screening for increased sensitivity to 5µM ABA compared to Zak wild-type. The Zak*ERA8* line was selected for closer study and mapping because it had strong ABA sensitivity and seed dormancy that was lost rapidly through after-ripening (Schramm *et al*., 2013; Martinez *et al*., 2016). Moreover, the ABA hypersensitive Zak*ERA8* showed higher PHS tolerance in spike-wetting tests (Martinez *et al*., 2016). Thus, mapping of an *ERA8*-linked SNP would make it easier to deploy *ERA8* as a source of PHS tolerance in white or red wheat. *ERA8* maintained increased dormancy and ABA sensitivity at 15 to 20 °C but lost dormancy at 10 °C, suggesting that the *ERA8* allele should reduce sprouting under moderately low field temperatures. ABA signaling can be controlled both at the level of hormone accumulation and at the level of the ABA sensitivity. Endogenous embryo ABA measurements showed that ABA content decreased similarly in *ERA8* and Zak WT after-ripening, and suggested that the

phenotype resulted from increased ABA signaling rather than increased ABA content. Thus, our working hypothesis is that the *ERA8* allele is a gain-of-function mutation in a positive regulator of ABA signaling.

Quantitative trait loci (QTL) for PHS and dormancy have been identified throughout the allohexaploid wheat genome. Major QTLs for PHS tolerance have been found on chromosomes 2B, 3A, 3D, 4A, 6D, and 7B (Zanetti *et al*., 2000; Ogbonnaya *et al*., 2008; Munkvold *et al*., 2009; Jaiswal *et al*., 2012; Kulwal *et al*., 2012; Zhou *et al*., 2017; Martinez *et al*., 2018a). While homologues of ABA signaling genes have been found on wheat chromosomes 3A, 4A, and 5A, only *Viviparous-1*/*ABA-insensitive3* (*VP1/ABI3*) has been linked to differences in wheat preharvest sprouting tolerance (Nakamura *et al*., 2001; McKibbin *et al*., 2002; Nakamura *et al*., 2007). To date, 17 dormancy and 13 PHS studies have identified the QTL on the long arm of chromosome 4A referred to as the *Phs1* locus (Liu *et al*., 2008; Liu *et al* 2014; reviewed in Mares and Mrva, 2014; Martinez *et al*., 2018a). The majority of these 4A QTL co-localize with the cloned preharvest sprouting *Triticum aestivum* gene now called *Mitogen-Activated protein Kinase Kinase 3* (*TaMKK3-A*) (Nakamura *et al*., 2016; Torada *et al*., 2016; Shorinola *et al*., 2017). Another major preharvest sprouting tolerance QTL on chromosome 3A was cloned from Japanese and U.S. germplasm, the wheat gene *MOTHER OF FLOWERING TIME AND TFL1* (Ta*MFT1*) (Nakamura *et al*., 2011; Liu *et al*., 2013). The *ERA8* gene may co-localize near one of these loci and/or it may be a homologue of ABA signaling genes identified in other plant species. Mapping *ERA8* will be the first step towards answering that question.

Fine mapping and map-based cloning of wheat genes is challenging due to the very large size (~17 Gb) and high redundancy of the allohexaploid bread wheat or common wheat genome (*Triticum aestivum*) (Yan *et al*., 2003; Gupta *et al*., 2008; Shewry, 2009). The large size of the wheat genome is due to the fact that it contains about 80 % noncoding DNA, and to the fact that it is derived from three diploid progenitors that each contributed seven homoeologous chromosomes referred to as the A, B, and D genomes (2n = 6x = 42, genomes AABBDD). The 21 chromosomes are numbered 1 through 7, followed by the genome designation (ex. 1A, 1B, 1D, etc.). The A genome donor is believed to be *Triticum uratu* (A^uA^u), a relative of the ancient diploid crop plant einkorn *Triticum monococcum* (A^mA^m)

(Gill and Jiang, 1994; Huang *et al*., 2002; Gu *et al*., 2004; Feldman and Levy, 2012). The B genome donor is an unknown relative of *Aegilops speltoides* (SS). The allotetraploid progenitor of wheat is *Triticum turgidum* (AABB) or durum wheat that is grown for making pasta. Finally, the D genome donor is *Aegilops tauschii* (DD). While the three genomes are highly similar and carry many of the same genes, during meiosis the A genome only pairs with the A genome, the B with the B, and the D with the D.

The hexaploid wheat reference genome sequence of Chinese Spring wheat has enabled the application of high-throughput sequencing methods to mapping and cloning strategies in wheat (IWGSC RefSeq v1.0, http://www.wheatgenome.org). Even so, the very large size of the wheat genome is still a challenge when using high-throughput sequencing methods. Several methods have been developing to enrich for relevant region of the wheat genome when resequencing. When mapping is performed using the genotyping-by-sequencing (GBS) method, restriction enzyme digests of genomic DNA are used to generate the library for next generation sequencing, thereby enriching for sequences that begin at the same positions (Poland *et al*., 2012; Truong *et al*., 2012). Bulked segregant analysis (BSA) coupled with next generation sequencing is a powerful approach to clone by sequencing, in which pooled-DNA samples from plants with contrasting phenotypes are sequenced to identify SNPs co-segregating with the trait of interest (Michelmore *et al*. 1991). In organisms with smaller genomes, it is now common to clone mutagen-induced mutations using BSA contrasting whole genome DNA sequence of bulked wild-type and mutant backcross populations (reviewed in Thole and Strader, 2015). However, the wheat genome is too large for this approach. Wheat genes have been cloned by BSA, however, using RNAseq instead of DNAseq to enrich for the expressed portion of the genome (Liu *et al*., 2012; Trick *et al*., 2012; Ramirez-Gonzalez *et al*., 2015a). This approach, however, has the drawback that different genes are expressed at different levels, leading to the possibility that the gene of interest might be underrepresented in the RNAseq experiment. Exome capture has recently been developed as a method to affinity-purify the known coding regions of wheat before performing whole exome resequencing (Allen *et al*., 2013; Henry *et al*., 2014; Mo *et al*., 2017; Uauy *et al*., 2017). This approach has been used to characterize the genome-sequence of EMS-mutagenized TILLING populations. Since this method was effective in identifying

EMS-induced mutations, this study used exome capture in a BSA-exome sequencing approach to map EMS-induced mutations linked to the *ERA8* allele.

This study first identified multiple QTL, including one on chromosome 4A, contributing to ABA hypersensitivity in a traditional RIL population. *ERA8* was again localized to chromosome 4A in a backcross population using bulk-segregant analysis to map relative to EMS-generated polymorphisms characterized by high throughput DNA sequencing of exome genomic DNA. Fine-mapping with a larger backcross population was used to co-localize *ERA8* with mutations in and near the *TaMKK3-A* gene that was previously cloned as a gene regulating wheat preharvest sprouting tolerance. This information is being used to introgress *ERA8* into soft white winter wheat to improve PHS tolerance by increasing ABA sensitivity.

Materials and Methods

Plant material

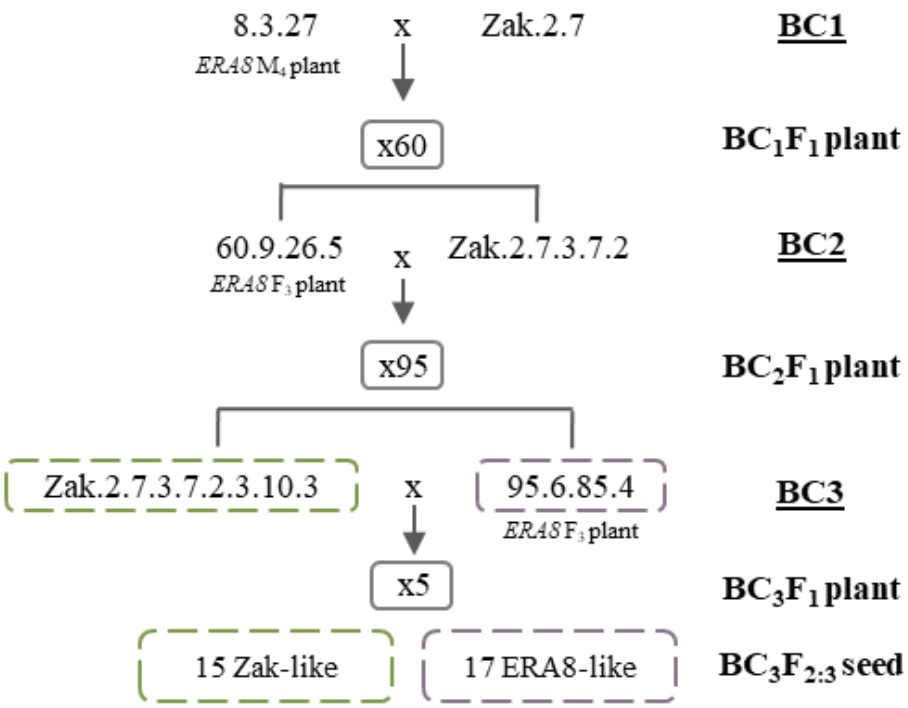
Allohexaploid wheat (*Triticum aestivum* L.) soft white spring wheat cultivars were used in this study. 'Louise' (PI 634865) was selected from the cross 'Wakanz' (PI 506352)/ 'Wawawai' (PI 574538) (Kidwell *et al*., 2006). 'Zak*ERA8*' (PI 669443) is derived from the cultivar 'Zak' (PI 612956) via the ethyl methanesulfonate (EMS)-induced *ERA8* mutation resulting in enhanced response to ABA during seed germination (Kidwell *et al*., 2002; Schramm *et al*., 2013; Martinez *et al*., 2014). A recombinant inbred line (RIL) population was derived from F_2 seeds from ten independent F_1 plants from the cross of Zak*ERA8* to Louise, with Louise as the female parent. The population was developed without selection through single seed descent of 225 recombinant inbred lines (RIL) from the F_2 generation to the F_5 generation. During each generation, plants were grown in a controlled greenhouse environment under a 16 h photoperiod and 15-17 °C for night cycle and 21-23 °C for day. During the F_5 generation, single plants were grown in 3 L pots in the Plant Growth Facility at Washington State University, Pullman, WA to increase seed. F_6 seeds were harvested at physiological maturity (environment 1 or E1). Thirty F_6

seeds from E1 were planted in headrows at the Washington State University Spillman Research Farm, Pullman, WA in 2014 and 2015 to produce F_7 grain from the E2 and E3 environments, respectively.

A series of backcross (BC) populations were derived by crossing the ABA hypersensitive Zak*ERA8* to the mutagenesis parent, soft white spring wheat Zak (Schramm *et al*., 2013). In the first backcross (BC_1F_1; X60.9), Zak wild-type (Zak isolate 2.7) was crossed onto a Zak*ERA8* M_4 plant (isolate 0.3.27), with Zak*ERA8* as the female parent (Supplemental Figure 1). An *ERA8*/*ERA8* homozygous BC_1F_3 (60.9.26.5) was obtained by single plant selection for ABA hypersensitive germination, and then backcrossed (BC_2F_1; X95) to Zak wild-type (Zak 2.7.3.7.2), with Zak*ERA8* as the female parent (Martinez *et al*., 2014). Again, a homozygous *ERA8/ERA8* BC_2F_3 individual was selected (X95.6.85.4) and backcrossed (BC_3F_1; X5) to Zak wild-type (Zak 2.7.3.7.2.3.10.3), with Zak as the female parent. A single F_1 seed (TH X5.2) was advanced. The ABA hypersensitive phenotype was examined by plating at both the BC_3F_2 to the F_3 generations. Genomic DNA was prepared from leaf tissue of the BC_3F_3 generation, and used for bulk-segregant analysis. Additional $BC_3F_{1:3}$ seeds (X5.1 and X5.2) were phenotyped and used for fine mapping.

Genotyping by Sequencing

Genomic DNA was extracted from F_2:F_5 leaf tissue from the Louise/Zak*ERA8* RILs on the Qiagen BioSprint-96. Genotyping-by-sequencing (GBS) was performed on a Thermo-Fisher Scientific Ion-Proton system (Poland *et al*., 2012; Truong *et al*., 2012). Briefly, to construct GBS libraries, DNA was digested with PstI and MspI, then ligated to barcode adapters using T4 DNA ligase. Samples were purified, pooled, and sequenced. The USDA-ARS Western Regional Small Grains Genotyping Lab SNP marker pipeline was used to analyze the sequencing reads. This pipeline used built-in Linux commands and routines written in the Perl5 programming language (http://www.perl.org) to remove extraneous bases 5' to the PstI site, trim the sequence reads to uniform length "tags" of 70 or 100 bases, then identify tags unique to each of the parent lines. These tags were aligned to the RefSeqv1.0 wheat genome (IWGSC RefSeq v1.0, http://www.wheatgenome.org/) using SOAP2 allowing 0-2 mismatches (Li *et al*., 2009b).

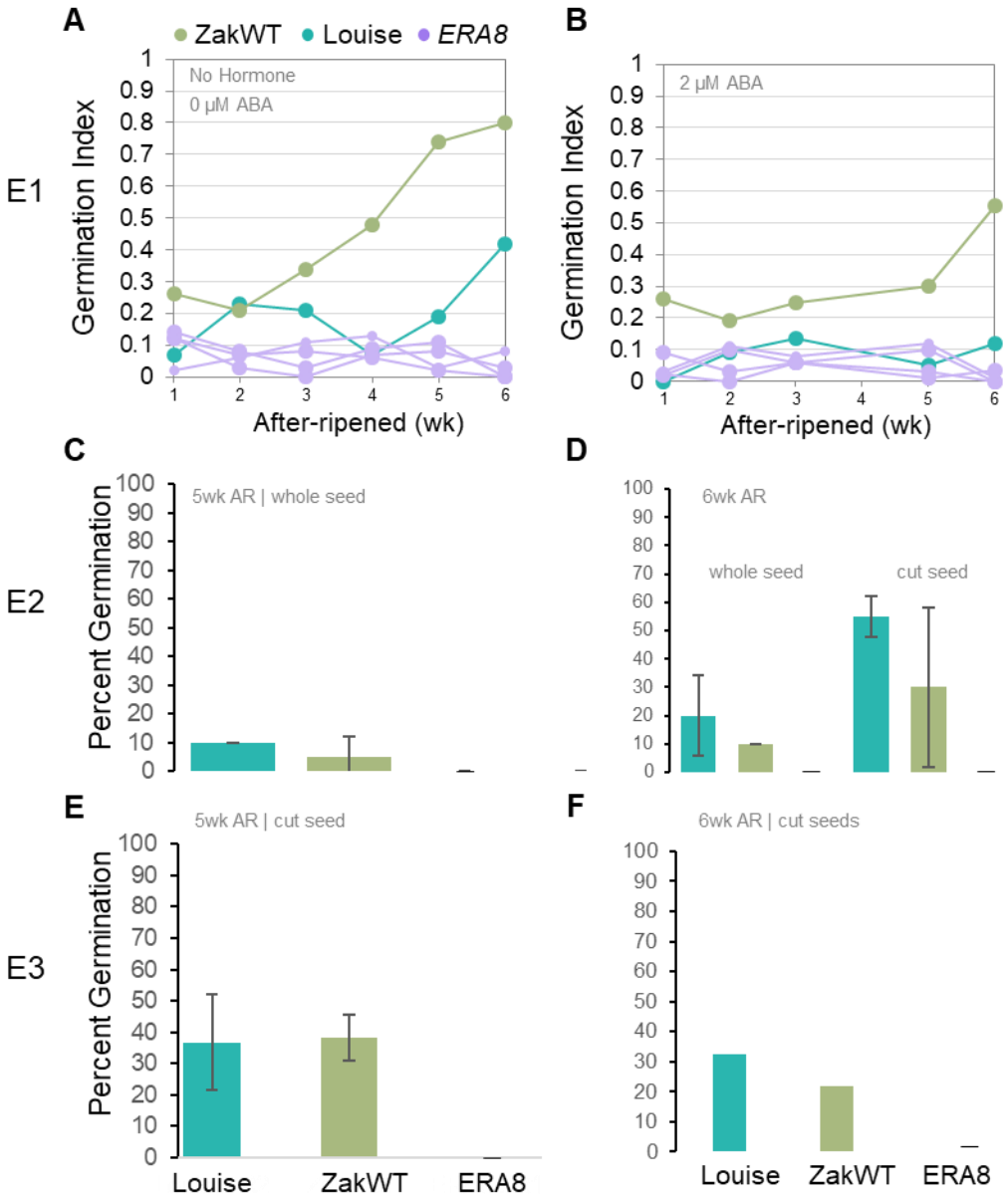


Supplemental Figure 1 – Crossing strategy for the Zak/Zak*ERA8* backcross (BC) population used in the bulk segregant analysis. The Zak WT parent (Zak2.7.3.7.2.3.10.3), the *ERA8* Parent (95.6.85.4), *ERA8*-like bulk, and Zak-like bulk samples indicated by the dashed green and purple boxes were used in the exome resequencing.

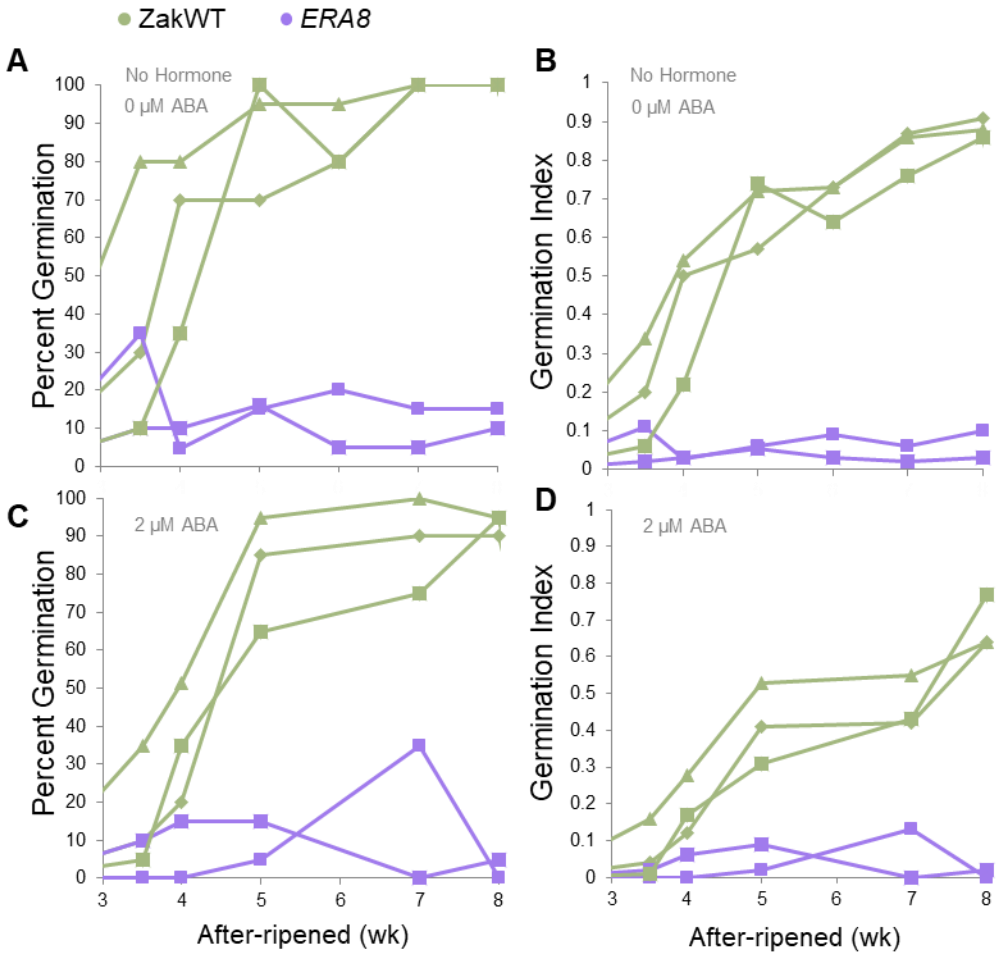
Tags that aligned to two or more chromosomes were discarded, leaving only tags that aligned to 0 (unknown) or 1 chromosome. A Perl5 routine was then used to identify pairs of these filtered tags that differed by one or two SNPs between the parents, Louise and Zak*ERA8*, possibly representing alternate alleles. Linux built-in commands were then used to score the presence of those markers in the progeny population and a Perl5 routine was used to convert the presence/absence data to genotype calls. Markers that were scored as missing (neither allele present) in more than 75 % of the progeny were removed from the data set. An iterative imputation of missing data was calculated using the 'missforest' package in R (Stekhoven and Bühlmann, 2012). If a marker appeared to be heterozygous for 21 or more of the 207 RILs, then that marker was excluded from analysis to reduce false positive results.

Germination assays

Plating assays were used to compare ABA sensitivity during seed germination using experimentally determined conditions that best differentiated between wild type and *ERA8*/*ERA8* parents for each population or after-ripening time point (Supplemental Figure 2 and 3). Seeds were harvested at physiological maturity, hand threshed, then dry after-ripened at room temperature and humidity (15-30 %) for 6-7 weeks (as in Martinez *et al*., 2016). Population parents were plated on varying concentrations of ABA weekly from 4-8 weeks to determine the after-ripening time point and ABA concentration with the optimal difference between parents (Supplemental Figure 2 and 3). Seeds were stored at -20 °C until the optimal dry after-ripening time point was selected. They were then after-ripened and plated on ABA as indicated (Tables 1; Supplemental Table 1). Note that the fine-mapping using a larger population of X5 BC_3F_3 grain was performed using seeds that were stored for longer periods at -20 °C. Seeds (30-90) were sterilized in a 50 mL tube with 20 mL of 10 % bleach/ 0.01 % SDS for 15 min, then rinsed 3x with 40 mL of sterile deionized water. Thirty seeds were plated on 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 2 µM, 5 µM, or 10 µM (+/-)-ABA (PhytoTechnology Laboratories). Germination was scored daily over 5 days of incubation at 30 °C



Supplemental Figure 2 – An after-ripening time course of the Louise (blue), *ERA8* (purple), and ZakWT (green) parents from environment 1 (greenhouse grown in 2013; **A**, **B**), environment 2 (field grown in 2014; **C**, **D**), and environment 3 (field grown in 2015; **E**, **F**). Germination assays were conducted with 2 µM ABA after 5 days of imbibition, except **A** was conducted with 0 µM ABA. Percent germination (**C**, **E, D**, **F**) after 5 days of imbibition at 30°C and germination index (**A**, **B**) were calculated from 30 whole seed germination assays.



Supplemental Figure 3 - An after-ripening time course of the *ERA8* (purple) and ZakWT (green) parents from the Zak/Zak*ERA8* was conducted without ABA (No Hormone; **A**, **B**) and with 2 µM ABA (**C**, **D**). Percent germination (**A**, **C**) after 5 days of imbibition at 30 °C and germination index (**B**, **D**) were calculated from 20 whole seed germination assays.

Supplemental Table 1 - ABA sensitivity for the Zak/Zak*ERA8* $BC_3F_{2:3}$ population across different seed lots.

Location	n [a]	b rep [b]	ABA (µM)	AR (days)	Imb. [c] (days)	Mean ± SD [d] PG	Mean ± SD [d] GI
5.2 BSA	15	3	2	35	5	12.6 ± 8.5	-
	17	3	2	35	5	79.9 ± 13	-
	ERA8	16	2	35	5	8.3 ± 7.2	-
	Zak	16	2	35	5	90.8 ± 15	-
5.2 Group 1	242	2	2	35	5	42.6 ± 21	0.21 ± 0.1
	ERA8	8	2	35	5	8.3 ± 4.3	0.03 ± 0.0
	Zak	8	2	35	5	90.8 ± 12	0.55 ± 0.0
5.1	122	1	10	42, 59	4	43.2 ± 25	0.29 ± 0.1
	ERA8	5	10	42, 59	4	54.3 ± 15	0.14 ± 0.1
	Zak	5	10	42, 59	4	89.3 ± 5.5	0.40 ± 0.1
5.2 Group 2	60	3	5	41	4	68.5 ± 17	0.42 ± 0.1
	ERA8	12	5	41	4	12.7 ± 5.2	0.08 ± 0.0
	Zak	12	5	41	4	90.5 ± 3.5	0.62 ± 0.0

[a] number (n) of backcross lines tested

[b] technical replicates per backcross or parental line.

[c] The number of days imbibed (Imb.) that was used for bulk segregant analysis (BSA) or fine mapping

[d] Raw mean and standard deviation (SD) across the backcross population, Zak, or *ERA8*.

(Supplemental Figure 4). Seeds were considered germinated upon embryonic root emergence. Germination index (GI), a value weighted for speed of germination, was calculated over 5 days 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 0-1, where a GI of 1 indicates that all of the seeds germinated on day 1 of imbibition. Raw means and standard deviations were calculated from three technical replicates of ten seeds. Zak/Zak*ERA8* BC_3F_3 lines were selected for an *ERA8*-like high ABA sensitivity or a Zak-like low ABA sensitivity phenotype after three biological replicates (3 plates of 30 seeds) on 2 µM ABA were conducted. Phenotypes were confirmed by progeny testing of the BC_3F_4. Chi-squared analysis of goodness-of-fit to Mendelian segregation models of F_3 seeds was performed as in Schramm *et al*. (2013; Supplemental Table 2). The $\chi 2$ statistic was calculated by $\Sigma [(O - E)2 / (E)]$ where O was the observed number of seeds germinated or ungerminated, and E was the expected number of seeds germinated/ungerminated based on the Mendelian segregation model and the F_2 germination on ABA. A $\chi 2$ distribution table was used to determine the p-values based on the $\chi 2$ statistic and the degrees of freedom = 1. The model fits the observed values when $p > 0.05$, and does not fit when $p < 0.05$.

Genetic Linkage Map and QTL Mapping

A linkage map of the Louise/Zak*ERA8* RIL population was constructed using SNP data from GBS using the maximum likelihood algorithm in JoinMap v4.0 (Van Ooijen 2006). Linkage groups of 5 or more markers were selected with a LOD above 7.0 (Supplemental Table 3; Supplemental Table 4; Supplemental Figure 5). Skewed markers with a segregation $p < 0.05$ were omitted from the analysis. Composite interval mapping (CIM) was performed with the R/qtl package in R v3.2.5 using the Haley-Knott Regression algorithm with step = 0 and step=1 (Haley and Knott, 1992; Broman *et al*., 2003). Significant LOD scores were determined by using the 'scanone' function in R/qtl with the Haley-Knotts method and 1,000 permutations for each trait. QTL graphs were constructed using the 'ggplot2' package v2.2.1.9000 in R v3.2.5 (Wickham, 2009).

Supplemental Table 2 - Segregation analysis of BC_3F_2 seed germination on 2uM ABA.

Genotype	n[a]	Gen.[b]	Germ.[c]	Not Germ.[c]	χ^2			p-value		
					3:1	1:3	1:2:1	3:1	1:3	1:2:1
5.2 segregating	300	BC_3F_2	154	146	140.7	36.6	1.62	<0.001	<0.001	**0.20**
+/+	60	parent[e]	54	6						
ERA8/*ERA8*	60	parent[e]	0	60						
+/*ERA8*	8	BC_2F_1	4	4						
F_2 Expected[d]	300				68	203	143			

[a] Number of seeds tested for germination, after-ripened for 5 weeks past physiological maturity; df = 1

[b] Generation of seeds tested

[c] Number of seeds that had germinated (Germ.) and not germinated (Not Germ.) after 5 days of imbibition on 2µM (+/-) ABA.

[d] Number of seeds expected to germinate after 5 days of imbibition for each single gene segregation ratio.

[e] Zak (+/+) and *ERA8* (*ERA8*/*ERA8*) parental lines were used to generate cross 5; Parents were grown at the same time as the F_1 plants.

Supplemental Table 3 – Summary of the Louise/Zak*ERA8* RIL population GBS linkage groups.

Chrm	No. Groups [a]	Total Number of Markers	Map Distance (cM)
1A	1	231	534
1B	2	141	334, 88
1D	2	57	73, 193
2A	2	98	46, 272
2B	1	49	270
2D	1	57	483
3A	4	180	296, 89, 33, 118
3B	1	64	241
3D	2	46	97, 153
4A	3	224	279, 134, 30
4B	2	130	294, 55
4D	1	11	122
5A	2	62	543, 42
5B	3	84	138, 117, 38
5D	2	27	239, 113
6A	2	169	323, 168
6B	2	39	167, 72
6D	3	34	62, 167, 23
7A	2	238	458, 272
7B	2	206	227, 40
7D	3	61	268, 78, 116
unknown	1	20	105
total	45	2,234	-

[a] Linkage groups were assigned to chromosomes based on the majority of markers aligned to one chromosome on the RefSeqv1.0 wheat genome.

Exome Capture and Sequencing

The Zak/Zak*ERA8* BC_3F_3 population genomic DNA was extracted using a phenol/chloroform-based DNA extraction method followed by an RNAse A digestion to degrade contaminating RNA (as in Yu *et al*., 2017). For bulk DNA extraction, a 2.5 cm long leaf from each BC_3:F_3 plant was ground together with lines that comprised each bulk. For each individual parent, 10-15 cm long leaf samples were ground for DNA.

Whole genome DNA resequencing of the large wheat genome (16 Mb) for the sake of bulked-segregant analysis is not economically feasible (Uauy *et al*., 2017). Exome capture was used to restrict genomic DNA sequencing to those regions encoding predicted genes (as in Krasileva *et al*., 2017). DNA samples of the Zak*ERA8* parent (95.6.85.4; *ERA8/ERA8*), Zak wild-type parent (Zak2.7.3.7.2.3.10.3; +/+), Zak*ERA8*-like bulks (15 bulked BC_3F_3; *ERA8/ERA8*; *ERA8*_b), and Zak-like bulks (17 bulked BC_3F_3; +/+; Zak_b) were sent to the Earlham Institute, UK for separate library preparation with unique adapter sequences, exome capture, and sequencing. The average insert sizes of *ERA8*, Zak, *ERA8*_b, and Zak_b libraries are 327, 345, 348, and 340 bp, respectively. All four libraries were captured on one exome-capture probe. Sequencing was conducted on a single Illumina HiSeq 2500 lane producing 125-bp paired end reads. The quality of the reads was analyzed using FastQC and the reads with a base quality score ranging from 20-40 were retained (Supplemental Table 5; Babraham Bioinformatics, 2012). Adapter sequences were removed using the Trimmomatic program with the IILLIMINACLIP (Truseq3-PE), CROP (125 bp), and MINIMUMLENGTH (50 bp) options (Bolger *et al*., 2014).

Two different short read alignment tools, BWA and Bowtie 2, were used to align the sequenced reads to the reference genome (Langmead *et al*., 2009; Li and Durbin, 2009). For both alignments, the Chinese Spring (CSS) IWGSC WGAv0.4 (160509_Chinese_Spring_v0.4_pseudomolecules_parts.fasta) was used as reference. BWA aln algorithm was used followed by sample analysis of the paired-end reads. The Bowtie 2 local alignment algorithm was used to maximize read alignment by performing soft clipping of the ends of reads thus allowing the alignment of reads which would not have aligned due to

Supplemental Table 5 - Sequencing read count and quality statistics from exome capture.

Sample Name	Number of Reads	Mean Q30 to base Read 1	Mean Q30 to base Read 2
ERA8 Parent	60,514,766	125	125
*ERA8*_Bulk	57,332,604	126	125
WT Parent	48,792,615	126	125
WT_Bulk	67,097,197	126	125

the mismatches located at read end. This algorithm resulted in a high number of read alignments (> 99 %) for all the samples. This alignment was followed by a Bowtie end-to-end alignment hereafter referred to as etex. The sensitivity of the alignment was also increased by adjusting the minimum score threshold parameter (-score-min). Score-min controls the minimum alignment score for which a read alignment is considered valid and is reported. The default score-min setting is L, -0.6, -0.6, that is ((-0.6 x length of read) - 0.6). For example, a read with length 100, will have a minimum score of -60.6, depending on the base quality score. This default setting will allow as many as 10 mismatches in this 100-bp read alignment. The min-score setting was reduced to L, 0, 0.15. This reduced the minimum score to 18.75 allowing 2-3 mismatches depending on the base quality score. The SAM raw alignment files produced in each of the alignment described above were converted into BAM files (binary form of SAM file) and were sorted and indexed using SAMtools view, sort, and index commands, respectively (Li *et al*., 2009a). PCR amplification during the library preparation often gives rise to redundant duplicate reads which affect accurate estimation of read depth coverage. This is especially important because accurate read depth values are important for identifying sites enriched for EMS mutations in the mutant bulk (*ERA8*_b) compared to wild-type bulk (Zak_b). PCR duplicates were removed using the Picard Markduplicate tool (http://broadinstitute.github.io/picard). This was done for the BAM files produced from BWA, Bowtie 2 local, and Bowtie 2 etex alignments. SAMtools faidx with the –f option was used to properly pair reads and allowed for the selection of the reads with specific tags (Li *et al*., 2009a). BAM files from 'Bowtie 2 local' and 'Bowtie 2 etex' analyses were filtered for properpair and were renamed as noDup-Sample - properpair. The 'Bowtie 2 etex' alignment was re-aligned to the current Chinese Spring IWGSC RefSeqV1.0 (http://www.wheatgenome.org/) once it became available and this alignment was used in the downstream analyses.

Bulk Segregant Analysis

Exome single nucleotide polymorphisms (SNPs) were first identified between the Zak and *ERA8* parents. SNP variants differing between the *ERA8*-like (*ERA8*_b) and Zak-like (Zak_b) bulked genomic sequences

were identified using SAMtools mpileup and Bcftool view commands for variant calling (Li *et al*., 2009a). The options used for SAMtools mpileup were -A (means also include non-paired reads in variant calling), -u (produced uncompressed output), -Q 30 (means only use bases with base quality > 30 for SNP calling), -f (the FASTA file for the CSS ref), -q 7 (use only reads with a mapping quality score (MAPQ) >= 7), -g (compute genotype likelihoods and output them in the binary call format (BCF)), and -D (output per sample read depth). The options for bcftools were -O v (output in VCF format), -c (call variants using Bayesian inference), and -v (output variant sites only). A program called snpsift (snpEFF) was used for the analysis, manipulation, and interactive filtering of the VCF files (Cingolani *et al*., 2012). Variants included deletions, insertions, or single nucleotide polymorphisms (SNPs). The first alignment, BWA, was discontinued because the mapping percentages were low (~ 50 %), suggesting the loss of a lot of the potentially good sequencing reads (Supplemental Table 6). The poor mapping might have been due to the use of the BWA 'aln' algorithm that was designed for shorter reads than the 125 bp reads used in the current study. The second alignment, Bowtie 2 local, resulted in some bad alignments identified during visual inspection. These bad alignments could have led to calling false positive SNP sites downstream. The third alignment (Bowtie 2 etex) increased the sensitivity and stringency of mismatches resulting in the percentage of read alignments slightly reduced but improved alignment quality. A higher number of variants were called from the Bowtie 2 local alignment compared to the Bowtie 2 etex alignment (Supplemental Table 7). Analysis was concentrated mainly on those SNPs known to be caused by EMS mutagenesis, C to T or G to A transitions. The percentage of EMS SNPs in the *ERA8* sequence compared to all identified variants was lower than expected (33-46 %). In the Bowtie 2 local alignment, selecting for sites with increased alternate allele depth (DV) improved the percentage of EMS SNP overall by only 6 % compared to the Bowtie 2 etex alignment, which improved it by 23 %.

Only SNPs with a "QUAL" (variant quality score) above 20 were selected. The filter for *ERA8*-specific variants was based on: a) the site was polymorphic between Zak and *ERA8* and b) the Zak variant was homozygous (because the *ERA8* mutation is semi-dominant). Variant calls supported by high

Supplemental Table 6 - Alignment statistics of the reads using different alignments and algorithms.

Tool/algorithm	General Mapping Statistic (% of total reads)	Samples			
		ZAK parent	**ZAK bulk**	***ERA8* parent**	***ERA8* bulk**
BWA aln/Sampe (%)	Reads mapped	49%	49%	49%	49%
	Reads mapped and paired	48%	49%	49%	48%
	Reads unmapped	51%	51%	51%	51%
	Reads properly mapped	36%	36%	33%	36%
Bowtie local very sensitive (%)	Reads mapped	100%	100%	100%	100%
	Reads mapped and paired	99%	99%	99%	100%
	Reads unmapped	0%	0%	0%	0%
	Reads properly mapped	97%	97%	98%	97%
Bowtie end-to-end with extra sensitive setting –etex (%)	Reads mapped	94%	95%	95%	94%
	Reads mapped and paired	91%	93%	92%	92%
	Reads unmapped	6%	5%	5%	6%
	Reads properly mapped	87%	90%	89%	88%

Supplemental Table 7 - Summary of variants call from both Bowtie local and Bowtie etex.

Variant Stats	Bowtie local			Bowtie etex		
	No of Var.	EMS [a]	% EMS	No of Var.	EMS	% EMS
All Variant	1,057,989	333,998	32%	3,619,251	1,061,469	29%
SNP with QUAL > 20	523,553	171,160	33%	473,539	170,884	36%
***ERA8* variant**	19,487	6,449	33%	4,396	2,025	46%
***ERA8* variants, >DV= 5** [b]	10,369	3,563	34%	2,417	1,159	48%
***ERA8* variants, DV>= 10**	6,322	2,264	36%	1,533	783	51%
***ERA8* variants, DV= 20**	2,985	1,124	38%	833	437	52%

[a] A C-T or G-A nucleotide change was considered an EMS mutation

[b] Alternate allele read depth (DV)

number of reads have a higher probability of being real. Therefore, variants with at least 3 different alternate allele reads were kept. Alternate allele read depth (DV) is the number of reads carrying the alternate (non-reference allele) for each sample, while DP is the total number of reads at that site. The DV depth used were >=5, >=10 and >=20 (Supplemental Table 7).

To reduce the noise signal arising from non-EMS sites, a more stringent criteria was used to select variants for the bulked segregant analysis. All EMS-type variants with QUAL score greater than 20 (about 170,884; Supplemental Table 7) in which Zak had zero DV (meaning the Zak sequence must be identical to the Chinese spring reference) but *ERA8* had greater than zero DV were retained. This filtering is more stringent than earlier filtering for *ERA8*-specific sites above as it excluded sites that were variant in Zak but invariant in *ERA8* relative to the Chinese Spring reference. Bulk variants were analyzed only for these EMS-derived Zak vs *ERA8* variants by specifying their location in the Mpileup command in SAMtools (Li *et al*., 2009a). Only 853 of the 1,320 SNP were called as variants in the bulk, 670 of which had read depth greater than five and were most like real *ERA8*-specific mutations. Subsequent bulked segregant calculation was done with this subset. The DP and DV for each sample were used to calculate the alternate allele frequencies at each variant site for *ERA8*_b and Zak_b using the formula BF = DV/DP were BF is the bulk allele frequency of either Zak_b or *ERA8*_b. The difference in the bulk allele frequency (BF_D) was also calculated between the two samples (BF_D = BF_*ERA8*_b - BF_Zak_b). Visual examination of SNP sites was conducted using the Integrative Genome Viewer program (Robinson *et al*., 2011). Annotation of the SNPs found between wild-type Zak and the *ERA8* mutant was done with the Variant effect predictor (Ve!P) tool with TGAC1 Wheat assembly hosted on EnsemblPlant (https://www.ensembl.org/vep; McLaren *et al*., 2016).

Specifically, EMS-type variants were selected across the genome where Zak had no alternate allele read (meaning the Zak sequence must be identical to the Chinese spring reference) and *ERA8* was allowed no less greater than 5 DV. Increasing the DV value increased the percentages of the EMS mutation in the etex files. The Bowtie etex alignment with a DV=20 was used in all subsequent analyses. There were 670 SNPs differing between Zak and *ERA8* identified.

Fine Mapping

Fine mapping was performed using Zak/Zak*ERA8* $BC_3F_{2:3}$ lines genotyped using Kompetitive Allele SNP PCR (KASP) markers. *ERA8*-linked KASP markers were developed based on SNPs identified through whole genome exome resequencing of Zak and Zak*ERA8* using PolyMarker with default settings (Supplemental Table 8; Ramirez-Gonzalez *et al*., 2015b). The KASP marker for the preharvest sprouting susceptible (A660) versus resistant (C660) alleles of *TaMKK3* were assayed as described in (Shironola *et al*., 2017). Genomic DNA was extracted from BC_3F_2 leaf tissue on the Qiagen BioSprint-96, DNA concentrations were determined using the BioTek Gen5 plate reader, and DNA was diluted to 50 ng/uL. A standard 10 µL KASP reaction contained 15 ng/uL gDNA, 1x KASP Master Mix (LGC Genomics; V4.0 2x Mastermix 96/384; Low Rox), and 0.14 µL of primer mix (Smith and Maughan, 2015). The primer mix contained 12 µL of the 100 mM Allele 1 forward primer, 12 µL of the 100 mM Allele 2 forward primer, 30 µL of the single reverse primer, and 46 uL of molecular grade water. KASP assays were performed on a thermocycler as follows: 1 cycle of 94 °C for 15 min, 10 cycles of 94 °C for 20 sec and 61-55 °C for 60 sec (starting at 61 °C, with 0.6 °C decrease in temperature per cycle), 26 cycles of 94 °C for 20 sec and 57 °C for 60 sec (T100, Bio-Rad, Hercules, CA, USA; Applied Bioscience Veriti, ThermoFisher Sci., Waltham, MA, USA; and Biometra Tadvance, Analytikjena, Jena, Germany).

Zak/Zak*ERA8* $BC_3F_{2:3}$ lines were used for fine mapping using EMS-induced exon mutations. A total of 2,381 BC_3F_2 lines were initially plated on ABA to obtain preliminary information about which showed a WT and which an *ERA8* phenotype. KASP assays were used to identify those lines containing recombination events within the *ERA8* region. Of these, 734 were advanced to BC_3F_3 and seeds were assayed for ABA sensitivity. Based on Chi-squared analysis $BC_3F_{2:3}$ from X5.1 (122 lines) and X5.2 (302 lines) showed Mendelian segregation, whereas $BC_3F_{2:3}$ from X5.3 (310 lines) did not (Supplemental Table 9). A linkage map of only the X5.1 and X5.2 Zak/Zak*ERA8* $BC_3F_{2:3}$ populations was constructed using the regression algorithm and Kosambi mapping function in JoinMap v4.0 (Kosambi, 1943; Van Ooijen 2006).

Supplemental Table 8 - *ERA8* primers created from the GBS and Exome capture.

Name	Allele	Sequence	Tag	Source
SNP_1	WT	GAAGGTGACCAAGTTCATGCTaCtctttctcatgagctctttgaG	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTaCtctttctcatgagctctttgaA	HEX	
	C	tcgcgttcatcatctacctG	-	
SNP_2	WT	GAAGGTGACCAAGTTCATGCTctgaaatgCctgcaagatgaG	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTctgaaatgCctgcaagatgaA	HEX	
	C	ggaaggccaaaatagcgacttA	-	
SNP_4	WT	GAAGGTGACCAAGTTCATGCTaaactcgacGaaaTtgcCaTC	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTaaactcgacGaaaTtgcCaTT	HEX	
	C	aatcaacaCcattttcatttcacaG	-	
SNP_5	WT	GAAGGTGACCAAGTTCATGCTgtggagtgTctgcaagatcG	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTgtggagtgTctgcaagatcA	HEX	
	C	tatgcactcaccacgccA	-	
SNP_6	WT	GAAGGTGACCAAGTTCATGCTCacaaactgtgctatcccgG	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTCacaaactgtgctatcccgA	HEX	
	C	tggtagcagttgtcttgacC	-	
SNP_7	WT	GAAGGTGACCAAGTTCATGCTggcttgcgggtaaaagagG	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTggcttgcgggtaaaagagA	HEX	
	C	tctttgttttataattgcagtgcaC	-	
SNP_8	WT	GAAGGTGACCAAGTTCATGCTtgTtAGatgtGaccttacctctcaG	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTtgTtAGatgtGaccttacctctcaA	HEX	
	C	cgagatgagcaaggaggtgg	-	
SNP_9	WT	GAAGGTGACCAAGTTCATGCTCggttctgcgttaccccatG	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTCggttctgcgttaccccatA	HEX	
	C	agcaaatgCcgCggatcG	-	
SNP_10	WT	GAAGGTGACCAAGTTCATGCTCggatcacgaTcgccttctcC	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTCggatcacgaTcgccttctcT	HEX	
	C	TgTgctcctcGtcCtGAC	-	
SNP_17	WT	GAAGGTGACCAAGTTCATGCTCCTCTGCTATTTGCTTTAATCTCTc	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTCCTCTGCTATTTGCTTTAATCTCTt	VIC	
	C	GGACTTGGCAGCATATGTCA	-	
SNP_20	WT	GAAGGTGACCAAGTTCATGCTCtcctgctcctgcttccgG	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTCtcctgctcctgcttccgA	HEX	
	C	cggcctcacttgcagaaaac	-	
SNP_29	WT	GAAGGTGACCAAGTTCATGCTgtgtacgcGcgCtactgC	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTgtgtacgcGcgCtactgT	HEX	
	C	ccatgatctccagcgacagA	-	
SNP_30	WT	GAAGGTGACCAAGTTCATGCTggtataaacatcagaatccctgtcC	FAM	Exome
	ERA8	GAAGGTCGGAGTCAACGGATTggtataaacatcagaatccctgtcT	HEX	
	C	ccggcatcttcTGtattaacatacA	-	
MKK3-A[a]	Res	GAAGGTGACCAAGTTCATGCTTTTTGCTTCGCCCTTAAGG	FAM	-
	Susc	GAAGGTCGGAGTCAACGGATTTTTTTGCTTCGCCCTTAAGT	HEX	
	C	GCATAGAGATCTAAAGCCAGCA	-	
A7575	WT	GAAGGTCGGAGTCAACGGATTCaAActacacactcgtcgggA	HEX	GBS
	ERA8	GAAGGTGACCAAGTTCATGCTCaAActacacactcgtcgggG	FAM	
	C	ccctgcagcagaggacatC	-	
A7946	*ERA8*	GAAGGTCGGAGTCAACGGATTCaAactacacactcgtcgggA	HEX	GBS
	Ref	GAAGGTGACCAAGTTCATGCTCaAactacacactcgtcgggG	FAM	
	C	ccctgcagcagaggacatC	-	

[a] *TaMKK3-A* KASP primers were from Shorinola *et al*. (2017)

[b] KASP tags are already included in the primer sequence

Supplemental Table 9 - Segregation analysis of Zak/*ZakERA8* BC_3F_2 X5.1, X5.2, and X5.3 germination on ABA.

Genotype	n[a]	Gen.[b]	Germ.[c]	Not Germ.[c]	χ^2			p-value		
				F_2	3:1	1:3	1:2:1	3:1	1:3	1:2:1
				F_3	0.625:0.375	0.375:0.625	0.375:0.25:0.375	0.625:0.375	0.375:0.625	0.375:0.25:0.375
A) 56d AR | 10uM ABA | 4d imb.										
X5.1	1311	BC_3F_3	588	723	18.6	39.8	0.08	<0.001	<0.001	0.78
WT/WT	54	parent[e]	39	15						
ERA8/*ERA8*	60	parent[e]	9	51						
WT/*ERA8*	8	BC_2F_1	4	4						
F_3 Expected[d]	1311				666	478	593			
B) 35d AR | 2uM ABA | 4d imb.										
X5.2 Group 1	7361	BC_3F_3	3974	3387	107.0	677.3	55.3	<0.001	<0.001	<0.001
WT/WT	120	parent[e]	109	11						
ERA8/*ERA8*	120	parent[e]	10	110						
WT/*ERA8*	8	BC_2F_1	4	4						
F_3 Expected[d]	7361				4409	2885	3655			
C) 49d AR | 5uM ABA | 5d imb.										
X5.2 Group 2	583	BC_3F_3	340	243	30.8	23.5	0.84	<0.001	<0.001	0.36
WT/WT	59	parent[e]	59	0						
ERA8/*ERA8*	52	parent[e]	9	43						
WT/*ERA8*	8	BC_2F_1	4	4						
F_3 Expected[d]	583				402	282	329			
D) 56d AR | 10uM ABA | 4d imb.										
X5.3[f]	1453	BC_3F_3	344	1109	427.5	102.8	272.2	<0.001	<0.001	<0.001
WT/WT	54	parent[e]	39	15						
ERA8/*ERA8*	60	parent[e]	9	51						
WT/*ERA8*	8	BC_2F_1	4	4						
F_3 Expected[d]	1453				738	530	657			

[a] Number of seeds tested for germination, after-ripened for 5 weeks past physiological maturity; df = 1

[b] Generation of seeds tested

[c] Number of seeds that had germinated (Germ.) and not germinated (Not Germ.) after 5 days of imbibition on 2µM (+/-) ABA.

[d] Number of seeds expected to **germinate** after 5 days of imbibition for each single gene segregation ratio.

[e] Zak WT (+/+) and Zak *ERA8* (-/-) parental lines used to generate cross 5; Grown at the same time as the F_1 plants.

[d] Note that X5.3 was not used in any analyses since it appeared to not have a 1:2:1 segregation.

Fine mapping was also conducted in the Louise/Zak*ERA8* F_5 population using genotype information from the *ERA8* KASP markers (Supplemental Table 8). Both populations were analyzed using composite interval mapping (CIM) performed with the R/qtl package in R v3.2.5 using the Haley-Knott Regression algorithm, and the Kosambi mapping function with step = 0 or 1 (Haley and Knott, 1992; Broman *et al*., 2003). Significant LOD scores were determined using the 'scanone' function in R/qtl with the Haley-Knotts method and 1,000 permutations for each trait. QTL graphs were constructed using the 'ggplot2' package v2.2.1.9000 in R v3.2.5 (Wickham, 2009).

RNA Sequencing

An RNASeq experiment (2.5 million reads) was performed on the original BC_3 parents Zak (Zak2.7.3.7.2.3.10.3) and Zak*ERA8* (BC_2F_3; 95.6.85.4). The RNA sequence was derived from dark-imbibed seed tissue instead of leaf tissue in order to reduce expression of transcripts of genes involved with photosynthesis. Seeds were imbibed on 5mM MES, pH 5.5 (2-[N-morpholino] ethane sulfonic acid, Sigma-Aldrich) for 8 hours. Embryos were harvested for RNA under a green safelight to avoid induction of photosynthesis genes. RNA was extracted from 20 dissected embryos per sample using a modified version of an RNA extraction technique which yielded an average of 2,300 ng/uL of RNA with a RIN > 8.0 (Onate-Sánchez and Vicente-Carbajosa, 2008; Nelson and Steber, 2017). Sequencing was performed using the Illumina HiSeq 2500 (WSU Genomics Core). Quality of the RNA reads were checked using FastQC and all samples had a quality score > 32 (Babraham Bioinformatics, 2012). Psuedoalignment and quantification of RNAseq reads was conducted with kallisto-0.42.3 using the quant command with options -b 100 and -t 8 and an addition option to output sequence alignment (SAM) files (Bray *et al*., 2016). The wheat RefSeqv1.0 transcriptome assembly (IWGSC RefSeq v1.0, http://www.wheatgenome.org/) was used as reference for this alignment. Samtools was used to sort and index the bam files v0.2.19 (Li *et al*., 2009a). Gene differential expression analysis was done using the 'sleuth' package in R (Pimenthel *et al*., 2017).

Introgression of *ERA8*

Zak*ERA8* BC_1 (60.1.27) was crossed into lines from the Washington State University (WSU) Winter Wheat breeding program whereas Zak*ERA8* BC_3F_3 (5.2) was crossed lines from the USDA-ARS Club Wheat breeding program (Appendix Table 3.10). For the WSU Winter Wheat breeding program introgression, Zak*ERA8* BC_1F_3 were crossed to 7 different cultivars or elite breeding lines (BC_1*ERA8*/male). Unselected F_2 plants were then crossed again to 5 different cultivars or elite breeding lines as the female (female//BC_1*ERA8*/male) with no recurrent parent. In addition, more unselected F_2 plants were then crossed again to 20 different cultivars or elite breeding lines as the male (BC_1*ERA8*/male//male) with no recurrent parent. For the USDA Club Wheat breeding program introgression, Zak*ERA8* BC_3F_3 seeds were crossed to 12 different PHS susceptible cultivars or elite breeding lines. Six of the female/BC_3F_3 *ERA8* crosses F_1 seeds were immediately backcrossed to the recurring female parent (female//female/BC_3F_3 *ERA8*). The remaining female/BC_3F_3 *ERA8* crosses F_1 seeds (16X0167 to 16X0172) were advanced to F_2 plants. Genomic DNA was extracted from F_2 tissue at the 2-3 leaf stage using the Qiagen Biosprint-96. *ERA8* segregation was followed using linked KASP markers (Appendix Table 3.9).

Results

***ERA8* mapping by QTL analysis of a recombinant inbred line population**

The Louise/Zak*ERA8* RIL population of 225 lines was created to map the *ERA8* ABA hypersensitive germination phenotype using quantitative trait locus analysis. This QTL analysis was performed using grain harvested at physiological maturity from three environments referred to as E1, E2, and E3 (Table 1). Because dormancy and wheat ABA sensitivity vary with environment, parental plating assays were used to determine the after-ripening time point and ABA concentration resulting in the strongest difference between parental Louise and Zak*ERA8* germination (Figure 1; Supplemental Figure 2). These conditions were then used for the segregation analysis of the RIL populations harvested from each environment (Table 1). It proved difficult to find conditions that differentiated between Louise and Zak*ERA8* because

Table 1 - ABA sensitivity for the Louise/Zak*ERA8* RIL population was tested across three environments.

Location	Year	n[a]	t rep[b]	ABA (µM)	AR (days)	Mean ± SD[c]					
						Day 1	Day 2	Day 3	Day 4	Day 5	GI
Greenhouse	2013	225	3	5	49	48.9 ± 29	71.9 ± 25	76.0 ± 22	78.8 ± 21	81.2 ± 19	0.71 ± 0.2
(E1)		*ERA8*	9	5	49	26.7 ± 21	68.9 ± 21	74.4 ± 18	78.1 ± 16	81.1 ± 16	0.66 ± 0.2
		Louise	9	5	49	70.4 ± 12	81.9 ± 13	86.3 ± 12	88.9 ± 10	91.9 ± 8	0.84 ± 0.1
Field	2014	181	3	2	42	22.3 ± 19	52.5 ± 25	63.3 ± 25	69.8 ± 25	74.9 ± 25	0.56 ± 0.2
(E2)		*ERA8*	3	2	42	0.0 ± 0	5.6 ± 5	13.3 ± 12	17.8 ± 13	20.0 ± 12	0.11 ± 0.1
		Louise	3	2	42	24.3 ± 8	44.4 ± 13	69.8 ± 18	77.8 ± 22	87.0 ± 11	0.58 ± 0.2
Field	2015	190	3	2	48	23.1 ± 22	71.5 ± 27	78.2 ± 24	82.2 ± 23	84.8 ± 21	0.68 ± 0.2
(E3)		*ERA8*	3	2	48	5.6 ± 2	37.8 ± 23	43.3 ± 30	50.0 ± 27	53.3 ± 30	0.38 ± 0.2
		Louise	3	2	48	27.8 ± 4	81.1 ± 10	86.7 ± 6	87.8 ± 7	88.9 ± 5	0.75 ± 0.1

[a] number (n) of recombinant inbred lines (RIL) tested

[b] technical replicates per RIL or parental line

[c] Raw mean and standard deviation (SD) across the RIL population, Louise, or *ERA8*.

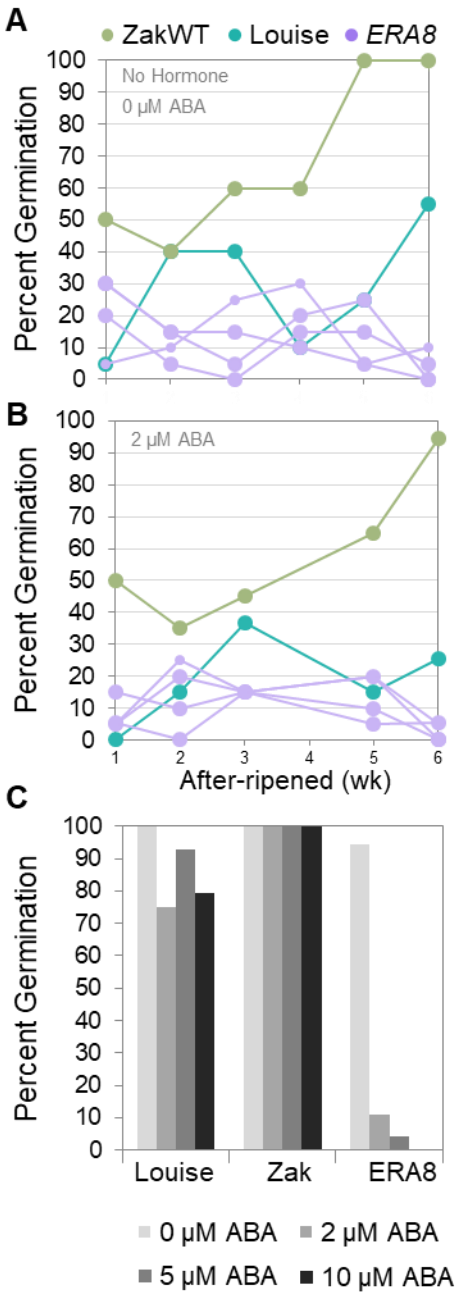


Figure 1 - An after-ripening time course of the Louise (blue), *ERA8* (purple), and Zak WT (green) parents in environment 1 (greenhouse grown in 2013) that was conducted **A**) without ABA and **B**) with 2 µM ABA. Percent germination after 5 days of imbibition at 30 °C were calculated from 20 whole seed germination assays. **C**) Cut seed assays were performed on 20 seeds per parent without (0 µM ABA) and with 2, 5, and 10 µM ABA after 7 weeks of after-ripening and 5 days of imbibition.

Louise had more ABA sensitivity than the original wild-type Zak mutagenesis parent. When whole-seed germination was examined, *ERA8* was only slightly more ABA sensitive than Louise (5.6 % versus 25.6 % germination at 6 weeks of after-ripening; Figure 1B). A stronger difference between Louise and Zak*ERA8* was observed when grains were cut and the embryos were plated on ABA, such E1 Louise and *ERA8* showed 92 % and 4 % germination, respectively, with 7 weeks of after-ripening and in the presence of 5 µM ABA (Figure 1C). Cutting the seeds increased the apparent difference in ABA sensitivity by stimulating wild-type germination by breaking seed coat imposed dormancy. For E1, the Louise and *ERA8* parents showed the strongest difference in germination with one day of imbibition (Table 1). E2 and E3 had overall low germination within the RILs and parents during days 1 and 2 of imbibition but increased by day 5. While there was better differentiation between the parents using germination index for E2 and E3, the GI distribution of the RILs was more skewed towards the non-dormant parent Louise whereas percent germination showed a more normal distribution (Table 1; Figure 2B; Supplemental Figure 4). Thus, both percent germination and GI were used in the QTL analysis.

QTL analysis revealed that both Louise and *ERA8* contributed multiple loci impacting ABA sensitivity during germination. GBS identified 2,609 single nucleotide polymorphisms (SNP) between Louise and *ERA8*. Of these, 2,234 SNPs grouped into one of 45 linkage groups spanning all 21 chromosomes (Supplemental Table 3; Supplemental Figure 5). QTL mapping for ABA sensitivity identified seven significant loci on chromosomes 2D, 4A, 4B, and 7B (Figure 3; Table 2). None of the QTL were significant in multiple environments. Louise contributed higher ABA sensitivity due to the *QABA.wsu-2D* and *QABA.wsu-4A.3* loci (Supplemental Figure 6C, D). In fact, the *QABA.wsu-2D* had the highest LOD score of 5.69 (Table 2). *ERA8* contributed higher ABA sensitivity due to the *QABA.wsu-4A.1*, *QABA.wsu-4A.2*, *QABA.wsu-4B*, *QABA.wsu-7B.1*, and *QABA.wsu-7B.2* loci (Supplemental Figure 6B, E, F). The two major *ERA8*-associated loci were on chromosome 4A, with a LOD scores of 5.32 and 5.05, respectively (Table 2).

To understand the effects of each ABA sensitive allele, haplotypes were compared separately and pyramided together across environments in which they were significant (Figure 4; Supplemental Figure

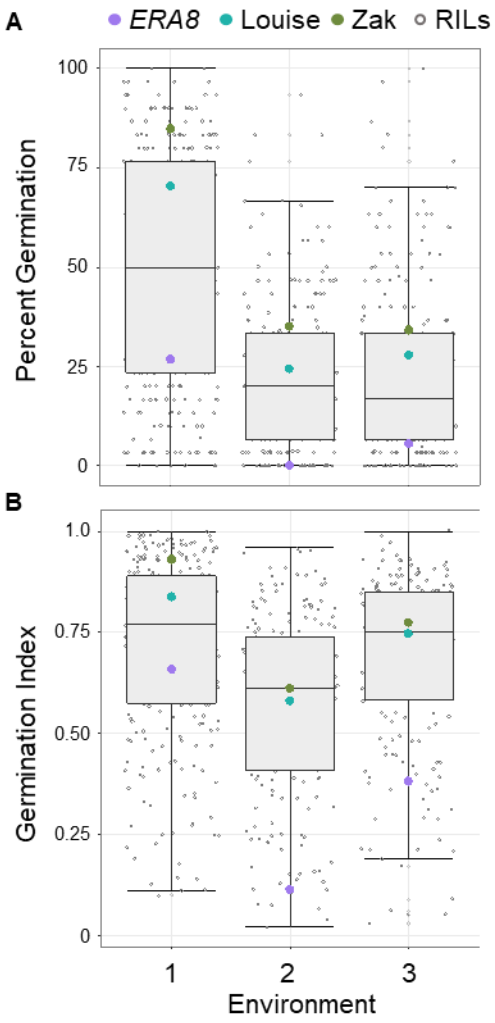
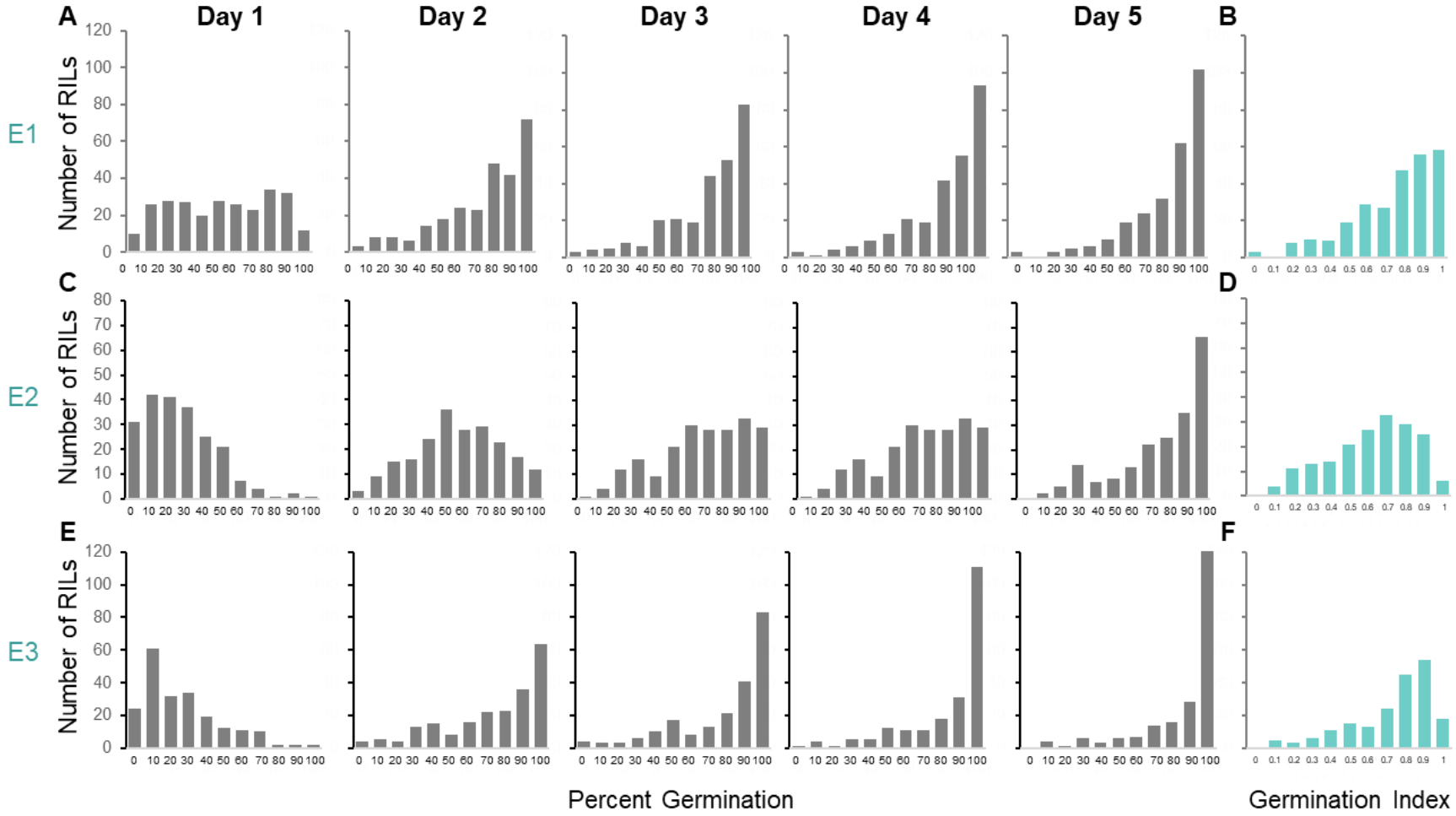
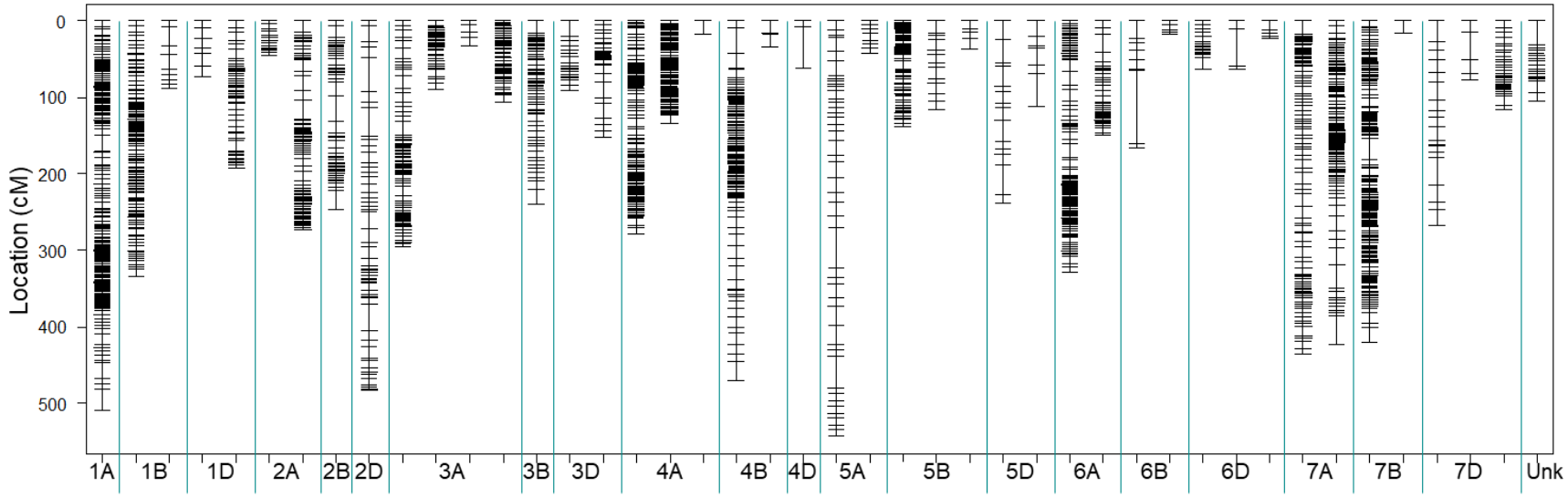


Figure 2 – Phenotypic distributions of all three environments 1: Greenhouse grown in 2013; 2: Field grown in 2014; and 3: Field grown in 2015. A) Percent germination after 1 day of incubation and B) Germination index on 5µM, 2µM, and 2µM of ABA are reported across 225 RILs (open grey), ERA8 (solid purple), and Louise (solid blue), respectively. Zak WT is shown in green.



Supplemental Figure 4 – Phenotypic distributions of the Louise/Zak*ERA8* RIL population across all three environments E1: Greenhouse grown in 2013 (**A**, **B**); E2: Field grown in 2014 (**C**, **D**); and E3: Field grown in 2015 (**E**, **F**). Percent germination is shown after 1, 2, 3, 4, and 5 days of imbibition (**A**, **C**, **E**) and germination index (**B**, **D**, **F**) at 30C on ABA across 225 RILs.



Supplemental Figure 5 - Linkage map was constructed with genotyping-by-sequencing (GBS) markers identified in the Louise/Zak*ERA8* F5 RIL population. Each chromosome has at least one or more linkage groups. Groups were annotated if more than 75 % of the markers within a group aligned to the same reference genome (RefSeq v1.0).

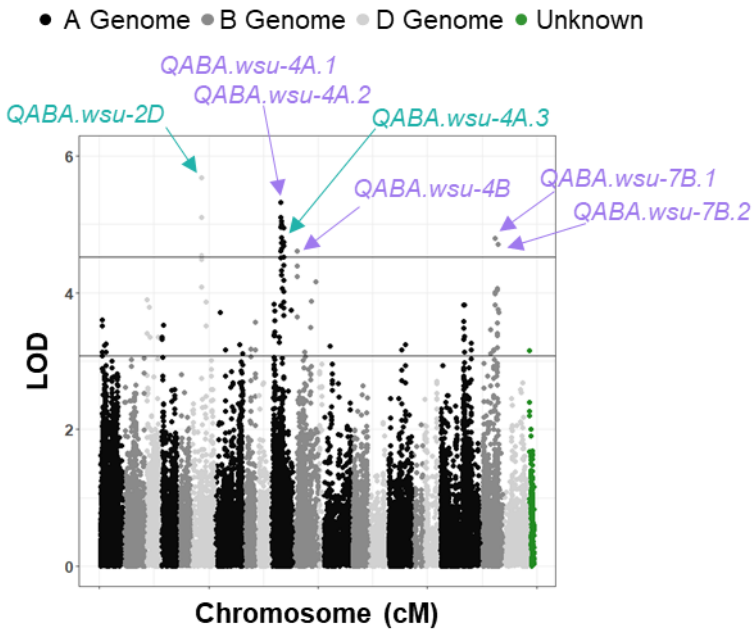


Figure 3 – A Manhattan plot of the Louise/Zak*ERA8* RIL population. LOD scores across all chromosomes (cM) for ABA sensitivity during germination. Significant QTL threshold of $p < 0.10$ for *ERA8* ABA sensitivity (purple) and Louise ABA sensitivity (blue) are shown.

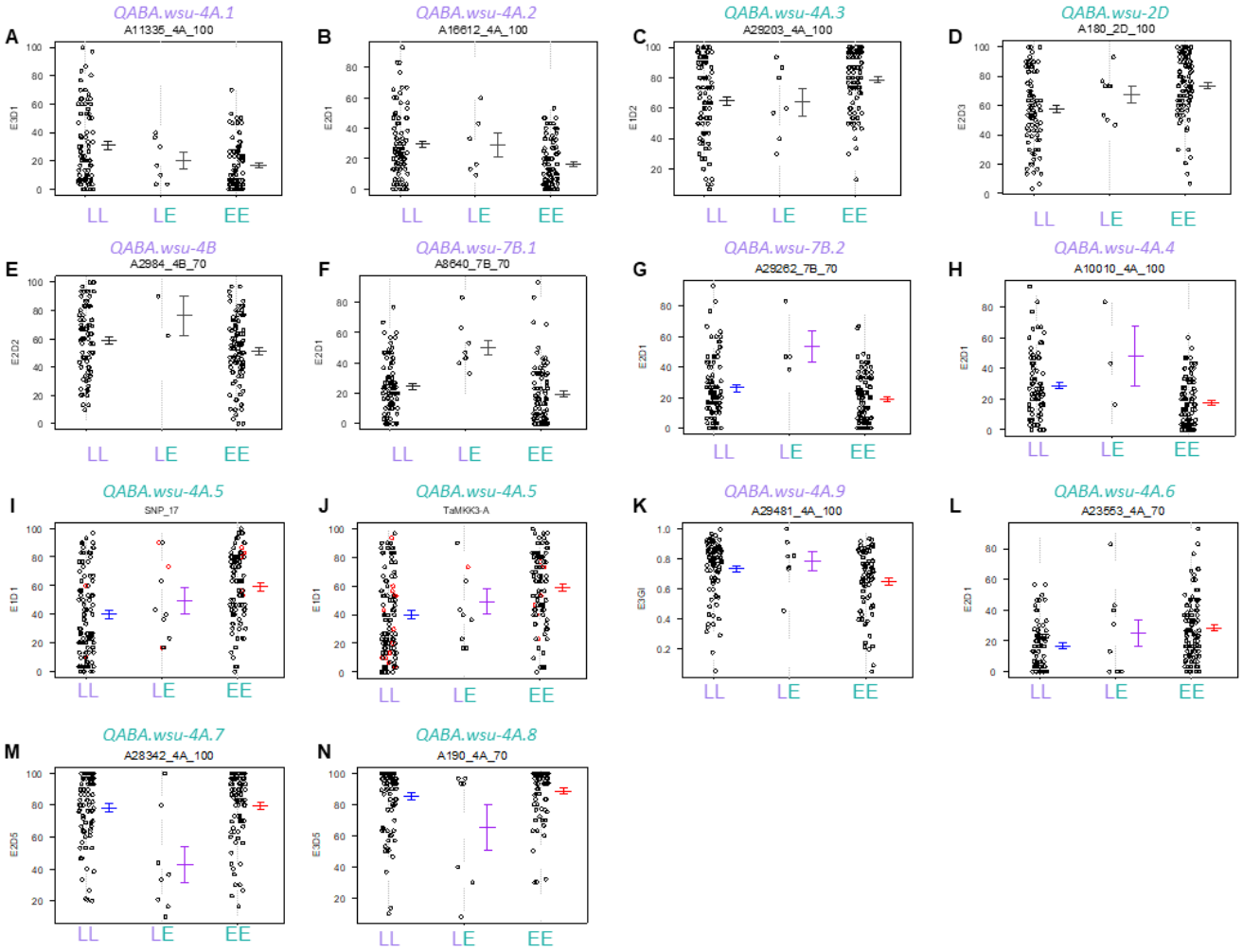
Table 2 - Significant QTL found in the Louise/Zak*ERA8* RIL population.

QTL Name	Marker	Chrm	Pos (cM)	Start [a]	End [a]	SNP Position	LOD [b]	Trait	Favorable Allele [c]
QABA.wsu-2D	A180	2D	198.9	36,271,198	36,271,099	54	5.69**	E2 D3	**C**/T
QABA.wsu-4A.1	A1512	4A	194.9	43,348,134	43,348,233	60	4.96*	E3 D1	G/**A**
	A11335	4A	199.7	83,469,184	83,469,281	85	5.32*	E3 D1	C/**T**
QABA.wsu-4A.2	A16612	4A	224.2	238,226,439	238,226,538	35	5.05*	E2 D1	G/**C**
QABA.wsu-4A.3	A28975	4A	266.8	29,794,783	29,794,714	27	4.79**	E1 D2	**C**/T
	A29203	4A	269.4	29,794,783	29,794,684	27, 88	4.69*	E1 D2	**C**/T
QABA.wsu-4B	A2984	4B	470.3	673,071,467	673,071,398	11, 15	4.16*	E2 D2	C/**T**
QABA.wsu-7B.1	A8640	7B	285.8	156,437,282	156,437,351	43	4.79*	E2 D1	C/**T**
QABA.wsu-7B.2	A29262	7B	365.7	416,37,734	41,637,803	11	4.71*	E2 D1	C/**T**

[a] The GBS markers was aligned to the RefSeqv1.0 reference genome and the start and end nucleotide position is reported with the SNP position within the sequence. The sequence for each marker is located in Supplemental Table 4.

[b] Significant LOD scores with a *p-value* < 0.05 and *p-value* < 0.10 are indicate with ** and *, respectively

[c] Favorable alleles decrease germination percent or index. Louise or *ERA8* contribute to the first or second allele, respectively.



Supplemental Figure 6 – Significant QTN allelic effects in the Louise/Zak*ERA8* RIL population. Homozygous Louise (**LL**) and homozygous *ERA8* (**EE**) alleles are labeled in purple and blue, respectively. ABA sensitivity QTL are shown for *QABA.wsu-4A.1- QABA.wsu04A.9* (**A**-**N**). Favorable alleles increase ABA sensitivity (*i.e.*, reduce percent germination in the presence of ABA). Each QTL is shown against the significant environment (E#) and day (D#) it was mapped to. Marker names (A####), chromosome position (##), and number of base pairs identified in the GBS (70 or 100) for that marker are labeled in addition to the QTL name. Note that red dots correspond to inferred genotypes from Rqtl (Broman *et al*., 2003).

3). When a RIL has both the *QABA.wsu-2D* and *QABA.wsu-4A.3* Louise alleles, overall percent germination decreases in the presence on ABA (Figure 4A, B). RILs that have the *QABA.wsu-4A.1, QABA.wsu-4A.2,* and *QABA.wsu-4A.3 ERA8* alleles appear to have lower percent germination on ABA compared to RILs without the *QABA.wsu-4A.1, QABA.wsu-4A.2,* and *QABA.wsu-4A.3 ERA8* alleles (Figure 4C). The decrease in ABA sensitivity effect multiplies when RILs also have the *QABA.wsu-4B* and *QABA.wsu-7B ERA8* alleles (Figure 4D-F). We also examined whether any of the same QTL were detected based on heading date (*Qhd.wsu*) and final plant height (*Qhei.wsu*) (Supplemental Figure 7). *Qhd.wsu-4A* for heading date was found on chromosome 4A, but the locus did not co-localize with any of the *QABA.wsu* loci (Supplemental Figure 7C). *Qhei.wsu-7B* was found on Chromosome 7B, but was 30 cM away from *QABA.wsu-7B.1*. Louise and Zak both have the wild-type *Rht-B1b* allele (Kidwell *et al*., 2006; Martinez *et al*., 2018b; S. Rynearson, unpublished). Note that height was taken from a single plant and not multiple plants across a plot.

ERA8 mapping by bulked segregant analysis of a backcross population

Because it was difficult to unambiguously map the *ERA8*-causative allele in the Louise/Zak*ERA8* RIL population, we decided to examine whether *ERA8* could be mapped in the Zak/Zak*ERA8* backcross population where the only DNA polymorphisms impacting ABA sensitivity would be those resulting from the EMS-mutagenesis of Zak (Figure 5; Supplemental Figure 1). *ERA8* segregates as a single semi-dominant Mendelian trait in backcross populations when the phenotype is examined at the optimal ABA concentration and after-ripening time point (Schramm *et al*., 2013; Martinez *et al*., 2014). Consistent with this, the Zak/Zak*ERA8* BC_3F_2 (5.2) population showed segregation as a single semi-dominant gene on 2 µM ABA at 5 weeks of after-ripening ($\chi 2 = 1.62$, p-value = 0.20; Supplemental Table 2). The germination of the 300 $BC_3F_{2:3}$ lines exhibited a normal distribution in the presence of 2 µM ABA, and the corresponding, Zak WT and *ERA8* controls showed an average of 66.9 % (SD ± 16.3) and 16.8 % (SD ± 8.8) germination after 5 days of imbibition, respectively (Figure 5D). Thus, an $F_{2:3}$ line was considered to be homozygous WT (+/+) if the percent germination was above 73 % and to be homozygous *ERA8*

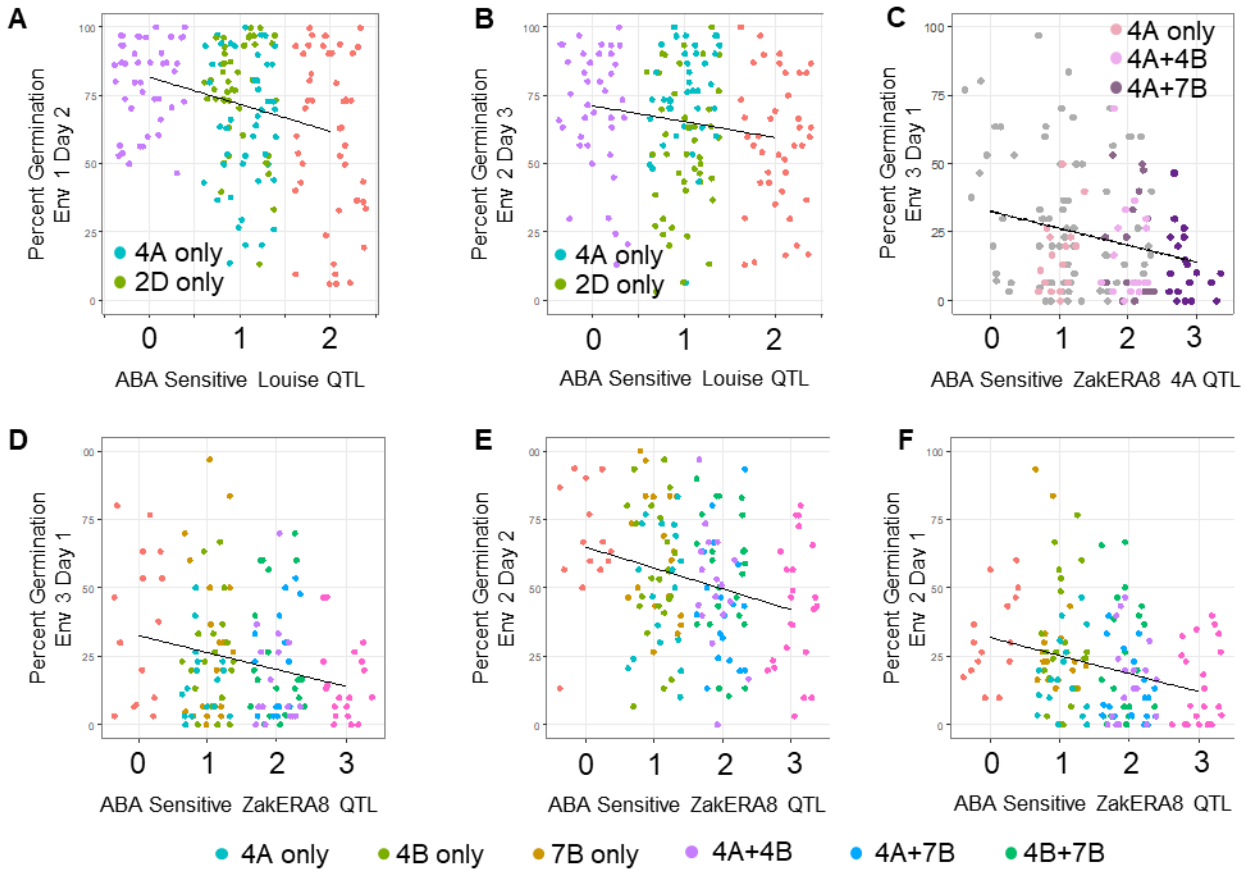
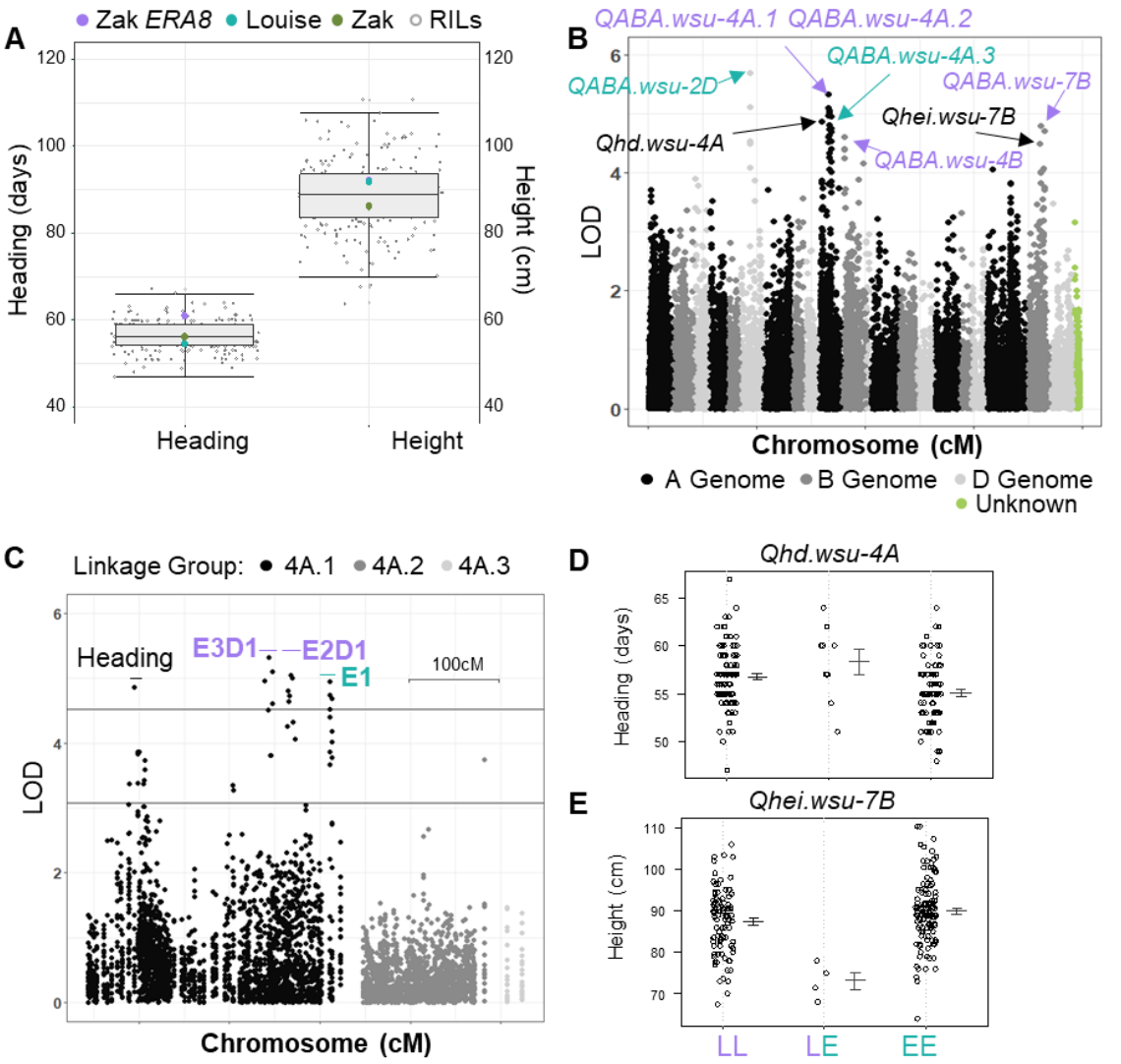


Figure 4 - Significant QTN allelic effects in the Louise/Zak*ERA8* RIL population. ABA sensitivity QTL contributed by Louise include *Qwsu.ABA-4A.3* and *Qwsu.ABA-2D* and are shown in the **A**) Environment (Env) 1 Day 2 and **B**) Env 2 Day 3. The major ABA sensitivity QTL contributed by *ERA8* on chromosome 4A, *Qwsu.ABA-4A.1*, is shown across **C**) Env 3 Day 1 and indicated by RILs with (purple) and without (grey) the 4A QTL Zak*ERA8* allele in combination with the 4B and 7B Zak*ERA8* QTL. The environment significant for *Qwsu.ABA-4A.1*, **D**) Env 3 Day 1, is shown across with all 3 Zak*ERA8* QTL alleles. **E**) Env 2 Day 2 and **F**) Env 2 Day 1 are shown with significant environments for *Qwsu.ABA-4B* and *Qwsu.ABA-7B*, respectively.



F

QTL Name	Marker	Chromosome	Pos. (cM)	LOD	Trait
Qhd.wsu-4A	A23913	4A	50.93	4.87	Heading
Qhei.wsu-7B	A2003	7B	252.3	4.49	Height

Supplemental Figure 7 - QTL analysis of heading and height data (**A**) taken on the Louise/Zak*ERA8* RIL population. LOD scores across **B**) all chromosomes (cM) and **C**) only for chromosome 4A are shown for height (*Qhei.wsu*), days to heading (*Qhd.wsu*), and ABA sensitivity (*QABA.wsu*). Significant QTL with a threshold of $p < 0.10$ are shown for height and heading (**black**), *ERA8* ABA sensitivity (purple), and Louise ABA sensitivity (blue). Alleles were compared between heading date (**D**, days) and height (**E**, cm) differences in the RIL population separated by Louise (LL) and *ERA8* (EE) alleles. **F**) QTL are considered significant if the LOD scores have a $p < 0.05$.

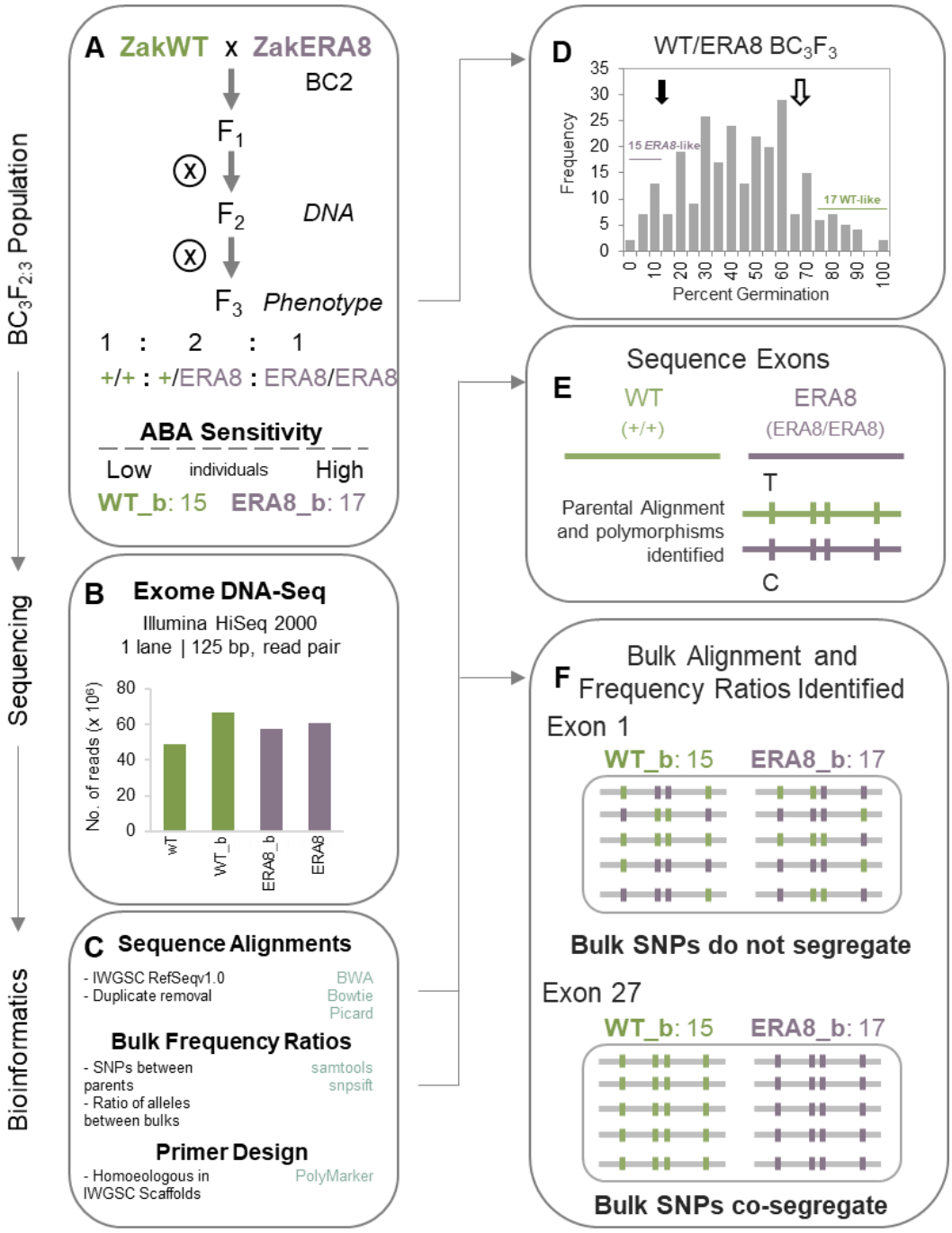


Figure 5 – An overview of the bulk segregant analysis method. **A**) The $BC_3F_{2:3}$ population was developed by crossing Zak wild-type (WT) and Zak*ERA8* BC_2 (*ERA8*). Genomic DNA was extracted from the BC_3F_2 2-3 leaf stage for exome capture. The percent germination on ABA of the $BC_3F_{2:3}$ seed (grey), *ERA8* (black arrow), and WT (hollow arrow) **D**) 15 individuals had low ABA sensitivity during germination, WT-like bulks (WT_b), and 17 individuals had high ABA sensitivity during germination, *ERA8*-like bulks (*ERA8*_b). **B**) The number of reads generated from the WT parent, WT_b, *ERA8*_b, and *ERA8* parent are reported. **C**) Multiple programs were used to align the sequences, calculate the bulk ratios, and design the SNP primers. An example of an exon alignment and bulk frequency ratios are shown (**E**-**F**).

(*ERA8*/*ERA8*) if below 24 %. These individuals were tested on ABA two more times to confirm the phenotype. The 15 $BC_3F_{2:3}$ lines that consistently showed high percent germination on ABA were defined as WT-like and the 17 individuals that had consistently showed low percent germination on ABA were defined as *ERA8*-like (Figure 5A, D). The WT-like and *ERA8*-like phenotypes were confirmed through F_4 progeny testing (Appendix Figure 3.15). Bulked segregant analysis was performed using bulked genomic DNA from the *ERA8*-like (*ERA8*_b) and WT-like (WT_b) $BC_3F_{2:3}$ lines.

This is the first study to map an EMS-generated allele in wheat using bulked segregant analysis of high throughput exome DNA sequence. In order to maximize the number of informative sequences, genomic DNA was enriched for coding sequence using exome capture before performing high-throughput DNA sequencing on the Illumina HiSeq 2500 (48-67 million reads per sample; Figure 5B; Supplemental Table 5). C to T and G to A polymorphisms between Zak WT and *ERA8* were identified by aligning to the RefSeqv1.0 wheat genome and variants were identified using three different methods (Figure 5C, E). EMS-induced SNPs identified between WT and *ERA8* were then screened against the *ERA8*-like and WT-like bulked exome sequences. If a region of the chromosome was not associated with the ABA hypersensitive phenotype, then a polymorphic SNP should segregate randomly, appearing in about 50 % in the WT bulk reads and 50 % in the *ERA8* bulk reads (Figure 5F). Conversely, if a region of the chromosome was associated with the ABA hypersensitive phenotype, then an EMS SNP within the *ERA8*-like bulk should result in nearly 100 % *ERA8* allele and 0 % WT allele. A large region of chromosome 4A showed strong enrichment for EMS-induced mutations in the *ERA8*-like bulk sequences (Figure 6). This region spanned most of chromosome 4A (33-613 Mb), and included 75 EMS-induced SNPs in the coding regions of 46 genes. Of these, 42 EMS-induced SNPs in the coding region of 27 genes were located on the long arm of chromosome 4A (Table 3). Interestingly, both the BSA exome sequencing and the Louise/Zak*ERA8* QTL mapping concluded that *ERA8* was located on the chromosome 4A.

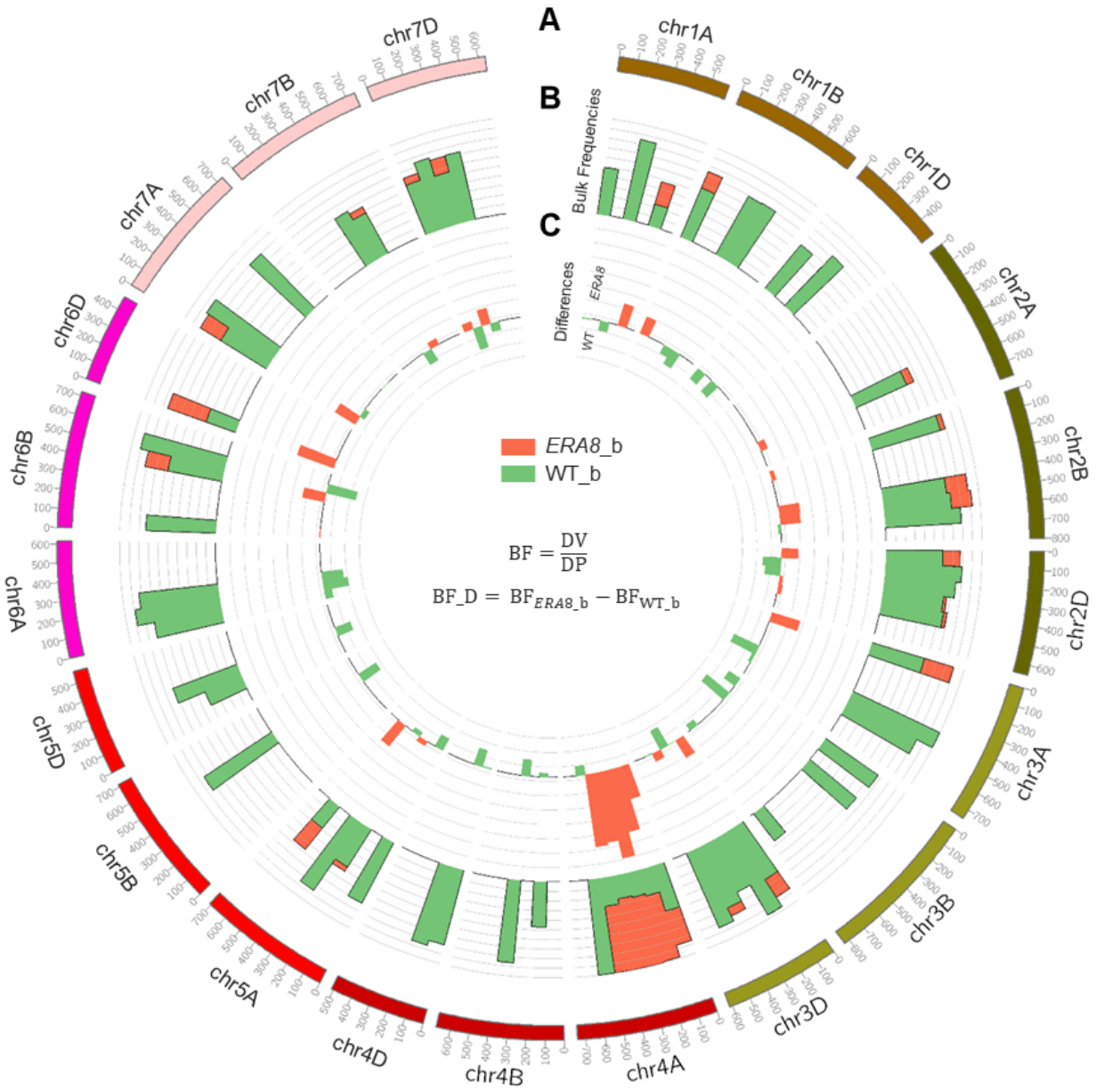


Figure 6 – Bulk frequencies aligned to the wheat genome. **A**) The RefSeqv1.0 wheat reference genome sequence spans the three (A, B, D) genomes with seven homologous (1-7) chromosomes each. **B**) Bulk frequencies (BF) of the *ERA8*_b (red) and WT_b (green) were aligned to the reference genome and were calculated as a fraction of the alternate allele read depth (DV) over the total number of reads. **C**) The BF differences (BF_D) were calculated from the differences between the BF of *ERA8*_b and the BF of WT_b.

Table 3 - Unique genes with SNPs identified between Zak and Zak*ERA8* in the BSA Exome capture found on the chromosome 4A long arm.

Gene Model Name [a]	Annotation	SNP Position
TraesCS4A01G164300	Retrotransposon protein, putative, Ty3-gypsy subclass	379,028,350
TraesCS4A01G172100	Pectinesterase	436,828,695
TraesCS4A01G184200	DEAD-box ATP-dependent RNA helicase 20, Uncharacterized protein	462,216,491
TraesCS4A01G192900	Phototropic-responsive NPH3 family protein	473,941,968
TraesCS4A01G193300	Methyl esterase 17	474,743,604
TraesCS4A01G216500	O-fucosyltransferase family protein	515,464,915
TraesCS4A01G217700	DEAD/DEAH box RNA helicase family protein	517,408,530
TraesCS4A01G220100	DNA polymerase	522,920,126
TraesCS4A01G224300	-	532,074,134
TraesCS4A01G225100	Sucrose-phosphate synthase	533,004,875
TraesCS4A01G225500	PHD finger protein	533,446,137
TraesCS4A01G227600	ABC transporter C family member 8, Uncharacterized protein	535,859,159
TraesCS4A01G232700	Endonuclease III homolog	542,033,771
TraesCS4A01G232800	ATP-dependent Clp protease ATP-binding subunit, Uncharacterized protein	542,033,771
TraesCS4A01G393100LC	Transposon protein CACTA, En/Spm sub-class	551,208,584
TraesCS4A01G250800	Endoplasmic oxidoreductin-2, Uncharacterized protein	562,286,039
TraesCS4A01G266600	Dynamin-related protein 1C, Uncharacterized protein	578,543,168
TraesCS4A01G278800	nucleobase-ascorbate transporter 7	586,452,395
TraesCS4A01G281700	BHLH family protein, putative, expressed, protein, Putative centromere protein	589,604,960
TraesCS4A01G284500	rRNA methyltransferase	590,988,110
-	Pollen allergen KBG 41, Uncharacterized protein	591,417,336
TraesCS4A01G299700	High affinity cationic amino acid transporter 1, Uncharacterized protein	597,908,536
TraesCS4A01G303900	3-ketoacyl-CoA synthase	599,613,160
TraesCS4A01G311100	Poly(A) RNA polymerase GLD2-A, Uncharacterized protein	603,446,405
TraesCS4A01G312200	GSK1 transcription factor 1	603,532,130
TraesCS4A01G319100	2OG-Fe- II - oxygenase superfamily	608,044,262
TraesCS4A01G325400	Pentatricopeptide repeat-containing protein	613,268,437

[a] Gene names with an LC at the end are from Low Confidence genes in the RefSeqv1.0 annotation. The remaining genes are High Confidence genes.

Fine Mapping of *ERA8* in RIL and Backcross Populations

Fine mapping of the *ERA8* mutation was performed using mutagen-induced SNPs identified in the BSA-exome-seq assayed as KASP markers (Supplemental Table 8). When the Louise/Zak*ERA8* RILs were assayed for an initial ten KASP markers (SNP_1 through SNP_10), *ERA8*-linked QTL appeared to localize to the chromosome 4A centromeric region between SNP_2 and SNP_5 (Table 2; Figure 7A). However, the LOD scores were fairly low, all below 4.0. In order to fine map in the backcross population, an additional 2,381 Zak/Zak*ERA8* were advanced from BC_3F_2 to F_3. Based on an initial random 145 Zak/Zak*ERA8* $BC_3F_{2:3}$ lines, the *ERA8* phenotype was not associated with the SNP_2 to SNP_5 region, but rather was most strongly associated with the most centromere-distal marker, SNP_6 (Figure 7B). Because this result suggested that *ERA8* is further from the centromere, we examined KASP markers more distal from the centromere (SNP_20, SNP_17, SNP_29, and SNP_30). Based on 424 Zak/Zak*ERA8* $BC_3F_{2:3}$ lines, the *ERA8* QTL was strongly associated with SNP_20, SNP_17, and SNP_29 (Figure 8A-C; Supplemental Figure 8D). The SNP_20 to SNP_29 interval contains 70 predicted genes (Table 4). SNP_17 had the highest LOD score, ranging from 16.51 to 5.24 depending on the population (X5.1 or X5.2, respectively) and plating experiment (Figure 8A-C). The X5.2 population likely segregated for a weaker phenotype because it lost more dormancy due to longer storage in the freezer before plating. Interestingly, SNP_17 is an *ERA8*-linked missense mutation in the cloned PHS gene, *TaMKK3-A*.

A comparison of phenotype to the location of recombination events was used to fine map *ERA8* relative to the four mutagen-induced SNPs in the *ERA8*-linked region (SNPs 6, 20, 17 and 29; Table 4). Both heterozygous (Figure 9A, B) and homozygous (Figure 9C, D) lines were placed into haplotype groups (I to VII) based on the location of recombination events. When the location of recombination events (Figure 9A, C) was compared to germination phenotype on ABA (Figure 9B, D), those groups carrying the *ERA8*-allele of SNP_17/*MKK3* appeared to have the most *ERA8*-like germination phenotype. Unfortunately, our ability to fine-map *ERA8* is severely limited by the fact that only four genes in the *ERA8* region contained mutagen-induced SNPs. If additional markers were available, then individual

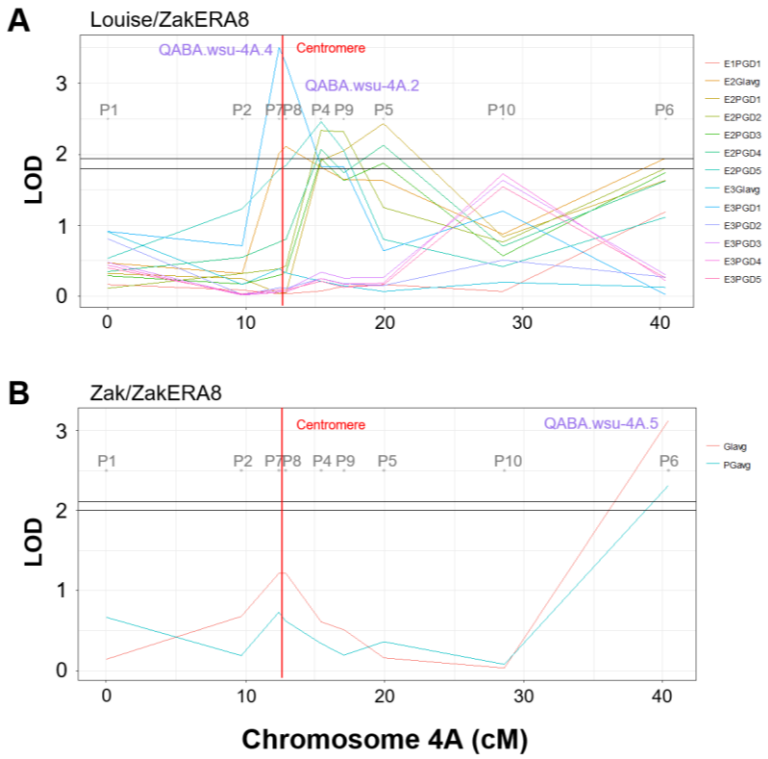


Figure 7 – QTL analysis of the *ERA8* region using the **A)** Louise/Zak*ERA8* RILs and the **B)** Zak/Zak*ERA8* $BC_3F_{2:3}$ population across the EMS-induce SNP markers (P1-10). The three Louise/Zak*ERA8* environments (E1, E2, E3) percent germination (PG) are shown across days (D1-D5) of imbibition and germination index (GI). An additional 145 Zak/Zak*ERA8* $BC_3F_{2:3}$ were initially screened against the EMS-induced SNP markers. The lowest and highest significant threshold ($p < 0.05$) of all the environments or traits are indicated by the black lines. The centromere is indicated by the red vertical line.

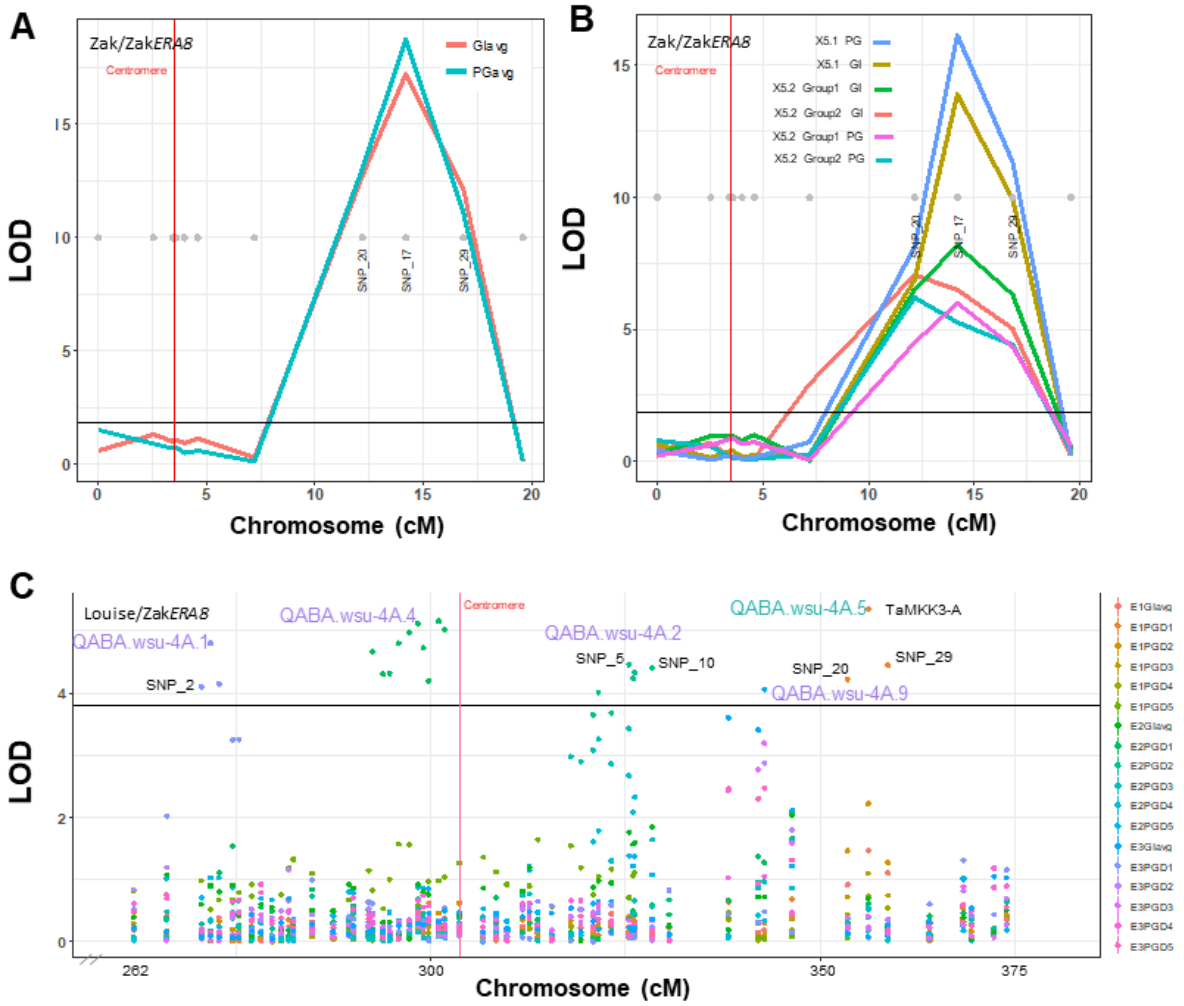
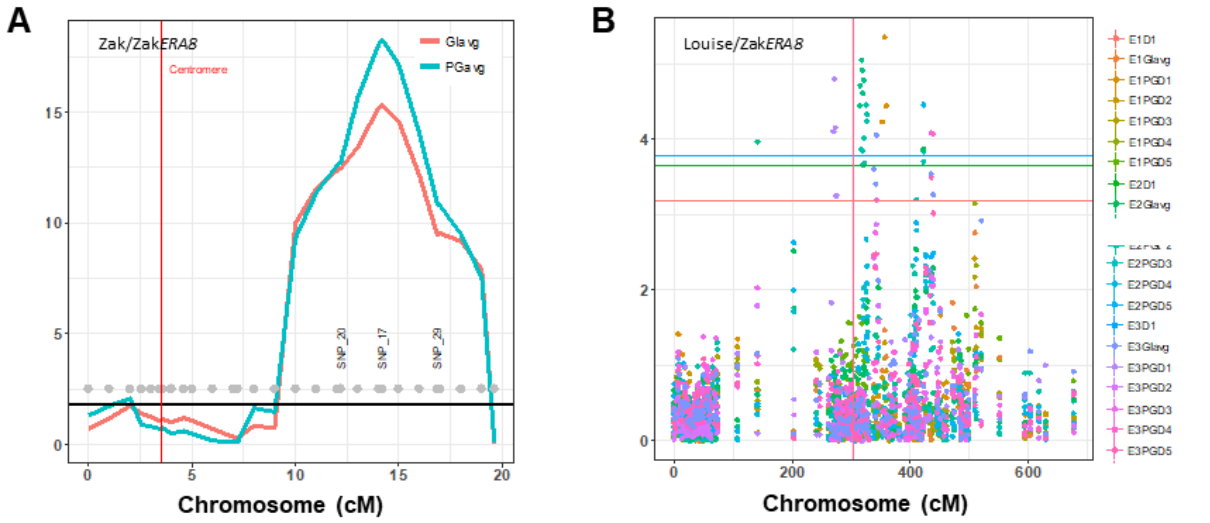
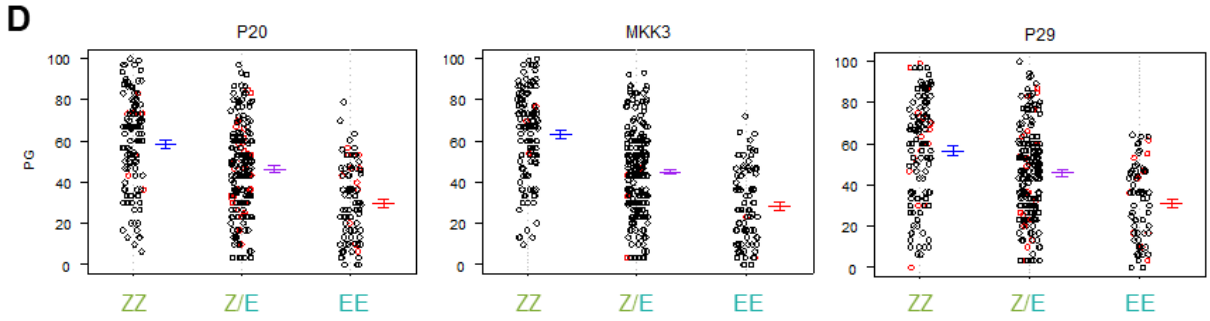


Figure 8 – Fine mapping *ERA8* on chromosome 4A. QTL analysis of percent germination (PG) and germination index (GI) on ABA in the Zak/Zak*ERA8* $BC_3F_{2:3}$. $F_{2:3}$ families are shown combined (**A**) and separated (**B**) by 5.1, 5.2 group 1, and 5.2 group 2. rQTL was used to calculate LOD scores across each KASP marker indicated in grey. **C**) QTL analysis was conducted on the Louise/Zak*ERA8* RIL population using both the GBS and EMS-induce SNP markers, which included Ta*MKK3*-A, and is shown across the 262 cM to 375 cM region.



C

QTL Name	Marker	Chromosome	Pos. (cM)	LOD	Trait	Favorable Allele
QABA.wsu-4A.4	A10010	4A	301.0	5.16	E2D1	G/**A**
QABA.wsu-4A.5	SNP_17	4A	356.2	5.35	E1D1	**G**/A
	MKK3-A	4A	356.2	5.35	E1D1	**C**/A
QABA.wsu-4A.6	A23553	4A	141.1	3.96	E2D1	**G**/A
QABA.wsu-4A.7	A28342	4A	422.9	4.46	E2D5	**A**/C
QABA.wsu-4A.8	A190	4A	439.1	4.08	E3D5	**G**/T
QABA.wsu-4A.9	A29481	4A	342.8	4.05	E3GI	T/**C**



Supplemental Figure 8 – Fine mapping *ERA8* on chromosome 4A. **A**) QTL analysis of percent germination (PG) and germination index (GI) on ABA in the Zak/Zak*ERA8* $BC_3F_{2:3}$. **B**) QTL analysis was conducted on the Louise/Zak*ERA8* RIL population using both the GBS and EMS-induced SNP markers, which included *TaMKK3-A*. **C**) QTL from Louise/Zak*ERA8* are considered significant if the LOD scores have a $p < 0.05$. Favorable alleles decrease germination percent or index. Louise or *ERA8* contribute to the first or second allele, respectively. **D**) Significant QTN allelic effects in the Zak/Zak*ERA8* backcross population for SNP_20, *MKK3* (SNP_17), and SNP_29. Homozygous Louise (**ZZ**) and homozygous *ERA8* (**EE**) alleles are labeled in green and blue, respectively.

Table 4 - Genes in the *ERA8* interval on chromosome 4A between SNP_20 and SNP_29 markers.

Gene Model Name	Annotation	Position	Orientation	EMS SNP	Primer
TraesCS4A01G299700	High affinity cationic amino acid transporter 1, Uncharacterized protein	597,908,536	+	C → T	SNP_6
	Poly(A) RNA polymerase GLD2-A, Uncharacterized protein	603,446,405		C → T	SNP_19
TraesCS4A01G311800	-	603,496,846	-	-	
TraesCS4A01G311900	-	603,504,474	+	-	
TraesCS4A01G312000	-	603,507,001	+	-	
TraesCS4A01G312100	-	603,510,936	-	-	
TraesCS4A01G312200	GSK1 transcription factor 1	603,530,872	-	C → T	SNP_20
TraesCS4A01G312300	C2 domain	603,533,711	-	-	
TraesCS4A01G312400	Domain of unknown function - DUF296 - PPC domain	603,539,435	-	-	
TraesCS4A01G312500	-	603,634,586	-	-	
TraesCS4A01G312600	Cyclophilin type peptidyl-prolyl cis-trans isomerase -CLD	603,637,101	+	-	
TraesCS4A01G312700	Major intrinsic protein	603,666,065	+	-	
TraesCS4A01G312800	NB-ARC domain	603,674,385	+	-	
TraesCS4A01G312900	NB-ARC domain	603,698,121	+	-	
TraesCS4A01G313000	-	603,699,642	-	-	
TraesCS4A01G313100	Leucine rich repeat	603,726,600	-	-	
TraesCS4A01G313200	YUCCA9	603,980,902	-	-	
TraesCS4A01G313300	PM19-A1	604,055,487	-	-	
TraesCS4A01G313400	PM19-A2	604,068,519	+	-	
TraesCS4A01G313500	Myosin-J-Protein	604,076,529	+	-	
TraesCS4A01G313600	Ubiquintin Congujating Enzyme E2-23	604,101,314	+	-	
TraesCS4A01G313700	ACC Oxidase -1 Like	604,104,116	-	-	
TraesCS4A01G313800	B3-domain-containing protein	604,107,899	+	-	
TraesCS4A01G313900	LRR receptor-Like Kinase Serine/threonine-protein Kinase	604,209,020	-	-	
TraesCS4A01G314000	ACC Oxidase -1 Like	604,594,773	-	-	
TraesCS4A01G314100	Agmatine coumaroyltransferase	604,659,700	+	-	
TraesCS4A01G314200	LRR receptor-like serine/threonine-protein kinase	604,672,359	+	-	
TraesCS4A01G314300	Disease resistance RPP13-like protein 3	604,772,917	+	-	
TraesCS4A01G314400	Disease resistance protein RPM1	604,849,387	-	-	
TraesCS4A01G314500	ERF-1B-Like	604,856,919	+	-	
TraesCS4A01G314600	ERF-1B-Like	604,940,912	+	-	
TraesCS4A01G314700	-	605,018,735	-	-	
TraesCSU01G167000	Ta*MKK3*-A [a]	-		G → A	SNP_17
TraesCS4A01G314800	Hypothetical protein	605,024,348	+	-	
TraesCS4A01G314900	Sugar Carrier protein A-Like	605,087,172	-	-	

Gene Model Name	Annotation	Position	Orientation	EMS SNP	Primer
TraesCS4A01G315000	PP1-like - serine/threonine-protein phosphatase	605,137,125	-	-	
TraesCS4A01G315100	Wall-associated receptor kinase galacturonan-binding; Protein kinase domain	605,559,326	-	-	
TraesCS4A01G315200	Myb -SANT-like DNA-binding domain	605,640,606	+	-	
TraesCS4A01G315300	Myb -SANT-like DNA-binding domain	605,644,924	+	-	
TraesCS4A01G315400	Plastocyanin-like	605,650,522	-	-	
TraesCS4A01G315500	Chaperonin Cpn60 -TCP-1 family	605,656,215	+	-	
TraesCS4A01G315600	-	605,663,094	-	-	
TraesCS4A01G315700	RNA polymerase sigma-70 region	605,711,154	+	-	
TraesCS4A01G315800	-	605,717,570	+	-	
TraesCS4A01G315900	ABC transporter-like	605,744,452	+	-	
TraesCS4A01G316000	-	606,116,294	+	-	
TraesCS4A01G316100	F-box domain	606,335,446	+	-	
TraesCS4A01G316200	Heavy-metal-associated domain	606,345,914	-	-	
TraesCS4A01G316300	RNA-binding protein Lupus La	606,361,307	+	-	
TraesCS4A01G316400	GDSL-like Lipase - Acylhydrolase	606,370,282	+	-	
TraesCS4A01G316500	GDSL-like Lipase - Acylhydrolase	606,397,403	+	-	
TraesCS4A01G316600	-	606,411,251	+	-	
TraesCS4A01G316700	Protein of unknown function	606,523,442	+	-	
TraesCS4A01G316800	WD domain - G-beta repeat	606,536,121	-	-	
TraesCS4A01G316900	-	606,585,463	+	-	
TraesCS4A01G317000	-	606,591,102	+	-	
TraesCS4A01G317100	-	606,593,057	-	-	
TraesCS4A01G317200	-	606,608,730	+	-	
TraesCS4A01G317300	-	606,643,500	-	-	
TraesCS4A01G317400	-	606,647,800	-	-	
TraesCS4A01G317500	Salt stress response -antifungal; Gnk2-homologous domain	606,761,092	+	-	
TraesCS4A01G317600	Protein kinase domain; ATP binding; protein phosphorylation; Salt stress response -antifungal; Gnk2-homologous domain	606,796,989	+	-	
TraesCS4A01G317700	-	606,811,790	+	-	
TraesCS4A01G317800	-	607,045,327	+	-	
TraesCS4A01G317900	LOG family; Possible lysine decarboxylase	607,177,351	-	-	
TraesCS4A01G318000	-	607,178,696	+	-	
TraesCS4A01G318100	LOG family; Possible lysine decarboxylase	607,261,062	-	-	

Gene Model Name	Annotation	Position	Orientation	EMS SNP	Primer
TraesCS4A01G318200	-	607,270,018	+	-	
TraesCS4A01G318300	-	607,270,941	-	-	
TraesCS4A01G318400	Domain associated at C-terminal with AAA; AAA-type ATPase - N-terminal domain	607,309,139	+	-	
TraesCS4A01G318500	-	607,374,419	-	-	
TraesCS4A01G318600	-	607,378,421	+	-	
TraesCS4A01G318700	-	607,417,280	+	-	
TraesCS4A01G318800	Domain of unknown function DUF303 - acetylesterase putative	607,427,390	-	-	
TraesCS4A01G318900	Domain of unknown function DUF303 - acetylesterase putative	607,432,144	+	-	
TraesCS4A01G319000	-	607,633229	+	-	
-	-	607,886,990		-	Barc170
TraesCS4A01G319100	-	608,043,459		G → A	SNP_29
TraesCS4A01G325400	Pentatricopeptide repeat-containing protein; Pyridine nucleotide-disulphide oxidoreductase	613,268,437		G → A	SNP_30

[a] Based on the physical mapping of *TaMKK3-A* in Shorinola *et al*. (2017), the gene should be located at the position in red.

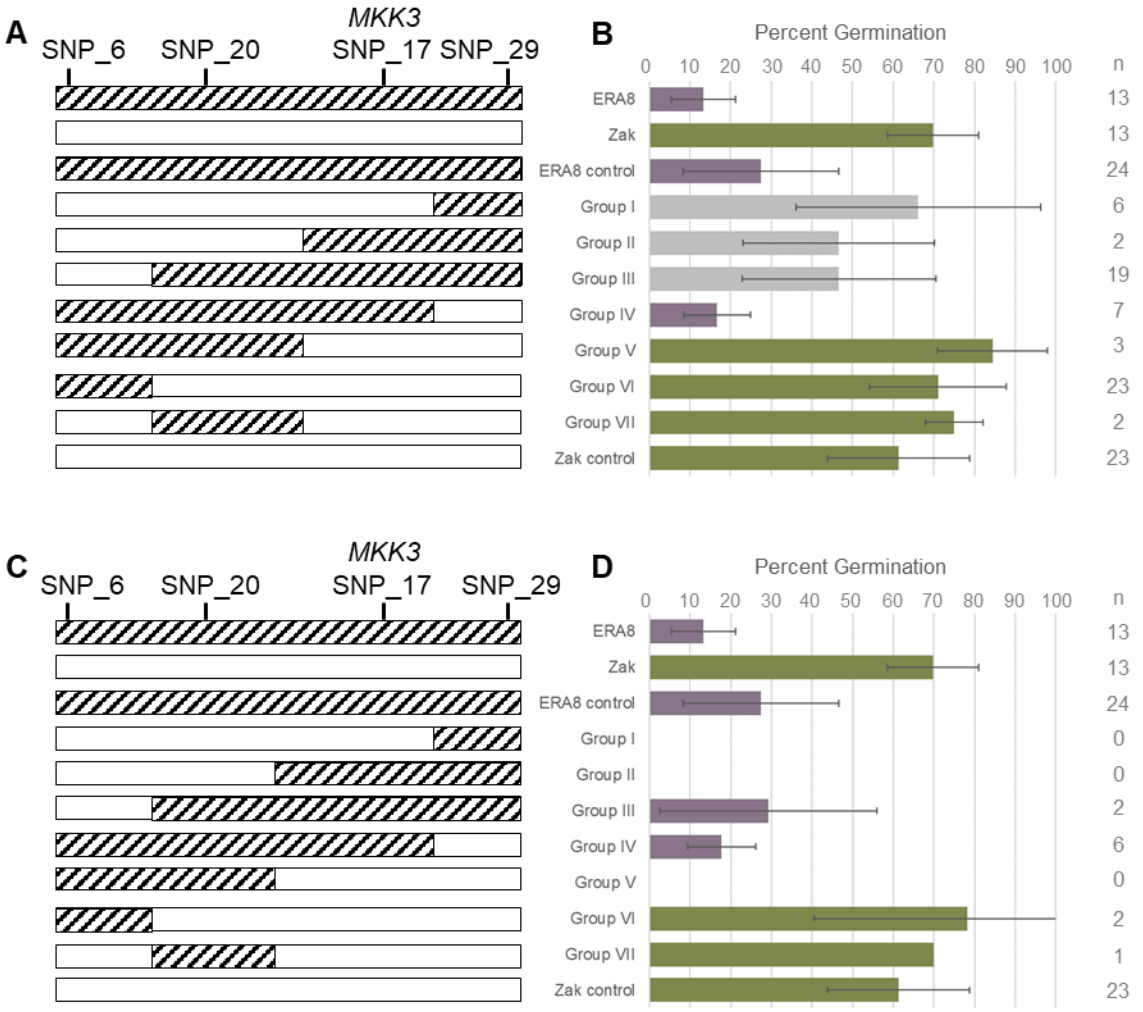
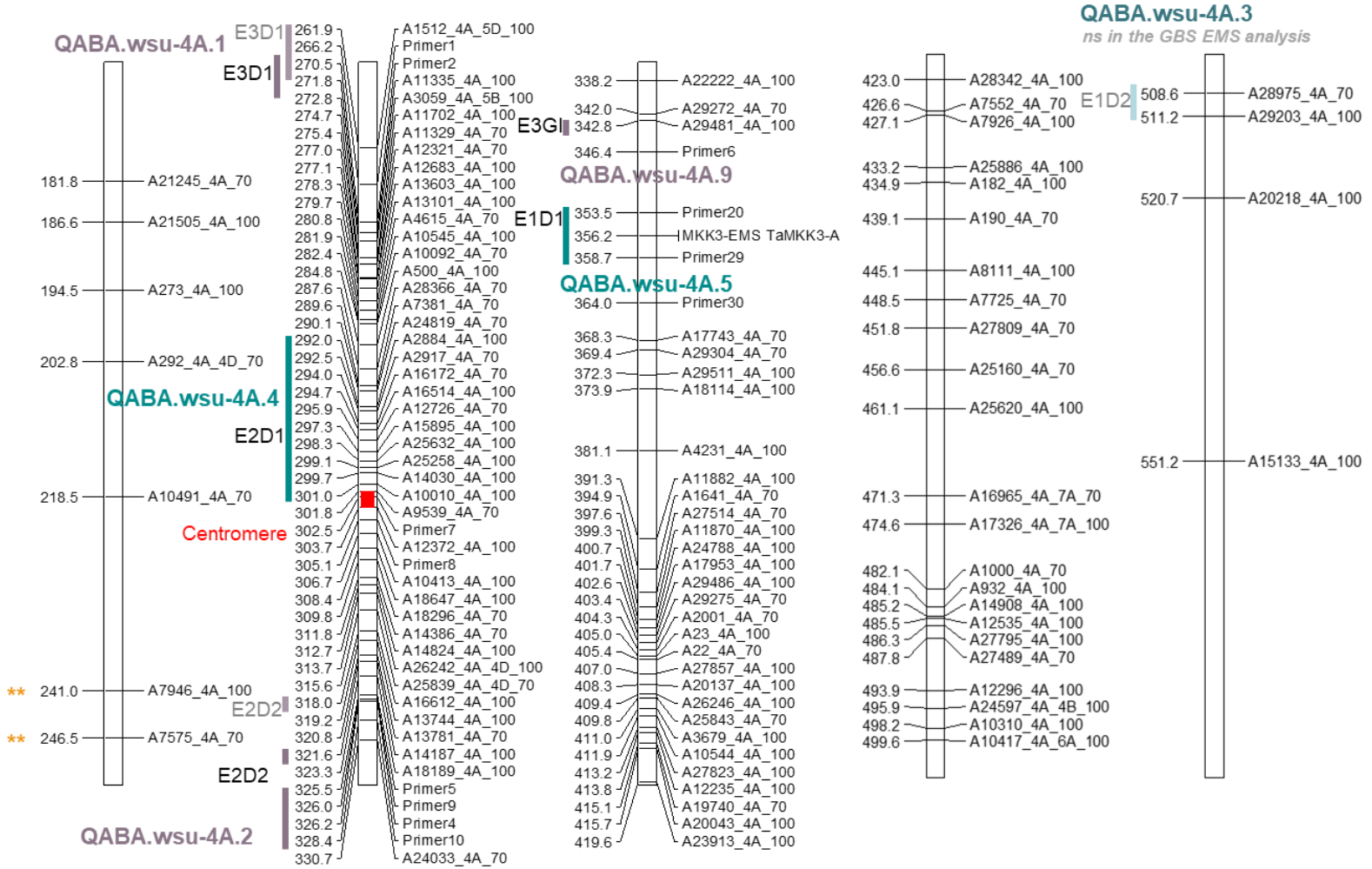


Figure 9 – Interval mapping of *ERA8* in Zak/Zak*ERA8* $BC_3F_{2:3}$ population. **A**) A linkage map of SNP_6, SNP_20, SNP_ 17 (*MKK3*), and SNP_ 29 markers across the *ERA8* interval where the *ERA8* and WT genotype are represented by filled or hollow bars, respectively. The genotypes of each group are aligned next to their percent germination phenotype on ABA (**B**). Some heterozygous *(+/ERA8)* genotypes were included in **A** and **B**. An analysis without heterozygotes are shown in **C** and **D**. ABA sensitivity of each backcross group is represented by *ERA8*-like (purple), Zak-like (green), or moderate (grey) compared to the parents. Error bars represent the standard deviation of the raw means.



Supplemental Figure 9 – Louise/Zak*ERA8* RIL population linkage map of the GBS (A###), EMS-induced (Primer##), and *TaMKK3* markers on chromosome 4A. QTLs identified are shown in black text across three environments (E) over different days of imbibition (D#) or germination index (GI). Favorable loci increasing ABA sensitivity by Louise or *ERA8* are denoted by blue or purple bars and text, respectively. QTL names, *QABA.wsu*, are listed in Table 2 or Supplemental Figure 8C. The centromere is indicated in red. QTLs were found in the initial analysis with only GBS markers (grey text). GBS Markers with polymorphisms between Zak and *ERA8* are indicated with an orange **.

recombination events in groups I, II, IV and V might be able to determine whether *ERA8* is most likely caused by the mutation in *MKK3* or in one of the neighboring genes.

To examine whether *ERA8* would map to the *MKK3* region in the Louise/Zak*ERA8* RIL population, the additional 13 mutagen-induced SNPs were genotyped on this population. Both the GBS and mutagen-induced markers were analyzed simultaneously to produce a more resolved linkage map (Supplemental Figure 9). QTL mapping identified eight significant QTL on chromosome 4A (Figure 8C; Supplemental Figure 8B, C). Unfortunately, none of the GBS polymorphisms were located in the SNP_20 to SNP_29 region, and so did not increase the marker density in the *ERA8* region. The *QABA.wsu-4A.3* locus contributed by Louise was no longer significant. While QTL analysis again detected QTL in the SNP_2 to SNP_5 region contributed by Zak*ERA8* (*QABA.wsu-4A.1*, *QABA.wsu-4A.2*, and *QABA.wsu-4A.4*), the strongest favorable QTL corresponding to SNP_17 (*QABA.wsu-4A.5*) was actually contributed by Louise (LOD 5.35). This QTL located at SNP_17 was only significant in one environment (E1) on the first day of imbibition. It was not detected in the other two environments, based on percent germination on other days of imbibition, nor based on germination index (Appendix Figure 3.16). Thus, the *ERA8* phenotype was not significantly associated with SNP_17 in the Louise/Zak*ERA8* RIL population. For some reason, the *ERA8* phenotype was not as strong in the Louise/Zak*ERA8* population as it was in the Zak/Zak*ERA8* backcross population. This is interesting in light of the fact that another RIL population Zak*ERA8*/Diva of 280 lines did not appear to segregate for ABA sensitivity (Martinez, unpublished; Appendix Figure 3.17). There are two possible explanations for this result, either there are other loci in the Louise background that suppress the *ERA8* phenotype, or both Louise and Zak*ERA8* contributed ABA-sensitive alleles of *TaMKK3-A* to the RIL population.

Alleles of *TaMKK3-A*

Based on the hypothesis that the *ERA8* phenotype may have resulted from a mutation in the *TaMKK3-A* coding region, we characterized the *TaMKK3-A* alleles of Zak, Zak*ERA8*, and Louise using KASP assays (Shorinola *et al*., 2017). The naturally-occuring PHS tolerant allele of *TaMKK3-A/Phs1* in wheat is

recessive, such that a C at position +660 is dormant and PHS tolerant whereas an A at position +660 is nondormant and PHS susceptible (Torada *et al*., 2016). The cultivar Zak, and consequently Zak*ERA8*, had the susceptible *TaMKK3-A*-A660^{S} allele (Figure 10; Supplemental Figure 10; Shorinola *et al*., 2017). In addition, the Zak*ERA8* line carries a G to A transition at position +1093 of *TaMKK3-A*. This missense mutation results in a change from a negatively-charged glutamic acid (E) to positively-charged lysine (K) at amino acid 365, within the conserved Nuclear Transport Factor 2 (NTF2) domain. Interestingly, KASP assays showed that Louise has the dormant *TaMKK3-A*-C660^{R} allele and the Zak-like *TaMKK3-A*-G1093^{S} allele (Figure 10B). Thus, it is possible that both Zak*ERA8* and Louise carry alleles of *MKK3* resulting in higher dormancy and ABA sensitivity. If true, this would explain why the *ERA8* phenotype was not strongly linked to SNP_17 in the Louise/Zak*ERA8* RIL population. In this case, the QTL mapped were due to contributions from other loci affecting ABA sensitivity in the population, or were due to one *MKK3* allele or the other contributing more seed dormancy in a particular environment. In this scenario, both the *ERA8* allele, *TaMKK3-A*-A1093^{R}, and the *TaMKK3-A*-C660^{R} allele from Louise are contributing higher seed dormancy and ABA sensitivity, while the Zak haplotype consists of both of the nondormant alleles, *TaMKK3-A*-A660^{S} and *TaMKK3-A*-G1093^{S}. Future work will need to determine whether the G1093A *MKK3* sequence variant results in the semi-dominant ABA-hypersensitive *ERA8* phenotype.

Differentially Expressed Genes in *ERA8*

RNAseq was used to identify *ERA8* candidate genes as *ERA8*-linked genes differentially expressed in imbibing Zak*ERA8* versus wild-type seeds. Based on fine mapping, we know that *ERA8* must be within the 4,597,054 bp between SNP_20 and SNP_29 (Figure 11; Table 4). This region contains 70 genes of which *TaMKK3-A* is the only gene carrying a mutagen-induced coding sequence mutation. We cannot conclude that *TaMKK3*-A1093^{R} is the *ERA8* allele because it is possible that *ERA8* results from a non-coding-region mutation in an *MKK3*-linked gene. If the semi-dominant *ERA8* phenotype is caused by a promoter mutation, then we might see strong up-regulation of a positive regulator of ABA signaling or

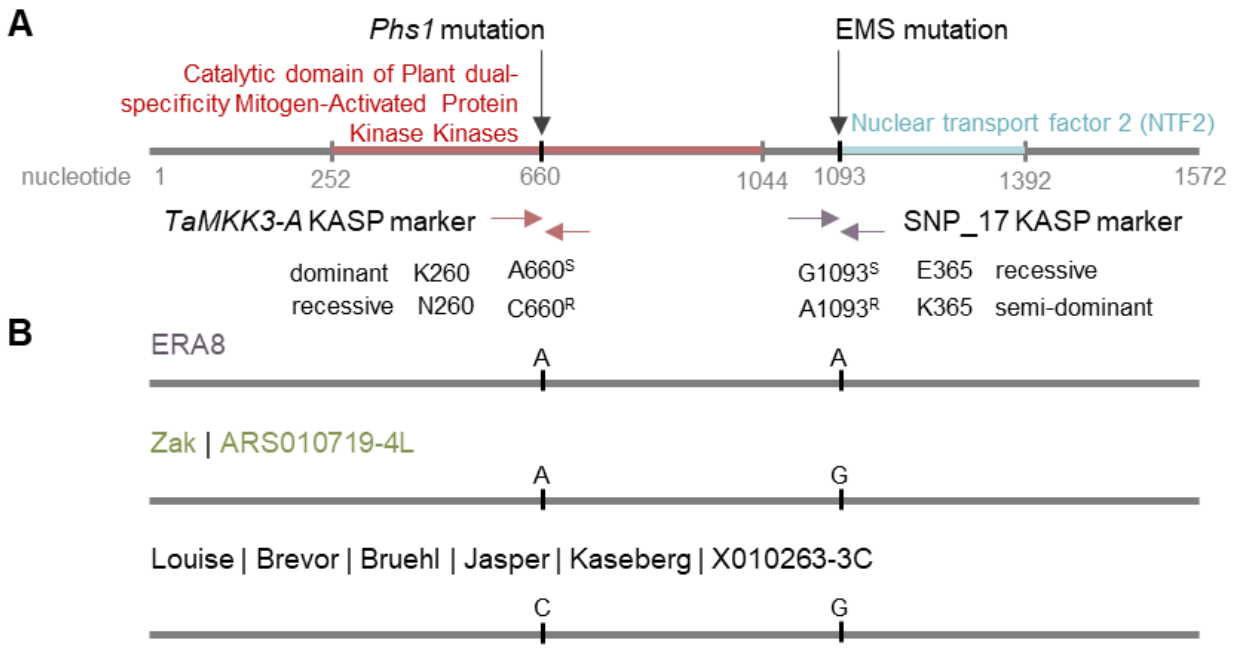


Figure 10 - A detailed diagram of the PHS tolerant *TaMKK3-A* gene and the alleles present in this study. **A**) *TaMKK3-A* includes the *TaPhs1* and EMS-induced mutations at the +660 and +1093 nucleotides, respectively, and are denoted with either an S (susceptible) or an R (resistant). This schematic includes the protein domains, KASP marker positions, SNP dominance, and the amino acid change. **B**) The genotypes at both mutation sites of *ERA8*, Zak, Louise, and other breeding material are shown.

Supplemental Figure 10 –The *TaMKK3-A* gene sequence of Zak wild-type and *ERA8*. **A**) Coding region nucleotide sequences include the *TaMKK3-A* C660A mutation (blue) found in Torada *et al*. (2016) and the *TaMKK3-A* G1093A EMS mutation (purple) found between Zak WT and *ERA8*. **B**) The protein sequence of *TaMKK3-A* contains a Catalytic domain of Plant dual-specificity Mitogen-Activated Protein Kinase Kinases (light blue) and a Nuclear transport factor 2 (NTF2; green). Note this is reverse complement relative to the reference scaffold.

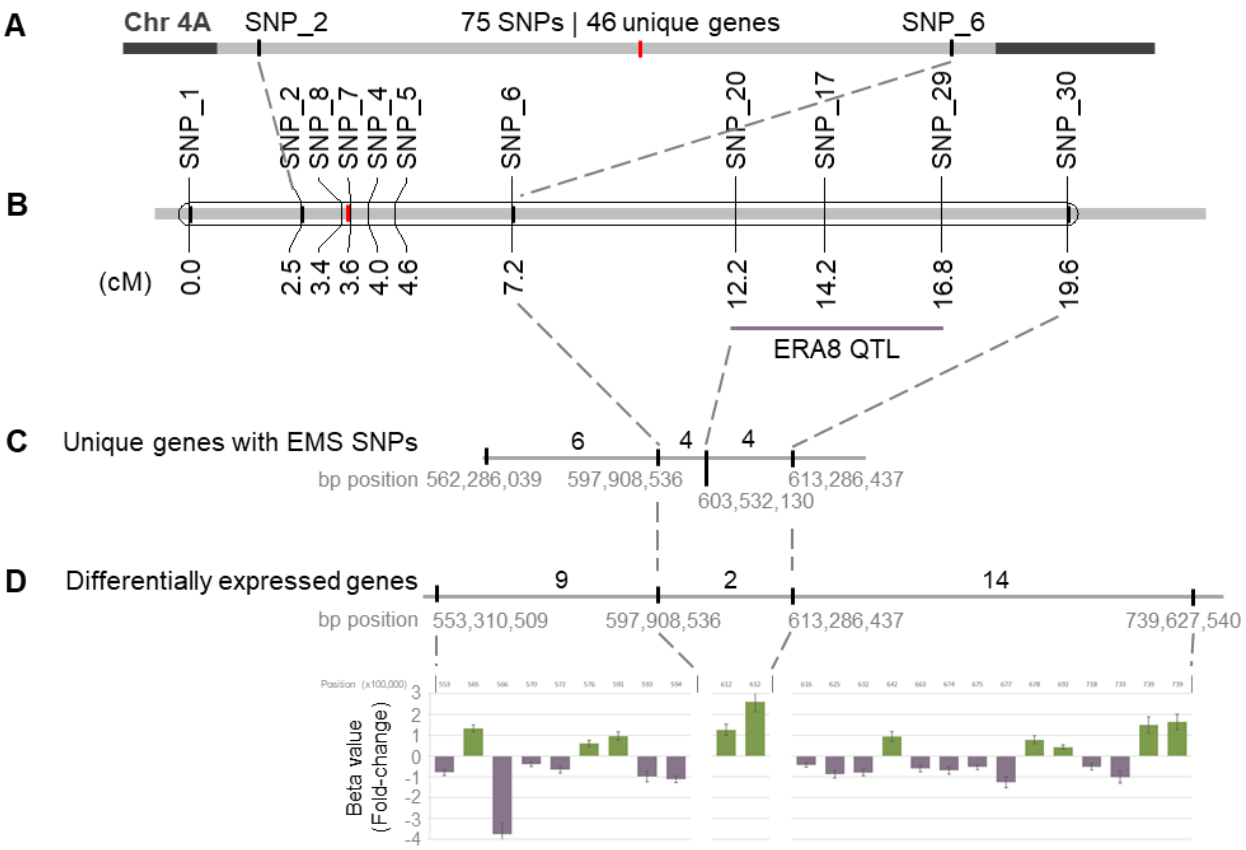


Figure 11 - The *ERA8* interval. **A**) Initial BSA exome sequencing identified the *ERA8* region (light grey line) on chromosome 4A (dark grey line) using a total of 32 bulks. The centromere is indicated with red. **B**) An additional 424 $BC_3F_{2:3}$ and 225 RILs were used to identified *ERA8* in a 4.6 cM region using EMS-induced primers (SNP_#). **C**) BSA identified 13 unique genes with C-T or G-A EMS SNPs between *ERA8* and WT. **D**) Comparison of WT versus *ERA8* from 1.5 yr after-ripened seeds imbibed 8 hr within the region differentially expressed 25 gene, such that positive values are relatively higher in WT than *ERA8*. The beta value in 'sleuth' is analogous fold change where a beta value > 1.5 is considered a significant change. Error bars represent the standard error of beta. Positions were determined by the Refseqv1.0 wheat reference genome.

down-regulation of a negative regulator. The semi-dominant germination phenotype would then result from a change in the dosage of a regulatory gene. *ERA8* might also result from a gain-of-function amino acid change altering the structure and function of the *ERA8* protein (i.e. *MKK3*). In such a scenario, the *ERA8* mRNA level may be down-regulated if the plant has a negative feedback response to increased function. Such a negative feedback response may be particularly apparent in long after-ripened *ERA8* seeds showing higher germination efficiency. Future work may also be able to detect *ERA8* as a positive-regulator of ABA signaling regulated by ABA or by after-ripening. mRNA was prepared from Zak*ERA8* BC_2F_3 and Zak WT seeds that had been after-ripened for 1.5 years (Figure 12). Seeds were imbibed for 8 hrs without ABA in the dark. Embryos were harvested into liquid nitrogen under a green safe light in order to enrich for transcripts involved in regulating seed germination while avoiding the induction of photosynthesis transcripts that may represent as much as 50 % of RNAseq reads from photosynthetic tissue (Trick *et al*., 2012).

The most significantly differentially regulated genes were located on chromosomes 2B (*ERA8*-up/WT-down) and 2D (WT-up/*ERA8*-down) (Table 5). On chromosome 4A, there were four WT-up/*ERA8*-down genes including Beta-fructofuranosidase 1, LRR family protein, BED zinc finger/hAT family dimerization domain protein, and a Retrotransposon protein (Table 6). There were eight *ERA8*-up/WT-down genes including FAR1-RELATED SEQUENCE 5 protein, Sentrin-specific protease 1, S-adenosyl-L-methionine-dependent methyltransferases superfamily protein, MLO-like protein, Sphingoid base hydroxylase 2, and a Transposon protein. There were only two differentially regulated genes within the SNP_6 to SNP_30 interval. (Figure 11). Both of these genes are homologues of BED Zinc finger family proteins believed to be involved in stress response (Joly-Lopez *et al*., 2017). The BED Zinc finger family protein gene, TraesCS4A01G485500LC, was more highly *ERA8*-downregulated than its nearby homologue, TraesCS4A01G485400LC. This pattern of expression would be consistent with down-regulation of a negative regulator of ABA signaling. However, both BED Zinc finger homologues were located between SNP_29 and 30, indicating that they are not in the strongest *ERA8*-linked region between SNP_ 20 and SNP_29. Under these conditions, the *MKK3* gene itself did not show any significant change

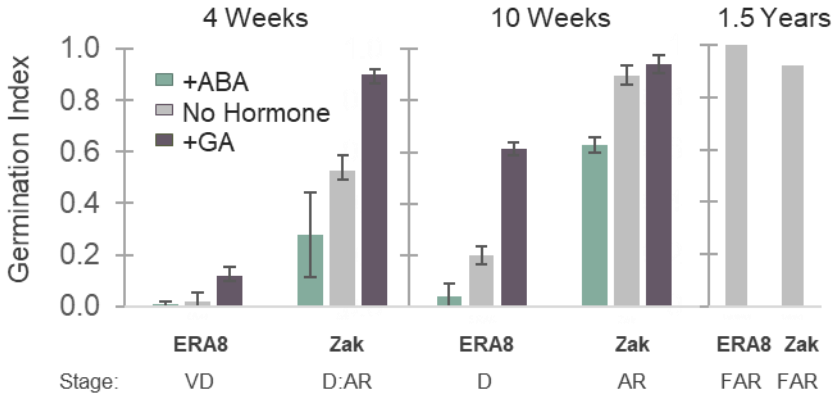


Figure 12 - The response of *ERA8* to exogenous hormones and after-ripening. **A**) The germination profile of Zak WT and *ERA8* on ABA (green), GA (purple) and no hormone (grey). Seeds were after-ripened for 4 weeks, 10 weeks, and 1.5 years. Stages of dormancy were based on the germination profile in the presences and absence of ABA and GA are very dormant (VD), dormant (D), after-ripened (AR), and fully after-ripened (FAR). There were three technical replicates of 10 seeds each during the 4 and 10 week samples, however the 1.5 year samples were only one replicate of 10 seeds, due to limited seed.

Table 5 - Unique genes differentially expressed between WT and *ERA8* throughout the wheat genome.

Gene ID	Annotation	Chrm	Start	End	beta [a]
TraesCS2B01G524400	-	2B	718,960,478	718,968,471	**-7.08**
TraesCS3B01G322600	-	3B	521,789,364	521,797,431	**-5.69**
TraesCS2D01G000600	Polycomb group protein VERNALIZATION 2	2D	278,541	286,171	**-5.26**
TraesCS6A01G338800	DNA (Cytosine-5-)-methyltransferase	6A	572,133,054	572,138,876	**-5.20**
TraesCS7D01G269200	Reticulocyte-binding protein 2 a	7D	253,341,441	253,348,906	**-5.05**
TraesCS7B01G229200	-	7B	430,829,130	430,842,036	**5.20**
TraesCS2D01G497100	Protein UXT-like protein	2D	593,215,038	593,217,521	**5.24**
TraesCS1A01G374800	Zinc finger protein-like	1A	549,837,796	549,847,148	**5.39**
TraesCS3B01G338100	-	3B	544,780,837	544,793,535	**5.42**
TraesCS7B01G484300	DUF789 family protein	7B	741,573,444	741,579,514	**5.57**
TraesCS2D01G500500	-	2D	595,157,820	595,161,251	**5.58**

[a] Beta value is analogous to fold-change in slueth. A fold change of an absolute value of 1.5 or higher is considered differentially expressed. A positive fold-change means there was more expression in WT compare to *ERA8*. A negative fold-change means there was more expression in *ERA8* compare to WT.

Table 6 - Unique genes differentially expressed between WT and *ERA8* on chromosome 4A.

Gene ID [a]	Annotation	Chrm	Start	End	beta [b]
TraesCS4A01G016000	Transcription factor	4A	10,164,187	10,165,250	1.09
TraesCS4A01G021100	F-box and associated interaction domains-containing protein	4A	14,340,239	14,344,018	-1.47
TraesCS4A01G021900LC	Protein yippee-like	4A	17,974,706	17,977,539	0.87
TraesCS4A01G022000LC	Retrotransposon protein, putative, unclassified	4A	17,978,744	17,981,991	**1.80**
TraesCS4A01G029300LC	Protein FAR1-RELATED SEQUENCE 5	4A	25,211,087	25,215,287	**-4.17**
TraesCS4A01G041700	-	4A	35,181,518	35,183,648	**-4.29**
TraesCS4A01G050500	SNP_1	4A	41,115,524	-	-
TraesCS4A01G052700LC	Sodium/hydrogen exchanger	4A	46,171,955	46,199,813	-0.94
TraesCS4A01G052800LC	Sentrin-specific protease 1	4A	46,173,178	46,174,094	**-2.92**
TraesCS4A01G079100	-	4A	80,840,552	80,844,726	**-4.10**
TraesCS4A01G087400	SNP_2	4A	91,795,916	-	-
TraesCS4A01G092100	Heat-shock protein, putative	4A	98,785,386	98,787,839	-0.80
TraesCS4A01G097400	Tryptophan synthase alpha chain	4A	108,369,445	108,371,437	1.43
TraesCS4A01G099900	Histone H2B	4A	112,767,092	112,767,812	0.51
TraesCS4A01G103900	SNP_13	4A	117,337,422	-	-
TraesCS4A01G121600	SNP_14	4A	150,302,500	-	-
TraesCS4A01G126600	8-amino-7-oxononanoate synthase	4A	163,345,723	163,347,867	1.01
TraesCS4A01G130600	NAC domain protein,	4A	173,630,224	173,632,115	0.69
TraesCS4A01G131600	SNP_7	4A	175,858,520	-	-
TraesCS4A01G131700	SNP_3	4A	176,543,353	-	-
TraesCS4A01G167000LC	Mitochondrial transcription termination factor family protein	4A	207,976,194	207,976,616	-1.32
TraesCS4A01G179300LC	2'-phosphotransferase	4A	230,997,232	231,003,186	0.67
TraesCS4A01G172100	SNP_8	4A	436,828,695	-	-
TraesCS4A01G248100LC	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	4A	340,489,977	340,490,856	**-2.02**
TraesCS4A01G160600	Phosphatidate phosphatase, Lipin	4A	345,835,861	345,844,921	1.17
TraesCS4A01G316400LC	Serine/threonine-protein kinase	4A	465,545,872	465,547,035	-0.81
TraesCS4A01G323300LC	Cysteine desulfurase, putative, expressed	4A	474,726,464	474,739,302	0.47
TraesCS4A01G194800	Non-specific serine/threonine protein kinase	4A	476,977,837	476,980,583	0.69
TraesCS4A01G202600	Carboxypeptidase	4A	492,530,884	492,534,660	0.70
TraesCS4A01G340900LC	MLO-like protein	4A	496,577,022	496,578,105	**-2.55**
TraesCS4A01G220100	DNA polymerase	4A	522,907,385	522,921,948	0.92
TraesCS4A01G221500	Sphingoid base hydroxylase 2	4A	527,112,529	527,113,641	**-2.65**
TraesCS4A01G224300	SNP_4	4A	532,074,134	-	-
TraesCS4A01G225500	SNP_9	4A	533,446,137	-	-
TraesCS4A01G232700, or TraesCS4A01G232800	SNP_15	4A	542,033,771	-	-
TraesCS4A01G243200	RING/U-box superfamily protein	4A	553,310,509	553,314,175	-0.79
TraesCS4A01G252800	Cathepsin B-like cysteine protease	4A	565,040,316	565,043,816	1.32
TraesCS4A01G411100LC	Transposon protein, putative, mutator sub-class	4A	566,101,663	566,104,738	**-3.76**
TraesCS4A01G258100	Lariat debranching enzyme	4A	570,981,287	570,985,759	-0.39
TraesCS4A01G259300	Anthocyanin 5-aromatic acyltransferase	4A	572,302,274	572,303,653	-0.65
TraesCS4A01G264600	UDP-glucose 6-dehydrogenase	4A	576,930,165	576,932,752	0.60

TraesCS4A01G278800	SNP_10	4A	586,452,395	-	-
TraesCS4A01G444900LC	Peptide transporter	4A	591,496,571	591,499,205	0.97
TraesCS4A01G288000	Argonaute protein	4A	593,194,838	593,200,481	-0.99
TraesCS4A01G290300	Ankyrin repeat family protein	4A	594,180,282	594,186,698	-1.12
TraesCS4A01G299700	SNP_6	4A	597,908,536	-	-
TraesCS4A01G311100	SNP_19	4A	603,446,405	-	-
TraesCS4A01G312200	SNP_20 GSK1 transcription factor 1	4A	603,532,130	-	-
TraesCSU01G167000	SNP_17 *MKK3*	unk	-	-	-
-	barc170	4A	607,886,990	-	-
TraesCS4A01G319100	SNP_29	4A	608,044,262	-	-
TraesCS4A01G485400LC	BED zinc finger,hAT family dimerization domain	4A	612,510,456	612,511,014	1.26
TraesCS4A01G485500LC	BED zinc finger,hAT family dimerization domain	4A	612,511,090	612,513,756	**2.60**
TraesCS4A01G325400	SNP_30	4A	613,286,437	-	-
TraesCS4A01G331900	Cinnamoyl-CoA reductase 4	4A	616,309,725	616,313,994	-0.44
TraesCS4A01G345800	Threonine synthase 1, chloroplastic	4A	625,029,910	625,030,335	-0.86
TraesCS4A01G359200	-	4A	632,150,159	632,155,404	-0.80
TraesCS4A01G370500	ABC transporter G family member	4A	642,209,939	642,213,850	0.94
TraesCS4A01G386400	Auxin repressed/dormancy associated protein	4A	663,986,105	663,987,373	-0.59
TraesCS4A01G593300LC	3'(2'),5'-bisphosphate nucleotidase 1	4A	674,553,838	674,556,604	-0.69
TraesCS4A01G401700	Cysteine synthase	4A	675,600,394	675,603,117	-0.53
TraesCS4A01G599200LC	Zinc finger MYM-type protein 4	4A	677,402,029	677,403,093	-1.26
TraesCS4A01G404800	arabinogalactan protein 5	4A	678,340,392	678,341,039	0.78
TraesCS4A01G421900	Histone H3	4A	692,358,749	692,359,852	0.43
TraesCS4A01G454800	Glutathione S-transferase	4A	718,848,586	718,850,423	-0.52
TraesCS4A01G473900	F-box protein	4A	733,670,552	733,672,307	-1.01
TraesCS4A01G485900	Beta-fructofuranosidase 1	4A	739,310,199	739,314,035	**1.50**
TraesCS4A01G486600	disease resistance family protein / LRR family protein	4A	739,627,540	739,630,396	**1.64**

[a] The genes model with LC are low confidence gene model and those without LC are High confidence gene models.
[b] Beta value is analogous to fold-change in slueth. A fold change of an absolute value of 1.5 or higher is considered differentially expressed and indicated in **bold**. A positive fold-change means there was more expression in WT compare to *ERA8*. A negative fold-change means there was more expression in *ERA8* compare to WT.

in expression in WT versus *ERA8*. Thus, the RNAseq data gave neither evidence suggesting an alternate *ERA8* candidate gene showing differential expression due to a potential promoter mutation, nor evidence confirming *MKK3* as a likely *ERA8* candidate gene.

Discussion

BSA-Exome-Seq as a Method to Map EMS-Induced Mutations

This is the first paper to demonstrate that bulked segregant analysis of exome-seq data can be used to rapidly map a gene in the large allohexaploid wheat genome relative to mutagen-induced polymorphisms. This approach combined two previously published methods. Exome capture was developed as a technique to characterize all of the coding-region EMS-induced mutations in a wheat TILLING population (Henry *et al*., 2014). This technique enables researchers to restrict whole genome resequencing to the predicted coding regions of the wheat genome. Previous work showed that BSA-RNAseq could be used to map genes relative to varietal DNA polymorphisms identified by RNAseq (Trick *et al*., 2012). We combined these two approaches to map a mutation using bulked-segregant analysis of exome sequence from bulked genomic DNA from WT-like and *ERA*-like individuals from a $BC_3F_{2:3}$ population. A similar approach was used to map tall versus short EMS-induced alleles from an M_3 individual of the tetraploid wheat Kronos TILLING population (Mo *et al*., 2017). BSA allows for mapping in a backcross population using a relatively small number of confidently phenotyped F_2 or F_3 individuals. While we used BC_3 populations, it is possible that this could be done in the first backcross to the mutagenesis parent, enabling rapid mapping of mutagen-induced genetic variants. In the case of the *ERA8* mutation, the mutant plants became healthier and the ABA hypersensitive phenotype more consistent with additional backcrosses. This may have occurred because background EMS mutations were removed by backcrossing. Thus, researchers interested in mapping subtle phenotypes may find it helpful to perform additional backcrosses before investing in BSA-exome-seq.

Comparison of *ERA8* Mapping by QTL Analysis and BSA-Exome-Seq

There are both advantages and disadvantages to mapping in an RIL population as opposed to in a backcross population. Creation of RIL populations may require more time than creating a population from a single backcross event since plants must be advanced by single seed descent to at least the F_5 generation. Furthermore, mapping in a backcross population decreases the impact of other genes seen in the QTL analyses, simplifying the genetics. While Louise was selected as an RIL population parent based on having dormancy characteristics similar to Zak, we were blind to the precise genetic alleles contributing to the dormancy or lack of dormancy in Louise (Figure 10). Thus, we were surprised when we learned that Louise carried a dormant allele of the *MKK3* gene; not an ideal choice given the suspicion that *ERA8* is a new allele of *MKK3*. However, both the Louise and Zak*ERA8* parents contributed other QTL associated with higher ABA sensitivity during germination to the RIL population (Table 2). These QTL are of potential use for PHS tolerance breeding in northwest U.S. wheat since pyramiding of multiple ABA-sensitive QTL provided increasing seed dormancy (Figure 4). BSA of the backcross population had the advantage that the ABA hypersensitive phenotype showed Mendelian segregation because *ERA8* was the major locus contributing the ABA hypersensitive phenotype (Supplemental Table 2; Martinez *et al*., 2014). One drawback to the mapping in the backcross population was the paucity of polymorphisms in the interval containing *ERA8*. We were unable to confidently localize *ERA8* to region smaller than 4,597,054 bp because we were unable to find additional polymorphisms between SNP_20 and SNP_29 (Table 4). A mapping population derived from a cross between near-isogenic lines containing varietal difference within one chromosome segment may have the advantage of having a higher density of mutations (Yan *et al*, 2003; Trick *et al*., 2012). But such a population has the drawback that there may be multiple sequence polymorphisms within a candidate gene(s), making it more challenging to identify the mutation causing the phenotype of interest (Shorinola *et al*., 2017). Future work will need to examine if *TaMKK3-A-ERA8* segregation can be better followed in a cross to a line carrying the PHS susceptible *TaMKK3-A-A660*^{S} allele. Clearly, the *ERA8* phenotype does not segregate

cleanly in crosses to cultivars other than Zak which is why we need molecular markers to deploy the gene.

ERA8 Candidate Genes

Several lines of evidence indicate that *MKK3-A* is the strongest candidate for the *ERA8* gene. First, SNP_17/*TaMKK3-A-A1093*^{R} is the strongest QTL mapped in either population, with a LOD as high as 16.51 (Figure 8A). Second, G1093A was the only apparent EMS-induced coding region mutation within the *ERA8*-linked interval between SNP_20 and SNP_29 (Table 4). Third, there were no genes in the SNP_20 to SNP_29 interval showing differential expression in Zak versus *ZakERA8* (Table 6). Thus, it does not appear that the *ERA8* phenotype could result a promoter mutation in this interval leading to altered expression of a regulatory gene. Fourth, is the notion that we were unable to map the *ERA8* phenotype in the Louise/Zak*ERA8* RIL population because both Louise and Zak*ERA8* carry dormant alleles of *TaMKK3-A* (Figure 8C).

The original exome-seq analysis selected only C to T and G to A transitions for mapping in order to filter out sequence differences that were not caused by EMS mutagenesis. Future work will need to reanalyze the Zak versus Zak*ERA8* exome sequence to determine if there are any additional *ERA8*-linked sequence polymorphisms within this 70-gene region that might explain the *ERA8* phenotype. The *TaMKK3-A-G1093A* polymorphism seems likely to cause a phenotype because it is predicted to cause an E365K amino acid change within the conserved NTF2 domain (Danquah *et al*., 2015). Among Map Kinases, the NTF2 domain is unique to *MKK3* gene family members and is believed to be involved in importing RanGDP into the nucleus (Quimby *et al*, 2000). It is unclear at this point why such an amino acid change would result in a semi-dominant phenotype. However, it is known that dormant alleles of *MKK3* can result from both gain and loss of function mutations because the dormant wheat *MKK3* K220N allele is recessive, whereas the dormant barley N260T allele is dominant (Nakamura *et al*., 2016; Torada *et al*, 2016). Both natural variants in wheat and barley are amino acid polymorphisms in the kinase domain. While sequence analysis suggests that *TaMKK3-A-G1093A* should result in altered gene

function, future work will need to determine if this allele results in an ABA hypersensitive germination phenotype when transformed into Zak or another variety carrying the PHS susceptible *TaMKK3-A-A660*^{S} allele.

There is one piece of evidence that is not consistent with the idea that *ERA8* is a new allele of *TaMKK3-A*. *TaMKK3-A* is expressed in grain, roots, cotyledons, and leaves of wheat (Torada *et al*., 2016). Thus, if *ERA8* is a mutation in *MKK3*, we would have expected it to result in a vegetative increase in wheat ABA sensitivity. Preliminary experiments looking at the effect of *ERA8* on leaf stomatal conductance, however, did not detect any statistically significant change (Schramm, 2010). In contrast, the ABA hypersensitive *Warm4* (Wheat ABA-responsive mutant 4) of Chinese spring exhibited both seed germination and vegetative phenotypes, resulting in decreased stomatal conductance (Schramm *et al*., 2010). This observation either suggests that *ERA8* is not an allele of *TaMKK3-A* or it suggests that the mutation in the NTF2 domain alters function in seeds but not vegetative function.

If transformation of the *TaMKK3-A-G1093A* into a *TaMKK3-A-A660*^{S} background fails to result in an ABA hypersensitive phenotype, then we will need to consider other candidate genes. None of the current data can rule out the possibility that the *ERA8* phenotype results from an intron splice junction mutation. If this were the case, then we would expect the affected transcript to be larger in *ERA8* than in Zak. The RNAseq data will be reanalyzed to determine if any of the 70 candidate genes in the SNP_20 to SNP_29 interval show such an increase in transcript size in *ERA8*. Alternatively, transcript sizes could be evaluated by PCR from cDNA or by northern analysis. If *ERA8* is not an allele of *MKK3*, then we would need to further investigate the BED zinc finger as a candidate identified as an *ERA8*-downregulated gene. Interestingly, previous work in Arabidopsis suggested that tandem zinc finger proteins are regulators of ABA signaling (Lin *et al*., 2011). However, the BED zinc finger family proteins are outside of our region of strongest linkage.

Breeding for PHS Tolerance with *ERA8*

Even if *ERA8* is not an allele of *MKK3*, the identification of SNP_17 as a strong *ERA8*-linked marker will have utility for breeding programs. However, screening of the natural *MKK3* mutation, A660C, also needs to be conducted in order to know which dormancy alleles will be segregating in the cross. Deploying an EMS-induced mutant is also advantageous since the marker associated with the gene will most likely be polymorphic in most outcrosses. Since *ERA8* was selected in a soft white wheat background, it is clear that this gene can be used to breed for PHS resistance regardless of seed coat color. Future breeding efforts will need to move *ERA8* into a hard wheat background to produce bread products versus soft wheat cake products. Previous work has also established that *ERA8* has sufficient dormancy to prevent preharvest sprouting at maturity, but loses dormancy rapidly enough through 8-10 weeks of after-ripening to allow fall planting of winter wheat without a strong negative impact on emergence or yield (Martinez *et al*., 2014, 2016). Farmers would need to be aware, however, that some storage time will be needed before replanting winter wheat. *ERA8* has also been shown to maintain increased seed dormancy compared to Zak at temperatures as low as 20 °C (Martinez *et al*., 2016). Since PHS occurs in both cool and wet conditions, maintaining dormancy at lower temperatures is beneficial. Future work will need to examine the relative utility of the *MKK3-A-A660C* and *-G1093A* alleles for preventing preharvest sprouting. Such a comparison would require the development of near-isogenic lines followed by careful characterization of seed dormancy, after-ripening time, ABA dose-response, and preharvest sprouting tolerance.

The potential role of *MKK3* signaling in controlling wheat grain dormancy and germination

If the *ERA8* phenotype truly results from the *TaMKK3-A-G1093A* mutation, then this result may tell us something about ABA signaling and seed dormancy in wheat. The recovery of *MKK3* alleles providing increased dormancy and PHS tolerance in wheat and barley based both on natural variation and chemical mutagenesis would suggest that *MKK3* is a key regulator of these responses in cereals. An understanding

of the literature about *MKK3* and ABA signaling can help develop testable models for this signaling pathway. Research has long suggested a connection between ABA signaling and MAPKs in cereals because ABA activated MAPKs in the barley aleurone system (Knetsch *et al*., 1996). The *MKK3* gene family is highly conserved in all eukaryotes, including plants such as Arabidopsis (*AtMKK3*), barley (*HvMKK3*), rice (*OsMKK3*), and tobacco (*NtNPK2*) (Shibata *et al*., 1995; Xiong and Yang, 2003; Hamel *et al*., 2006; Nakamura *et al*., 2016; Torada *et al*., 2016). The mitogen-activated kinase signaling cascade was initially characterized based on its role in controlling mitosis and cell growth in yeast and humans (reviewed in Rodriguez *et al*., 2010). MAP kinases function in a wide range of signaling pathways through protein phosphorylation. The Arabidopsis MAP kinase kinase *MKK3* was shown to perform jasmonic acid (JA)-stimulated phosphorylation of the MAP kinase *MPK6*, which in turn negatively regulates the MYC2/JIN1 transcription factor, a positive regulator of root growth (Takahashi *et al*., 2007). This is interesting given that JA hormone appears to stimulate germination in barley and in wheat (Barrero *et al*., 2009; Tuttle *et al*., 2015; Martinez *et al*., 2016; Xu *et al*., 2016). *MKK3* and *MPK6* also play a role in ABA signaling and seed dormancy in Arabidopsis, together with other map kinases *MPK3, MPK17, MPK18, MKK1, MKK9, MPK1, MPK*2, *MPK7*, and *MPK14* (Liu, 2012; Danquah *et al*., 2015). Loss of *AtMKK3* function in Arabidopsis led to ABA hypersensitive seed germination (Danquah *et al*., 2015). This can only be consistent with the observation that an *MKK3* gain-of-function mutation gives ABA hypersensitive germination in *ERA8* if the *TaMKK3-A-G1093A* allele is a dominant negative mutation in *ERA8*. Since the function of the NTF2 domain of *MKK3* is yet unclear, we cannot speculate as to the exact mechanism resulting in this phenotype. Future work will need to examine the known wheat and barley alleles in the context of a structure-function analysis of the *MKK3*. Such an analysis would need to examine both its activity as a kinase and its ability to function in seed germination and other ABA responses such as root elongation and stomatal closure.

There are other clues to the function of *MKK3* in hormone signaling. The ABA-insensitive mutant *ABI1-1*, a gain-of-function mutation in a protein phosphatase type 2C, increased *AtMPK6* activity associated with decreased seed dormancy (Leung *et al*., 2006). ABA hormone and the ABA receptor

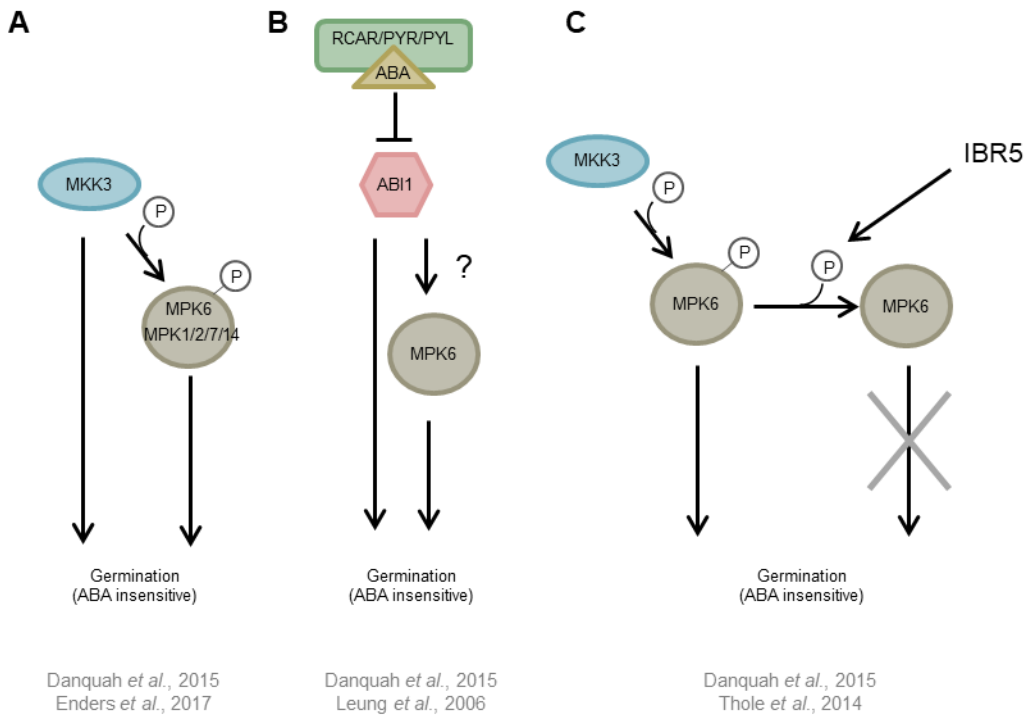


Figure 13 - Proposed model for *Mitogen-activated protein Kinase Kinase3* (*MKK3*) and seed germination based on previously published work. **A**) *MKK3* phosphorylates *Mitogen-activated Protein Kinases* (*MPK*) which results in ABA insensitivity and germination (Danquah *et al*., 2015; Enders *et al*., 2017). **B**) There is also interaction between an *ABA Insensitive1* mutant, *ABI1*, and *MPK6* resulting in ABA insensitivity in seeds (Danquah *et al*., 2015; Lueng *et al*., 2006). **C**) An auxin mutant, *Indole-3-Butyric acid Response5* (*IBR5*), has also been shown to act antagonistically with *MKK3* resulting in the lack of germination downstream (Danquah *et al*., 2015; Thole *et al*., 2014).

(*PYR/PYL/RCAR*) negatively regulate the ABI1 phosphatase via direct protein binding (Park *et al*., 2009; Santiago *et al*., 2009; reviewed by Cutler *et al*., 2010). Thus, one model is that the *MKK3* kinase and ABI1 phosphatase may act as positive regulators of seed germination by stimulating the phosphorylation of *MPK6* and other Map kinases (Figure 13). In this case, we would expect ABA to negatively regulate *MKK3*, which in turn would phosphorylate MPK6, thereby activating MPK6 as a positive regulator of seed germination. Conversely, ABA inhibits the ABI1 phosphatase which interacts with MPK6 protein. However, it is not known if ABI1 removes a phosphate group from MPK6. A candidate for a MAP kinase phosphatase that acts antagonistically to the *MKK3* kinase is, *Ibr5*. Loss of *IBR5* results in ABA-insensitive germination and reduced dormancy (Monroe-Augustus *et al*., 2003; Strader *et al*. 2008; Thole *et al*., 2014; Enders *et al*., 2017). This may result from failure to cleave the phosphate group from Map kinases like MPK6, leading to a constitutively active promoter of seed germination.

Supplemental Material

Supplemental Figure 1 - Flowchart of crossing strategy to generate the backcross mapping population

Supplemental Figure 2 - Louise and *ERA8* parental whole seed and cut seed germination assay

Supplemental Figure 3 - Zak and *ERA8* parental seed germination assay

Supplemental Figure 4 - Phenotypic distributions of the Louise/Zak*ERA8* RIL population

Supplemental Figure 5 - Linkage map of the Louise/Zak*ERA8* RIL population

Supplemental Figure 6 - Allelic effects for each individual QTL from Louise/Zak*ERA8* RILs

Supplemental Figure 7 - QTL analysis of Louise/Zak*ERA8* heading and height

Supplemental Figure 8 - QTL analysis of *ERA8* ABA sensitivity on chromosome 4A

Supplemental Figure 9 - Linkage map of Louise/Zak*ERA8* RIL GBS markers and Exome markers

Supplemental Figure 10 - Zak and Zak*ERA8* coding sequence of *TaMKK3.*

Supplemental Table 1 - Summary of Zak/Zak*ERA8* ABA phenotyping conditions

Supplemental Table 2 - Chi-squared analysis of Zak*ERA8* BC_3 5.2

Supplemental Table 3 - Summary of the linkage groups for the Louise/Zak*ERA8* RIL population

Supplemental Table 4 - The Louise/Zak*ERA8* GBS marker information

Supplemental Table 5 - Exome capture sequencing quality and statistics

Supplemental Table 6 - Alignment statistic of the read using different aligners and algorithms

Supplemental Table 7 - Summary of number and type of variants call for both Bowtie local and Bowtie etex.

Supplemental Table 8 - *ERA8* primers created from the GBS and Exome capture

Supplemental Table 9 - Chi-squared analysis of Zak*ERA8* BC_3 5.1, 5.2, and 5.3

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

Author Contribution Statement

SAM, OS, CMS, and CU designed the experiments. SAM and CMS developed the population. SAM and SC conducted the LZ8 ABA sensitivity, linkage map, and QTL analysis. DS and DS conducted GBS and analyzed the raw sequence data. SAM conducted the ZZ8 ABA sensitivity, *ERA8* fine mapping, and introgression *ERA8* into breeding lines. OS performed the exome capture, BSA, and developed KASP markers. SAM and OS conducted the RNA sequencing and *TaMKK3* alignment. CMS and CU provided resources. SAM and CMS wrote the manuscript.

Acknowledgments: The authors sincerely thank Emily Karlquist, Tracy Harris, and Kerry Balow for developing and maintaining the *ERA8* introgression crosses. Thanks to Nuan Wen, Stephanie Sjoberg, and Anna Pettyjohn for their expert assistance on the *ERA8* marker-assisted selections. The authors would like to thank Ben (Liu) Yiyong for his expertise in RNA sequencing. Thanks are due to members of the Pumphrey, Garland Campbell, and Steber labs assistance with planting, sampling, and harvesting. The authors also thank the International Wheat Genome Sequencing Consortium (IWGSC) for pre-publication access to the wheat genome RefSeqv1.0. A special thanks goes to the Garland Campbell,

Steber, and Uauy labs for helpful feedback on the research and manuscript. This work was funded the USDA-ARS project 424575 and USDA-ARS NIFA (2015-05798).

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CHAPTER FOUR: Registration of the Louise/Alpowa Wheat Recombinant Inbred Line Mapping Population.

(Published as a research paper in the Journal of Plant Registrations)

Abstract

A mapping population was developed from the cross of soft white spring wheat (*Triticum aestivum* L.) cultivars 'Louise' and 'Alpowa' for use in investigating the genetic architecture of drought tolerance in the US Pacific Northwest. The Louise/Alpowa (Reg. No. MP-8, NSL 520824 MAP) recombinant inbred line mapping population was developed through single seed descent from the F_2 generation to the F_5 generation. The population consists of 141 $F_{5:6}$ recombinant inbred lines, of which 132 were used to construct the genetic linkage map. The 32 linkages groups included 882 single nucleotide polymorphism markers and one simple sequence repeat marker spanning 18 of 21 chromosomes. The Louise/Alpowa population was characterized for variation in agronomic traits, phenology, and end-use quality traits. This population will be used for identification and introgression of multiple loci providing resistance to environmental stress such as drought, stripe rust, and high temperatures.

Introduction

The inland northwest of the United States is a major wheat (*Triticum aestivum* L.)–producing region comprising eastern Washington, eastern Oregon, Idaho, and parts of western Montana. Three-quarters of the water-limited dryland farmland in the western United States is located in the inland northwest (Schillinger and Young, 2004). The value of the 2016 wheat crop of Washington, Idaho, and Oregon was $1.27 billion (USDA–NASS, 2017). This environment is unique in that it has the precipitation pattern of a mediterranean climate (winter snow and rain and dry summers) combined with high latitude. The soils in this region retain as much as 70 % of annual precipitation, allowing the production of wheat on stored soil moisture. Nevertheless, wheat yield and grain filling remain particularly vulnerable to periodic and severe drought, causing an average yield loss of 10-20 %, with a value of $90 million per year (USDA–NASS, 2017). Climate models for the next 30 yr predict increasing incidence of drought in this region (Rehfeldt *et al.*,2006; Klos *et al.*,2014).

The inland northwest is an excellent source of drought-tolerant wheat germplasm. Historically, plant breeding programs have capitalized on stress-tolerant genes without knowing the genetic mechanisms contributing to environmental resilience. While “weighing the bag” provides an adequate target for selection, faster progress and greater efficiency could be gained through knowledge of the genetic architecture of stress tolerance and by mapping the contributing quantitative trait loci (QTL) for production characteristics. The soft white spring cultivar Alpowa (PI 566596; Konzak *et al.*,1994) was widely grown for over 14 yr from 1994 to 2008 in the dryland areas of eastern Washington in part because of its stripe rust (causal agent *Puccinia striiformis* Westend f. sp. *tritici* Erikss.) resistance, due to *Yr39* and other minor genes, and high grain yield potential (Chen *et al.*,2004; Lin and Chen, 2007). Although Alpowa was criticized at the time it was widely grown for slow early growth, it was one of few cultivars capable of yielding well under drought-stress conditions (Santra *et al.*,2009). The widely grown soft white spring cultivar Louise (PI 634865, PVP 200500311; Kidwell *et al.*,2006) has been one of the highest yielding cultivars in eastern Washington since 2006. It is known for its superior end-use quality,

high-temperature adult-plant (HTAP) resistance to local races of stripe rust, and high grain yield potential in both high and low rainfall zones (Kidwell *et al.*,2006; Carter *et al.*,2009). The Louise/Alpowa (Reg. No. MP-8, NSL 520824 MAP) recombinant inbred line (RIL) population was developed to examine the genetic components contributing to the superior environmental stress resilience of both cultivars and to provide progeny for breeder selection.

Louise and Alpowa are both well adapted to the inland northwest, but they differ in the loci controlling flowering time in response to vernalization (requirement for exposure to cold to flower) and photoperiod response (requirement for long days to flower) genes. This allows examination of the effect of phenology and flowering time on stress response. The photoperiod genes are major determining factors of climate adaptability in barley (*Hordeum vulgare* L.) and wheat (Worland *et al.*,1998; Cockram *et al.*,2007). The major locus controlling wheat photoperiod response is *Photoperiod-D1* (*Ppd-D1*) (Beales *et al.*,2007). Alpowa has the dominant *Ppd-D1a* allele, resulting in complete photoperiod insensitivity, early flowering, and lack of response to the circadian rhythm (Table 1). Louise has the recessive *Ppd-D1b* photoperiod sensitive allele. Winter wheat requires 4 to 8 wk of vernalization, or cold treatment, to induce flowering. The major loci that are segregating for vernalization response in cultivated wheat are the *Vrn-1* homeologous genes, *Vrn-A1*, *Vrn-B1*, and *Vrn-D1*, on the long arms of chromosomes 5A, 5B, and 5D, respectively (Cockram *et al.*,2007). The winter growth habit (vernalization required) requires recessive *vrn-1* alleles at all three loci, whereas the spring growth habit (no vernalization required) results from a dominant allele at any one of the three *Vrn-1* loci. Louise has two strong spring alleles, *Vrn-A1a* and *Vrn-B1a*, either of which eliminates the requirement for a cold period before initiation of flowering, whereas Alpowa has a single weaker spring *Vrn-B1b* allele, which still has a short (1–2 wk) requirement for a cold period.

This study describes the initial characterization of the Louise/Alpowa RIL population. The effect of varying photoperiod and vernalization loci on the timing of plant developmental events was examined. The population was also examined for variation in agronomic traits under irrigated and nonirrigated

conditions, for variation in resistance to stripe rust, and for end-use quality. Finally, we describe the genetic map of the Louise/Alpowa RIL population based on single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) markers examined in 132 $F_{5:6}$ generation RILs.

Materials and Methods

Parents

Louise was selected from the cross Wakanz (PI 506352)/Wawawai (PI 574538) (Kidwell *et al.*,2006). Wakanz has a pedigree of K-78504//K-7400195/Arthur-71/5/Henry/Karrn-90//Onas-52/4/Lemhi-66/3/Yaktana-54-A//Noring-10/Brevor, whereas Wawawai has a pedigree of ID-000190//Potam-70/Fielder/5/Tifton-3725/Walladay//Fielder/Potam-70/3/N-700315/ID-00065/4/ID-00065/Potam-70. Alpowa (PI 566596) is derived from the cross Fielder/Potam-70//Walladay/3/Walladay/Potam-70 (Konzak *et al.*,1994) and is closely related to Louise.

Population Development

The F_1 between Louise and Alpowa was made at the Washington State University Plant Growth Facility in 2004, with Louise as the female parent and Alpowa as the male. The hybrid (F_1) seed from one cross was harvested for population development. The F_2 seed was harvested from two to five F_1 plants. The exact number of F_1 plants was not recorded. The population was developed without selection through single seed descent of 141 RILs from the F_2 generation to the F_5 generation. During each generation, plants were grown in a controlled greenhouse environment under a 16-h photoperiod and 22 °C day/15 °C night cycle. The F_5 plants were grown in 3-L pots in the Plant Growth Facility at Pullman, WA, to increase seed. $F_{5:6}$ seeds were harvested, bulked, and planted at the Washington State University Spillman Research Farm, Pullman in 2010. The $F_{5:7}$ increase was planted in single replicate (2.44 m length × 1.68 m width) 4.1-m^2 plots. Subsequent generations were derived from this bulk plot harvested from the Pullman field location.

Table 1 - Alleles for major genes in the Louise and Alpowa wheat cultivars.

Gene	Chromosome	Parent	Allele†	Phenotype
Ppd-D1	2D	Louise	*Ppd-D1*b	sensitive
		Alpowa	*Ppd-D1*a	insensitive
Vrn-A1	5A	Louise	*Vrn-A1*a	spring
exon4		Alpowa	*vrn-A1b*	longer vrn
Vrn-B1	5B	Louise	*Vrn-B1*a	spring
		Alpowa	*Vrn-B1*b	weak spring
HTAP‡	2B	Louise	155	HTAP
(wmc474)		Alpowa	150	non-HTAP

† We analyzed the *Glu-D1* (5+10), *Rht-B1b* (wild-type), *Rht-D1b* (dwarf), *Pina-D1* (soft), and *Pinb-D1* (soft) and found no polymorphism between the two parents.

‡ HTAP, high-temperature adult-plant.

Population Phenotyping

The Louise/Alpowa mapping population was characterized in the field over 5 yr, 2010 to 2014. Three climates were studied including "rainfed," "irrigated," and "drought" conditions. The rainfed treatment was planted in Pullman, a higher rainfall area receiving an average of 53-cm annual precipitation (AgWeatherNet, weather.wsu.edu). Irrigated and nonirrigated (drought) plots were grown side-by-side in Prosser (about 20 cm of annual precipitation) and Lind, WA, (<30 cm of annual precipitation). Plant development was compared at 60 d after planting by rating Zadoks growth stage in Prosser, 2011 (Figure 1; Zadoks *et al.*,1974). Stripe rust ratings of intensity and percentage severity were collected in Pullman in 2012 without any fungicide protection (unprotected) and in Pullman in 2014 under a single early (Zadoks stages 30–33) fungicide application of propiconazole at the labeled rate (Figure 2; Line and Qayoum, 1992). Plant height was determined after senescence based on the average distance from the soil surface to the top of the canopy on a plot basis in Lind in 2012. Grain yield was measured during harvest with a Wintersteiger Classic Plot Combine equipped with the Harvest Master Grain Gage mobile harvesting system (Wintersteiger Inc.). Fertilizer and herbicide treatments were applied according to Washington State University Extension guidelines for eastern Washington in all years unless otherwise stated (Koenig, 2005).

Population Genotyping

Genomic DNA was prepared from leaf tissue harvested from the F_6 generation from each of the 132 RILs advanced by single seed descent from individual F_2 plants. Tissue was immediately frozen in liquid nitrogen, stored at -80 °C, and then lyophilized. Genomic DNA was extracted using a Sarkosyl lysis buffer protocol, as in Thompson *et al.* (2015). After extraction, DNA concentration was determined with the NanoDrop 2000c (Thermo Scientific). Single nucleotide polymorphic markers were genotyped using the Illumina Infinium iSelect 9k SNP assay for wheat (Cavanagh *et al.*,2013). Marker data were manually curated in GenomeStudio (Illumina), and 1031 polymorphic markers identified. The Louise/Alpowa population was assessed for known alleles at major wheat loci, including *vernalization-A1* (*Vrn-A1*, *Vrn-*

B1) (Yan *et al.*,2003; Zhu *et al.*,2014), the puroindolines (*PinA-D1* and *PinB-D1*) (Morris, 2002), reduced height (*Rht-B1b* and *Rht-D1b*) (Ellis *et al.*,2002), photoperiod (*Ppd-D1*) (Beales *et al.*,2007), high molecular weight glutenins (*Glu-D1*) (Liu *et al.*,2008), and the Louise HTAP stripe rust resistance QTL, *QYrlo.wpg-2BS* (Tables 2 and 3, Supplemental Table 2; Carter *et al.*,2009). All genes except for *QYrlo.wpg-2BS* were assayed using Kompetative Allele Specific polymerase chain reaction (KASP) markers (LGC Genomics; MASWheat, 2017). We did not examine alleles at the *Vrn-2* (Yan *et al.*,2004) and *Vrn-3* (Yan *et al.*,2006) vernalization genes because our population is not polymorhphic at these loci. The QTL at *QYrlo.wpg-2BS* was examined using the wmc474 SSR marker as described in Carter *et al.* (2009).

Genetic Linkage Map

Linkage maps were created using JoinMap4 (Van Ooijen, 2006). Linkage groups were determined using the grouping parameter of the test for independence with a minimum logarithm of odds score of 3.0. The Kosambi mapping function was applied to calculate the distances in centimorgans between markers. The maximum likelihood mapping algorithm was used to build the map. Linkage groups were assigned to specific chromosomes based on the previously published consensus map of the iSelect 9k SNP Assay (Cavanagh *et al.*,2013). The visual of the linkage map in Supplemental Figure 1 was created using the rQTL package in R (Broman *et al.*,2003).

Characteristics

The genetic map for the Louise/Alpowa population consists of 32 linkage groups, covering 18 of the 21 (1*n*) wheat chromosomes (Table 2). Of the 1031 polymorphic SNP markers, 882 markers were assigned to a linkage group. The haplotype data and genetic map are found in Supplemental Table 1 and are formatted for rQTL.

Several known genes were added to the linkage map, including the *Ppd-D1* alleles on chromosome 2D (LGML17) at 55.3 cM; the *Vrn-A1* Exon4 SNP on chromosome 5A (LGML30) at 0.0 cM; and the

Table 2 - Description of the 32 linkage groups mapped in the Louise/Alpowa wheat mapping population.

Linkage group†	Assigned chromosome‡	Map distance§	Number of markers per group	Total number of markers
		cM	——— no. ———	
LGML1	1AS	9.3	9	9
LGML2,3	1BL, 1BS	73.7, 26.4	32, 9	41
LGML4	1DL	8.1	12	12
LGML5,6	2A, 2AL	105.22, 188.9	66, 60	126
LGML14,15,16	2B, 2BL, 2BL	51.8, 62.2, 21.0	36, 41, 11	88
LGML17	2DSL	145.7	36	36
LGML19,20,21	3AS, 3AL, 3AL	44.3, 11.5, 37.6	14, 17, 28	59
LGML22,23,24	3B, 3BS, 3BL	18.6, 83.2, 22.6	9, 46, 48	103
–	3D	–	–	–
LGML25	4AS	61.0	14	14
LGML26	4BL	27.6	15	15
–	4D	–	–	–
LGML27,28,30	5AS, 5A, 5A	40.6, 37.3, 158.7	15, 18, 75	108
LGML31,32	5B	110.1, 221.4	71, 41	112
–	5D	–	–	–
LGML33,34	6AS, 6AL	27.3, 5.2	15, 9	24
LGML35,36	6BS, 6BL	11.5, 42.7	18, 21	39
LGML37	6DL	29.6	23	23
LGML38,39	7AS	23.4, 11.5	12, 8	20
LGML40	7BL	114.2	47	47
LGML41	7D	70.3	7	7
Total	18/21			883 SNP, 1 SSR

† Linkage using the maximum likelihood (LGML) mapping function were selected using the following criteria: a minimum logarithm of odds (LOD) of 3.0 and a minimum of seven markers per linkage group.

‡ The 32 linkage groups were assigned to chromosomes (S, short arm; L, long arm) based on previously published consensus map of the iSelect 9k single nucleotide polymorphism Assay (Cavanagh *et al*., 2013).
§ Map distance indicates the total length on a linkage group.

Table 3 - Number of recombinant inbred lines (RILs) containing parental and heterozygous genotypes for six genes assayed across 132 individuals in the Louise/Alpowa wheat RIL population.

	Louise genotype	Heterozygous	Alpowa genotype	NA†
Vrn-A1 exon4‡	50	10	70	2
Vrn-B1‡	61	3	67	1
Ppd-D1‡	62	13	49	8
HTAP‡	66	4	58	4

† NA, markers could not be amplified for *n* RILs after multiple attempts.
‡ Louise and Alpowa alleles for each gene are, respectively, *Vrn-A1a* and *vrn-A1b*; *Vrn-B1a* and *Vrn-B1b*; *Ppd-D1b* and *Ppd-D1a*; high-temperature adult-plant (HTAP) resistant and HTAP susceptible.

Vrn-B1a and *Vrn-B1b* alleles on chromosome 5B(LGML32) at 204.9 cM and 211.3 cM, respectively (Supplemental Figure S1, Supplemental Table 1). The Wmc474 SSR marker that was linked to the QTL for HTAP resistance to stripe rust in Louise on chromosome 2BS was expected to link to the 2BS linkage group in our map (Carter *et al.*,2009). Its failure to fall into a linkage group suggests that there are gaps in the genetic map.

Effect of *Ppd-D1* and *Vrn* Alleles on Phenology

The population was segregating for two alleles for vernalization requirement at the *Vrn-A1* gene. The *Vrn-A1a* allele removes the requirement for vernalization and results in a strong spring growth habit while the recessive *vrn-A1* allele results in a winter growth habit requiring vernalization to flower. At the homeoelogous *Vrn-B1* gene, the population was segregating for two alleles, the strong spring *Vrn-B1a*, the weak spring *Vrn-B1b*, but not for the recessive allele. The two alleles at the *Ppd-D1* gene were the photoperiod insensitive *Ppd-D1a* and the photoperiod senstive *Ppd-D1b*.

We characterized the impact of the segregating photoperiod and vernalization alleles on the timing of RIL developmental events based on Zadoks growth stages (Figure 1). Lines with the photoperiod insentive *Ppd-D1a* allele showed faster development (higher growth stage values) at 60 d after planting than sensitive (*Ppd-D1b*) lines, regardless of whether they were associated with the stronger *Vrn-B1a* or the weaker *Vrn-B1b* alleles ($p < 0.001$; difference of 1.4 Zadoks stage; Figure 1A). Photoperiod insensitive lines (*Ppd-D1a*) also showed faster development when associated with the strong spring vernalization allele, *Vrn-A1a* ($p < 0.001$; difference of 3.1 Zadoks stage; Figure 1B, light blue dots).

The *Ppd-D1a* insensitive lines with the winter-type recessive *vrn-A1* allele (Figure 1B, gray dots) were all slower growing (lower growth stage; $p < 0.001$; difference of 3.1 Zadoks stage) than those containing the spring *Vrn-A1a* allele (Figure 1B, light blue dots). This same pattern was observed for lines with the *Ppd-D1b* photoperiod sensitive allele but there were not many lines with both the *Ppd-D1b* and *vrn-A1* alleles.

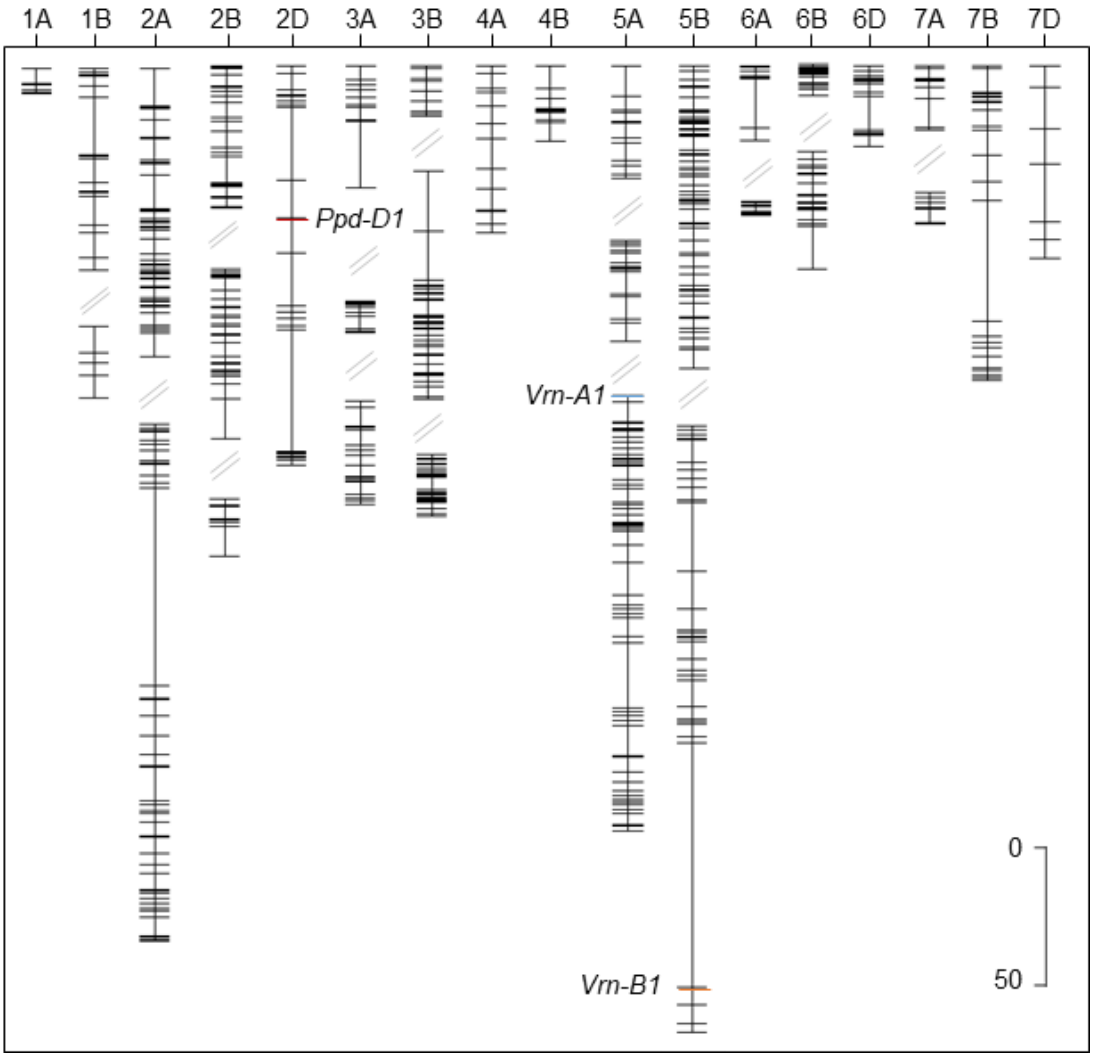
Supplemental Table 1 - The haplotype and genetic map data in rQTL format.

Dissertation Attachment: SMartinez_10857505_Chapter4_TableS1

Online Resource found at: https://dl.sciencesocieties.org/publications/jpr/supplements/0/jpr2017.08.0053crmp_supptableS1_supplement2.csv

Supplemental Table 2 - Marker information and sequence used in the Louise/Alpowa mapping population.

Trait	Gene	Chromosome	Sequence	Phenotype	Reference
plant height	RhtB1	4B	CCCATGGCCATCTCSAGCTG	tall	Ellis et al. 2002. Theor. Appl. Gen. 105:1038-1042
			CCCATGGCCATCTCSAGCTA	dwarf	
			TCGGGTACAAGGTGCGGGCG		
	RhtD1	4D	CATGGCCATCTCGAGCTRCTC	tall	
			CATGGCCATCTCGAGCTRCTA	dwarf	
			CGGGTACAAGGTGCGCGCC		
gluten strength	GluD1	1D	ATAGTATGAAACCTGCTGCGGAG	2+12 or others	Ishikawa et al. 2007. Wheat Inform. Service 103:1-4
			ATAGTATGAAACCTGCTGCGGAC	5+10	
			TACTAAAAAGGTATTACCCAAGTGTAACTT		
kernel texture	PinB	5D	CTCATGCTCACAGCCGCC	soft	Morris. 2002. Plant Molec. Bio. 48:633-647
			CCTCATGCTCACAGCCGCT	hard	
			GTCACCTGGCCCACAAAATG		
	PinA	5D	AACTGCCAACAACTTCGCTA	soft	
			TTGTCTAGTACCCCGCTCTG	hard	
			ATGAAGGCCCTCTTCCTCATAGG		
spring growth habit	Vrn-A1 exon4	5A	AGGCATCTCATGGGAGAGGATC	reduced Vrn requirement	Diaz et al. 2012. Plos one 7(3):1-11
			CAGGCATCTCATGGGAGAGGATT	regular Vrn requirement	
			CCAGTTGCTGCAACTCCTTGAGATT		
	Vrn-A1 exon7	5A	GAGTTTGATCTTGCTGCGCCG	reduced Vrn requirement	
			CTGAGTTTGATCTTGCTGCGCCA	regular Vrn requirement	
			CTTCCCCACAGCTCGTGGAGAA		
	VrnB1a	5B	GGCAGCTAATGTGGGGTAGTCA	winter	Milec et al. 2013. Euphytica 192(3):371-378
			GGCAGCTAATGTGGGGTAGTCT	spring	
			CACAGGCTTTCCTATCATTCGT		
	VrnB1b	5B	GCGCAAGCGGGAGCTACATG	winter	Santra et al. 2009. Plant Breed. 128:576-584
			GCGCAAGCGGGAGCTACATC	spring	
			GCCATGAACAACAAAGGGGGTGGT		
photoperiod-sensitivity	Ppd-D1	2D	CAAGGAAGTATGAGCAGCGGTT	insensitive	Beales et al (2007) TAG 115:721-733
			AAGAGGAAACATGTTGGGGTCC	sensitive	
			GCCTCCCACTACACTGGGC		



Supplemental Figure 1 - The genetic linkage map of the Louise/Alpowa RIL population across 18 of the 21 the wheat chromosomes. *Ppd-D1* (red), *Vrn-A1* (blue), and *Vrn-B1* (orange) are indicated on chromosomes 2D, 5A, and 5B, respectively. The linkage map length is in centiMorgans (cM). Grey breaks indicate different linkage groups within that chromosome and are not linked.

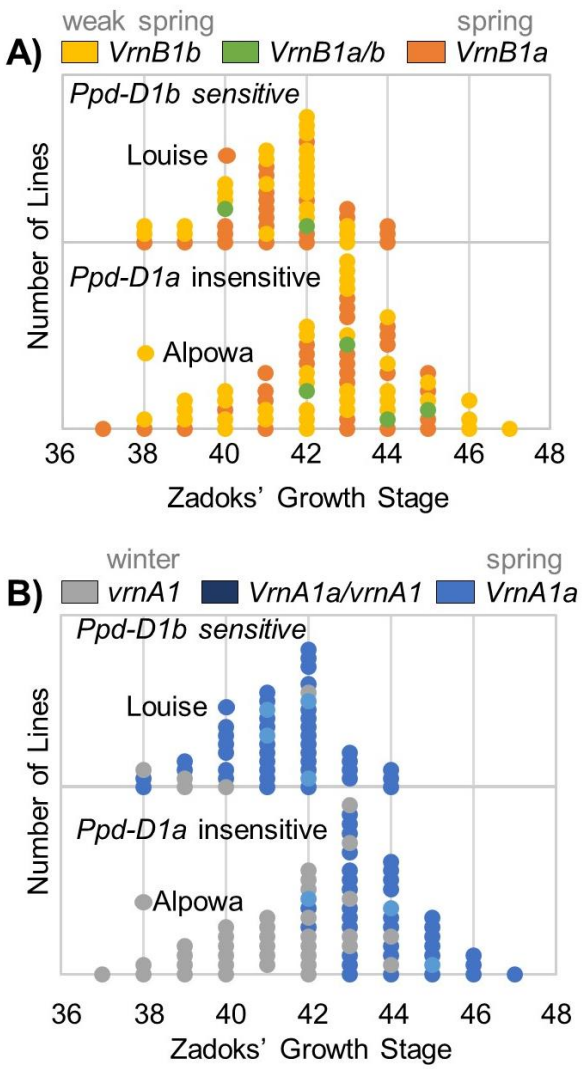


Figure 1 - Comparison of Louise/Alpowa recombinant inbred line (RIL) *Ppd-D1*, (A) *Vrn-B1*, (B) *Vrn-A1*, and the Zadoks growth stage rating during the booting stage in an irrigated trial at Prosser, WA., 2011. Each dot represents one RIL. The *y* axis represents the number of lines measured for growth stage 60 d after planting.

In contrast, the *Ppd-D1a* insensitive lines with the weaker *Vrn-B1b* allele (Figure 1A, yellow dots) did not differ in the average growth stage ($p < 0.44$) compared with *Ppd-D1a* insensitive lines containing the stronger *Vrn-B1a* allele (Figure 1A; orange dots). This suggests while the *Ppd-D1a* insensitive allele had an effect in the absence of the strong *Vrn-A1a* allele; the interaction with specific spring habit alleles at *Vrn-B1* was weak. When the *Ppd-D1a* insensitive lines with the *Vrn-B1a* allele (Figure 1A, orange dots) were compared to those with the *Vrn-A1a* allele (Figure 1B, light blue dots), the lines with the *Vrn-B1a* allele showed greater variation of growth stages (Zadoks growth stages 37–45) compared with lines with the *Vrn-A1a* allele (Zadoks growth stages 42–47). Thus, it appears that the presence of *Vrn-A1a* and *Ppd-D1* is associated with increased rate of development in this population. If a RIL had both *Vrn-A1a* and *Ppd-D1a* insensitive alleles, the development was accelerated, which may be too rapid for optimal grain filling and further downstream developmental processes.

In the greenhouse, the lines with carrying the recessive *vrn-A1* and the weak spring *Vrn-B1b* alleles need 2 wk of vernalization to flower synchronously in the greenhouse. This minimum vernalization requirement is also needed in the field and is usually met by reduced night temperatures.

Agronomic Characteristics

The agronomic characteristics of the RILs were examined under irrigated and nonirrigated (drought) field conditions. Under the drought conditions, grain yield was much lower (an average of 1485 kg ha^{-1} compared with 2480 kg ha^{-1} irrigated), and the population variance for grain yield was reduced (Figure 2A). Since there are RILs that have a higher yield compared with either parent, there is a potential to increase yield under stress through selection (irrigated: $p < 0.03$; drought: $p < 0.1$). Plant height also varied less under dry conditions (Figure 2B). The Louise/Alpowa population varied in plant height from 52.8 to 77.8 cm even though the RILs were not segregating for alleles at the major *Rht* (*Reduced height*) loci *Rht-B1* and *Rht-D1*. Both Louise and Alpowa carry both the *Rht-D1b* allele (Table 1), suggesting that other loci likely control the observed variation in plant height.

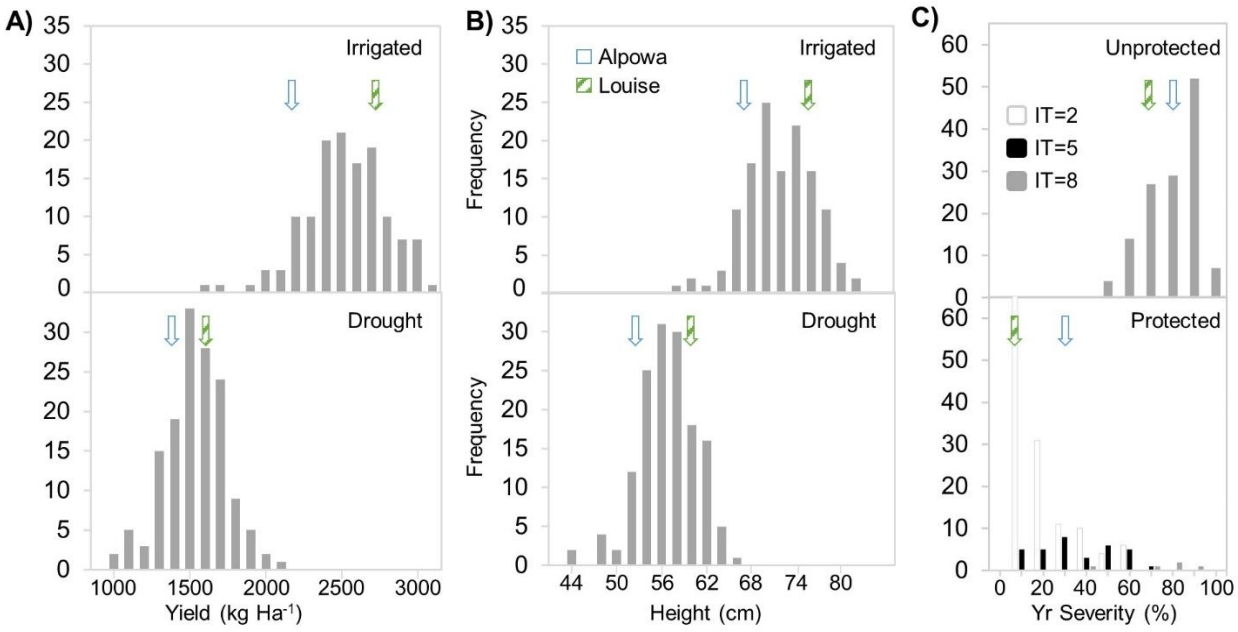


Figure 2 - Distribution of (A) yield and (B) height across the Louise/Alpowa recombinant inbred line (solid gray bars), Louise (green stripe arrow), and Alpowa (blue hollow arrow) parents under irrigated and drought conditions at Lind, WA, 2012. (C) Stripe rust ratings of intensity and percent severity in Pullman, WA, 2012, without any fungicide protection (unprotected) and in Pullman, WA, 2014, under fungicide protection (protected).

Genes for stripe rust resistance that have been mapped in Alpowa include one all-stage resistance gene, *Yr-Alp*, which was mapped to chromosome 1B but is no longer effective in the Pacific Northwest, and one named gene for HTAP resistance, *Yr39*, on chromosome 7BL (Lin and Chen, 2007; Rosewarne *et al.*,2013). The main stripe rust resistance locus in Louise is the QTL *QYrlo.wpg-2BS* for HTAP resistance (Table 2; Carter *et al.*,2009). Although the population segregated for resistance, there were no strongly resistant RILs, indicating that the all-stage resistance gene was ineffective and the RILs lcontain one, both, or no adult plant resistance genes. These breeding lines may provide sources of multigenic adult plant resistance to stripe rust that can be combined with seedling resistance to increase durability to that disease (Figure 2C).

Conclusions

Genes affecting developmental rate and flowering time can have a profound effect on drought tolerance. For example, the weak spring vernalization allele (*Vrn-B1b*) of Alpowa slows spring growth, allowing initial water conservation (Santra *et al.*,2009). Once the mild vernalization requirement of Alpowa is met, its photoperiod insensitivity (*Ppd-D1a*) results in rapid growth and possibly escape of drought-stress conditions. Louise, in contrast, has no vernalization requirement due to two strong spring *Vrn-A1a* and *Vrn-B1a* alleles and is photoperiod sensitive (*Ppd-D1b*). Thus, Louise must use different strategies to acclimate to drought. Preliminary results suggest that Alpowa and Louise differ for both root architecture and water use efficiency traits (K. Sanguinet, B. Ghimire, L. Murphy, unpublished data).

The Louise/Alpowa RIL population provides a resource for mapping and as breeding lines segregating for differences in stress tolerance and for genes governing flowering time and development. Because this population was derived from cultivars well adapted in the inland Northwest, it is an excellent resource for plant breeding in that region. It is also an interesting population to examine the impact of phenology on stress tolerance and for investigating the genetic control of plant height when alleles at the *Rht* loci are fixed.

Availability

Seed of the Louise/Alpowa population and the Louise and Alpowa parents used to generate the population will be maintained by the USDA-ARS wheat breeding program at Washington State University, Pullman, WA, 99164; small quantities of seed (<5 g) may be requested from the corresponding author for research purposes. Five hundred seeds from each RIL (F_8) have been deposited in the USDA–ARS National Laboratory for Genetic Resources Preservation, where they will become available 5 yr from date of publication. It is requested that appropriate recognition of the source be given when the population contributes to the research or development of new genetic stocks, molecular tools, germplasm, and improved cultivars.

Acknowledgements

Financial support was obtained by the Washington Grains Commission project no. 3019-6451 and 3019-7768 (to C.M. Steber and K.A. Garland Campbell and to C.M. Steber and K.A. Garland Campbell, and K.A. Sanguinet) and the USDA-ARS (in house) project 424575. Special thanks go to Stephen Johnson and Patricia DeMacon for their management and support during the field seasons. Thanks are due to Kimberlee Kidwell and Victor DeMacon for greenhouse assistance during population development and to Arron H. Carter for assistance with the linkage map development. Mention of trade names or commercial products in this registration publication is solely for the purpose of providing information and does not imply recommendation or endorsement by USDA, an equal opportunity provider and employer.

Supplemental Materials

Three supplemental files are available with this article:

Supplemental Figure 1: Visualization of the genetic linkage map with the known genes.

Supplemental Table 1: The haplotype and genetic map data in rQTL format.

Supplemental Table 2: Marker information and sequence.

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APPENDIX

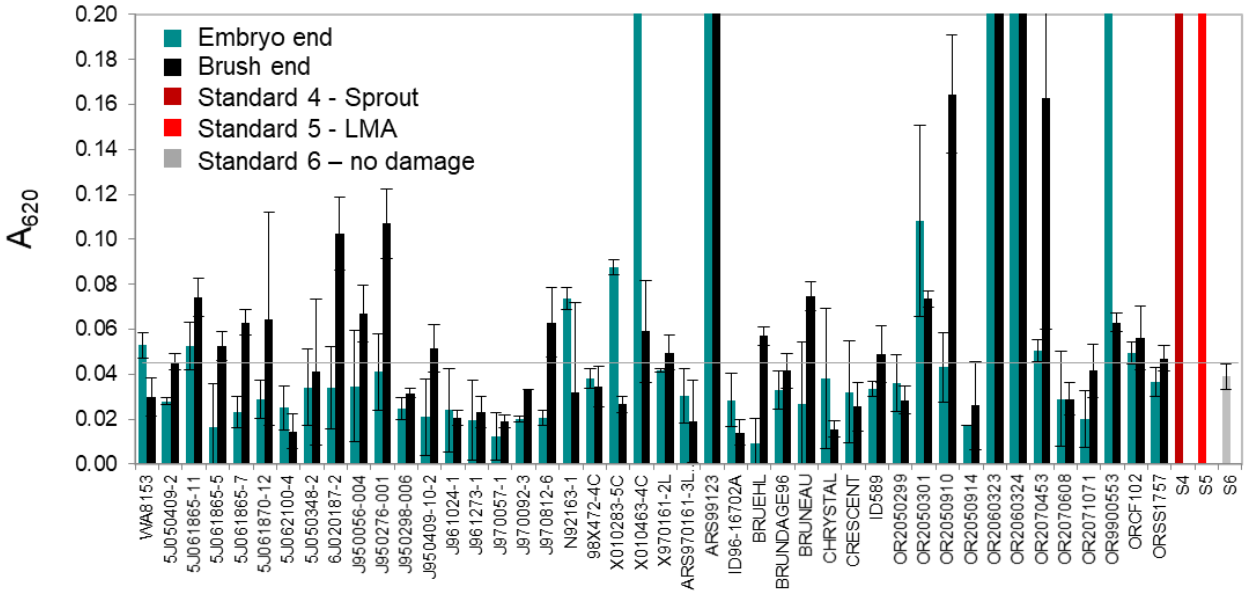

Figure 2.6 - α-amylase levels of a subset of the mapping panel from Central Ferry 2016. Seeds were separated by the embryo end (**blue**) and the brush end (**black**). Samples with a higher α-amylase levels in the embryo end are considered to have sprout damage. Samples with a higher or equal α-amylase levels in the brush end are considered to have LMA damage.

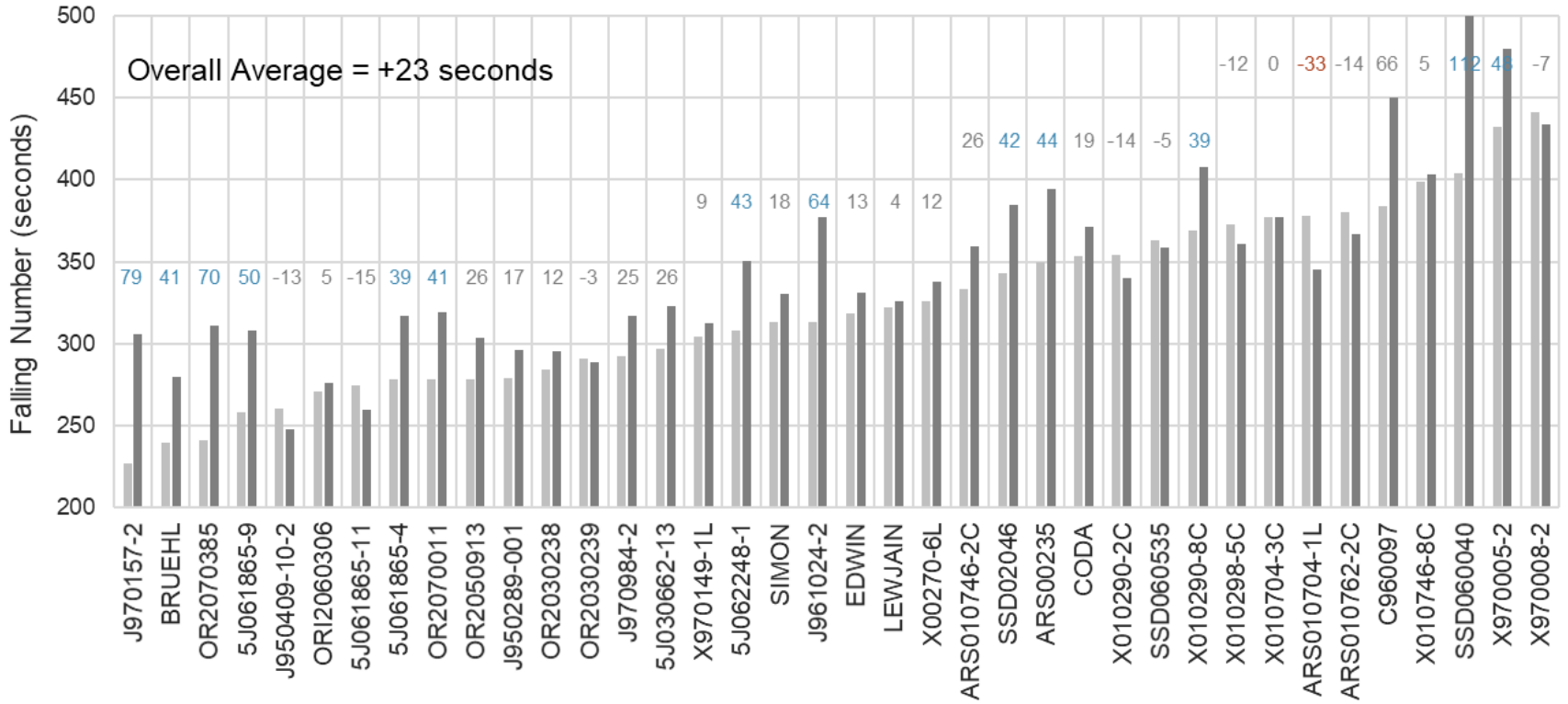

Figure 2.7. Falling number (FN) response to long-term storage at -15 °C. Samples were initially tested for FN (light grey bar) right after harvest July 21-22, 2016 and stored in the -15 °C freezer in brown papers bags in taped harvest bins. Samples were tested again for FN (dark grey bars) Dec 22, 2016- Jan 26, 2017, approximately 5-6 months in storage. The difference between the two tests are indicated above the bars. The FN machine has a typical standard deviation of 30 seconds, differences within that error range are in grey (values in seconds above bar). Samples that increased with cold storage are in blue and samples that decreased in cold storage are in red. The overall average change in FN in -15 °C storage is +23 seconds.

Table 3.7 - Summary of *ERA8* and WT bulk selections for the bulk segregant analysis (BSA).

ERA8-like Bulks

BSA	Name	Total G	No Seed	PG	B. rep	Set	Name	Total G	No Seed	PG	B. rep	Set	Name	Total G	No Seed	PG	B. rep
Y	5.2.292.	0	30	0	1	4	5.2.292.	0	30	0	2	3	5.2.292.	0	30	0.00	3
Y	5.2.265.	0	30	0	1	4	5.2.265.	1	30	3.33	2	3	5.2.265.	1	30	3.33	3
Y	5.2.40.	5	30	16.67	1	1	5.2.40.	2	30	6.67	2	3	5.2.40.	2	30	6.67	3
Y	5.2.224.	4	30	13.33	1	4	5.2.224.	5	30	16.67	2	1	5.2.224.	2	30	6.67	3
Y	5.2.21.	9	30	30	1	1	5.2.21.	8	30	26.67	2	3	5.2.21.	3	30	10.00	3
Y	5.2.22.	8	30	26.67	1	1	5.2.22.	5	30	16.67	2	2	5.2.22.	3	28	10.71	3
Y	5.2.277.	3	30	10	1	4	5.2.277.	0	30	0	2	2	5.2.277.	3	30	10.00	3
Y	5.2.24.	6	30	20	1	1	5.2.24.	4	30	13.33	2	4	5.2.24.	4	30	13.33	3
Y	5.2.140.	2	30	6.67	1	2	5.2.140.	3	30	10	2	3	5.2.140.	4	30	13.33	3
Y	5.2.210.	4	30	13.33	1	3	5.2.210.	7	23	30.43	2	3	5.2.210.	4	29	13.79	3
Y	5.2.281.	1	30	3.33	1	4	5.2.281.	5	30	16.67	2	1	5.2.281.	4	30	13.33	3
Y	5.2.48.	5	30	16.67	1	1	5.2.48.	0	30	0	2	4	5.2.48.	5	30	16.67	3
Y	5.2.113.	1	30	3.33	1	2	5.2.113.	5	30	16.67	2	4	5.2.113.	5	30	16.67	3
Y	5.2.299.	1	30	3.33	1	4	5.2.299.	6	30	20	2	2	5.2.299.	5	30	16.67	3
Y	5.2.246.	2	30	6.67	1	4	5.2.246.	4	30	13.33	2	3	5.2.246.	6	30	20.00	3
Y	5.2.207.	8	30	26.67	1	3	5.2.207.	5	30	16.67	2	4	5.2.207.	9	30	30.00	3
Y	5.2.223.	7	30	23.33	1	3	5.2.223.	3	30	10	2	2	5.2.223.	1	30	3.33	3
	5.2.118.	7	30	23.33	1	2	5.2.118.	8	30	26.67	2	4	5.2.118.	6	30	20.00	3
	5.2.242.	1	30	3.33	1	4	5.2.242.	8	30	26.67	2	4	5.2.242.	6	30	20.00	3
	5.2.4.	8	30	26.67	1	1	5.2.4.	6	30	20	2	4	5.2.4.	7	28	25.00	3
	5.2.245.	1	30	3.33	1	4	5.2.245.	4	30	13.33	2	3	5.2.245.	8	30	26.67	3
	5.2.78.	7	30	23.33	1	1	5.2.78.	8	30	26.67	2	4	5.2.78.	10	30	33.33	3
	5.2.231.	4	30	13.33	1	4	5.2.231.	6	30	20	2	4	5.2.231.	13	30	43.33	3
	5.2.291.	1	30	3.33	1	4	5.2.291.	5	30	16.67	2	3	5.2.291.	17	30	56.67	3

Zak WT-like Bulks

BSA	Name	Total G	No Seed	PG	B. rep	Set	Name	Total G	No Seed	PG	B. rep	Set	Name	Total G	No Seed	PG	B. rep
Y	5.2.153.	29	30	96.67	1	3	5.2.153.	30	30	100	2	3	5.2.153.	29	30	96.67	3
Y	5.2.286.	22	30	73.33	1	4	5.2.286.	30	30	100	2	3	5.2.286.	28	30	93.33	3
Y	5.2.287.	20	30	66.67	1	4	5.2.287.	27	30	90	2	3	5.2.287.	28	30	93.33	3
Y	5.2.60.	20	30	66.67	1	1	5.2.60.	27	30	90	2	3	5.2.60.	27	30	90.00	3
Y	5.2.178.	24	30	80	1	3	5.2.178.	23	30	76.67	2	4	5.2.178.	26	29	89.66	3
Y	5.2.162.	22	30	73.33	1	3	5.2.162.	28	30	93.33	2	1	5.2.162.	24	30	80.00	3
Y	5.2.234.	19	30	63.33	1	4	5.2.234.	27	30	90	2	1	5.2.234.	24	30	80.00	3
Y	5.2.173.	25	30	83.33	1	3	5.2.173.	27	29	93.1	2	3	5.2.173.	23	28	82.14	3
Y	5.2.272.	20	30	66.67	1	4	5.2.272.	27	30	90	2	2	5.2.272.	22	30	73.33	3
Y	5.2.54.	20	30	66.67	1	1	5.2.54.	26	30	86.67	2	2	5.2.54.	21	30	70.00	3
Y	5.2.64.	27	30	90	1	1	5.2.64.	25	30	83.33	2	2	5.2.64.	21	30	70.00	3
Y	5.2.44.	20	30	66.67	1	1	5.2.44.	22	28	78.57	2	3	5.2.44.	20	30	66.67	3
Y	5.2.144.	26	30	86.67	1	2	5.2.144.	21	30	70	2	4	5.2.144.	20	30	66.67	3
Y	5.2.267.	26	30	86.67	1	4	5.2.267.	26	30	86.67	2	1	5.2.267.	19	30	63.33	3
Y	5.2.68.	21	30	70	1	1	5.2.68.	24	30	80	2	2	5.2.68.	11	30	36.67	3
	5.2.102.	21	30	70	1	2	5.2.102.	25	30	83.33	2	1	5.2.102.	20	30	66.67	3
	5.2.222.	22	30	73.33	1	3	5.2.222.	23	30	76.67	2	3	5.2.222.	20	30	66.67	3
	5.2.130.	23	30	76.67	1	2	5.2.130.	22	30	73.33	2	2	5.2.130.	19	30	63.33	3
	5.2.101.	23	30	76.67	1	2	5.2.101.	27	30	90	2	2	5.2.101.	18	29	62.07	3
	5.2.169.	23	30	76.67	1	3	5.2.169.	26	30	86.67	2	3	5.2.169.	15	30	50.00	3
	5.2.3.	24	30	80	1	1	5.2.3.	21	30	70	2	3	5.2.3.	9	11	81.82	3
	5.2.104.	20	30	66.67	1	2	5.2.104.	23	30	76.67	2	2	5.2.104.	9	30	30.00	3

Table 3.8 – *MKK3* alleles in wheat and barley.

Gene	Original QTL	Phenotype		Nucleotide Position	Amino Acid	SNP domain
TaMKK3-A	*Phs1*	PHS Tolerant	Recessive	C660	N220	Kinase
		PHS Susceptible	Dominant	A660	K220	
TaMKK3-A-ERA8 [a]	*ERA8-EMS*	PHS Tolerant	Semi-Dominant	A1093	K365	NTF2
		PHS Susceptible	Recessive	G1093	E365	
MKK3	*Qsd2_Ak*	Seed Dormant	Dominant	C	T260	Kinase
		Non-dormant	Recessive	A	N260	

[a] Assuming the mutagen-induced SNP in *ERA8* caused the ABA hypersensitivity phenotype.

Table 3.9 - KASP marker profile of PNW elite breeding lines and cultivars against mutagen-induced SNPs, (1-30; labeled SNP_1 – SNP_30 in Chapter 3), GBS marker A7946, *TaMKK3-A*, and other chromosome 4A genes in the *TaPhs1* interval. Allele X and Allele Y (blue) of the KASP marker analysis correspond to the allele in rows 2 and 3.

Name	1	2	3	4	5	6	7	8	9	10	17	19	20	29	30	A7946	TaMKK3-A	ASC1-like	PPD1-like	LRR2	PM19-A1	PM19-A2	DNA Plate
Y:	EMS	EMS	EMS	EMS	EMS	EMS	EMS	EMS	EMS	EMS	EMS	EMS	EMS	EMS	EMS	EMS	PHS Susceptible	Alt	Alt	Alt	Alt	Alt	
X:	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	PHS Resistant	Ref	Ref	Ref	Ref	Ref	
X010735-0-2	X	X	X	X	X	X	X	X	X	-	X	X	-	-	-	-	-	-	-	-	X	-	LZ8_Plate3
2006X121-0-47C	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	X	-	-	-	X	-	LZ8_Plate3
X010770-0-1	-	-	X	-	-	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-	LZ8_Plate3
X010263-10-3C	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	X	-	-	-	Y	-	LZ8_Plate3
X06132-36C	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	X	-	-	-	Y	-	LZ8_Plate3
X06141-1L	-	-	-	-	-	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	X	-	LZ8_Plate3
X06141-22C	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	Y	-	-	-	Y	-	LZ8_Plate3
ARS990077-1C	X	X	X	X	-	X	X	X	X	-	X	X	-	-	-	-	Y	-	-	-	X	-	LZ8_Plate3
HS00293-2C-1	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	-	-	-	-	Y	-	LZ8_Plate3
X010746-4C?	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	Y	-	-	-	X	-	LZ8_Plate3
070048-0-0-45L	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	X	-	-	-	X	-	LZ8_Plate3
X060192-0-21C	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	Y	-	-	-	X	-	LZ8_Plate3
070051-0-0-20C	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	Y	-	-	-	X	-	LZ8_Plate3
X010301-4-2C	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	X	-	-	-	X	-	LZ8_Plate3
ARS010251-5C	X	X	X	X	X	X	X	X	X	-	X	X	-	-	-	-	Y	-	-	-	X	-	LZ8_Plate3
X06136-59C	X	X	X	X	X	X	X	X	X	-	X	X	-	-	-	-	Y	-	-	-	-	-	LZ8_Plate3
2006X123-0-16C	X	X	X	X	X	X	X	X	-	-	X	X	X	-	-	-	-	-	-	-	-	-	LZ8_Plate3
X06134-12C	X	X	X	X	X	X	X	X	X	-	X	-	X	-	-	-	X	-	-	-	-	-	LZ8_Plate3
070056-0-0-10C	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	-	-	-	-	X	-	LZ8_Plate3
X06137-2C	X	X	X	X	X	X	X	-	-	-	X	X	X	-	-	-	-	-	-	-	X	-	LZ8_Plate3
X010662-2C	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	Y	-	-	-	X	-	LZ8_Plate3
4J071246-1	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	Y	-	-	-	Y	-	LZ8_Plate3
ARS010729-1L	X	X	X	X	X	X	X	X	X	-	X	X	X	-	-	-	X	-	-	-	Y	-	LZ8_Plate3
Louise	X	X	X	X	X	X	X	X	X	X	X	X	X	-	-	-	X	-	-	-	Y	-	LZ8_Plate3
ARS010719-4L	X	X	-	X	X	X	X	X	-	X	X	X	X	X	X	X	Y	X	Y	-	X	X	ZZ8_431_Plate5
BREVOR	X	X	-	X	-	-	X	-	-	X	X	X	X	-	-	X	X	X	Y	-	Y	X	ZZ8_431_Plate5
BRUEHL	X	X	-	X	X	X	X	X	-	X	X	X	X	X	-	X	X	X	Y	-	-	X	ZZ8_431_Plate5
ERA8.5	Y	Y	-	Y	Y	Y	Y	Y	-	Y	Y	Y	Y	Y	Y	Y	Y	X	Y	X	X	X	ZZ8_431_Plate5
JASPER	X	X	-	X	X	X	-	X	-	X	X	X	X	X	X	X	X	X	Y	-	Y	X	ZZ8_431_Plate5
KASEBERG	X	X	-	X	X	X	X	X	-	X	X	X	X	X	X	X	X	Y	Y	-	Y	-	ZZ8_431_Plate5
X010263-3C	X	X	-	X	X	X	X	X	-	X	X	X	X	X	X	X	X	Y	Y	-	Y	-	ZZ8_431_Plate5
ZAK.5	X	X	-	X	X	X	X	X	-	X	X	X	X	X	X	X	Y	X	Y	X	X	X	ZZ8_431_Plate5

Table 3.10 - Introgression of *ERA8* into breeding material.

Breeding Crosses	*ERA8* Name	No. F1 seeds	No. F2 adv	Program [a]	Crossing Block Name
Kaseberg/*ERA8*//Kaseberg	5.2.40.8	65	6	KGC	Sprouting_*ERA8*
	5.2.265.13	60	4	KGC	Sprouting_*ERA8*
	5.2.277.9	124	11	KGC	Sprouting_*ERA8*
ARS010719-4L/*ERA8*//ARS010719-4L	5.2.40.8	69	5	KGC	Sprouting_*ERA8*
	5.2.265.13	96	8	KGC	Sprouting_*ERA8*
	5.2.277.9	51	6	KGC	Sprouting_*ERA8*
BREVOR/*ERA8*//BREVOR	5.2.40.8	88	5[a]	KGC	Sprouting_*ERA8*
	5.2.265.13	121	8	KGC	Sprouting_*ERA8*
	5.2.277.9	41	5	KGC	Sprouting_*ERA8*
BRUEHL/*ERA8*//BRUEHL	5.2.40.8	43	2	KGC	Sprouting_*ERA8*
	5.2.265.13	47	5	KGC	Sprouting_*ERA8*
	5.2.277.9	64	7	KGC	Sprouting_*ERA8*
Brevor/*ERA8*/Bruehl	5.2.40.8	-	-	KGC	Sprouting_*ERA8*
Jasper/*ERA8*//Jasper	5.2.40.8	32	3	KGC	Sprouting_*ERA8*
	5.2.265.13	63	7	KGC	Sprouting_*ERA8*
	5.2.277.9	64	4	KGC	Sprouting_*ERA8*
X010263-3C/*ERA8*//X010263-3C	5.2.40.8	48	4	KGC	Sprouting_*ERA8*
	5.2.265.13	130	7	KGC	Sprouting_*ERA8*
	5.2.277.9	22	4	KGC	Sprouting_*ERA8*
14x1124/*ERA8*	5.2.277.9	-	8	KGC	ARS_*ERA8*
14X1070/*ERA8*	5.2.277.9	-	8	KGC	ARS_*ERA8*
Brevor//Brevor/*ERA8*	5.2.40.8	6	-	KGC	Brevor_*ERA8*
	5.2.265.13	-	-	KGC	Brevor_*ERA8*
	5.2.277.9	-	-	KGC	Brevor_*ERA8*
MDM//*ERA8*/WA8097	60.1.27	-	2	AHC	HWW15
MDM//*ERA8*/MDM	60.1.30	-	2	AHC	HWW15
ERA8/WA8096//MDM	60.1.27	-	unk	AHC	HWW15

OR2080236H//*ERA8*/6J030184-2	60.1.27	-	unk	AHC	HWW15
ERA8/UIDarwin//OR2080236H	60.1.27	-	unk	AHC	HWW15
WA8158//*ERA8*/WA7940	60.1.27	-	unk	AHC	HWW15
WA8158//*ERA8*/WA8097	60.1.27	-	unk	AHC	HWW15
WA8183//*ERA8*/WA7940	60.1.27	-	unk	AHC	HWW15
WA8184//*ERA8*/WA7940	60.1.27	-	unk	AHC	HWW15
ERA8/UIDarwin//WA 8183	60.1.27	-	unk	AHC	HWW15
ERA8/UIDarwin//UI Lochsa	60.1.27	-	unk	AHC	HWW15
ERA8/WA7940//UI Lochsa	60.1.27	-	unk	AHC	HWW15
ERA8/WA8097//09x1990-T4	60.1.27	-	unk	AHC	HWW15
ERA8/WA8097//HWW13130-B	60.1.27	-	unk	AHC	HWW15
ERA8/WA8097//HWW13135-B	60.1.27	-	unk	AHC	HWW15
ERA8/WA8096//HWW13133-B	60.1.27	-	unk	AHC	HWW15
ERA8/UIDarwin//HWW13135-B	60.1.27	-	2	AHC	HWW15
ERA8/T-2003//2J081069-0-0-6W	60.1.27	-	unk	AHC	HWW15
ERA8/T-2003//HWW13129-B	60.1.27	-	unk	AHC	HWW15
ERA8/T-2003//HWW13133-B	60.1.27	-	unk	AHC	HWW15
ERA8/T-2003//HWW13134-B	60.1.27	-	unk	AHC	HWW15
ERA8/T-2003//HWW13142-B	60.1.27	-	unk	AHC	HWW15
ERA8/T-2003//X070146-0-0-15L	60.1.27	-	unk	AHC	HWW15
ERA8/WA7940//HWW13131-B	60.1.27	-	unk	AHC	HWW15
ERA8/WA7940//HWW13132-B	60.1.27	-	unk	AHC	HWW15
ERA8/WA7940//HWW13141-B	60.1.27	-	unk	AHC	HWW15
ERA8/6J030184-2//HWW13136-B	60.1.27	-	unk	AHC	HWW15
ERA8/MDM//HWW13137-B	60.1.30	-	unk	AHC	HWW15
Otis/*ERA8*	95.3.91.5	-	-	CMS	OtERA

[a] The breeding program crossing technicians are the following: E. Klarquist (KGC), K. Barlow (AHC), and T. Harris (CMS).

Table 3.11 – Zak WT, *ERA8* (BC_2F_2 or BC_2F_3), and Otis greenhouse grown yield traits. The number of tillers, number of heads (spikes), total grams, and total seed measurements are per individual plant then averaged across the number of plants tested. The X95.6.85.4 *ERA8* was the BC_2F_3 line used for the BC_3 cross.

Zak ERA8 BC3 Parental Yield Data G30 2013

Variety	Grams/Head	StEr		Seeds/Head	StEr		Height (cm)	StEr	
Zak ERA8	1.3948	0.02943	A	36.4734	0.6017	A	81.0488	1.0839	A
Otis	1.8264	0.1217	B	41.697	2.4876	B	86	4.007	A
Zak	1.296	0.05268	A	30.2727	1.0772	C	76.5	2.0035	B

Variety	No. Tillers	StEr		No. Heads	StEr NHStEr		Total Grams	StEr	
Zak ERA8	15.3659	0.3359	A	13.8049	0.3044	A	19.1871	0.4302	A
Otis	11.6667	1.2418	B	11	1.1253	B	20.09	1.5904	A
Zak	16.5	0.6209	A	14.5833	0.5627	A	19	0.7952	A

Variety	Total Seeds	StEr		No. Plants
Zak ERA8	501.73	15.5543	A	41
Otis	458.67	57.5018	A	3
Zak	444	28.7509	A	12

Geno	Grams/Head	StEr		Seeds/Head	StEr		Height (cm)	StEr		No. Plants
Otis	1.8264	0.1212	D	41.697	2.3488	E	86	3.6561	B	3
Zak.2.2	1.0741	0.1049	A	20.0682	2.0341	A	73.8333	3.6561	A	3
Zak.2.6	1.4056	0.07595	BC	37.369	1.4722	CDE	78.6667	2.5852	AB	6
Zak.3.10	1.3075	0.1005	AB	27.2083	1.9475	B	74.8333	3.6561	A	3
95.3.83	1.2687	0.1038	AB	33	2.0114	C	75.1667	3.6561	A	3
95.3.91	1.3637	0.1087	ABC	24.9024	2.1072	AB	73	3.6561	A	3
95.6.47	1.4659	0.1115	BC	37.3077	2.1606	CDE	80.3333	3.6561	AB	3
95.6.85	1.6155	0.1212	CD	33.5758	2.3488	CD	77.1667	3.6561	AB	3
95.6.87	1.2388	0.1062	AB	32.8605	2.0576	C	74.6667	3.6561	A	3
95.6.85.4	1.4155	0.05277	BC	37.7644	1.0229	DE	83.3462	1.7563	B	13
95.3.91.4	1.3949	0.05063	BC	39.7778	0.9814	E	84.5	1.7563	B	13

Geno	No. Tillers	StEr		No. Heads	StEr		Total Grams	StEr		Total Seeds	StEr	
Otis	11.6667	1.1967	A	11	1.0487	A	20.09	1.5573	AB	458.67	39.4161	CD
Zak.2.2	16	1.1967	D	14.6667	1.0487	BC	15.7533	1.5573	A	294.33	39.4161	A
Zak.2.6	16.3333	0.8462	DE	13.8333	0.7416	BC	19.6633	1.1012	B	523.17	27.8714	DE
Zak.3.10	17.3333	1.1967	DE	16	1.0487	C	20.92	1.5573	B	435.33	39.4161	BCD
95.3.83	17.3333	1.1967	DE	15	1.0487	BC	19.03	1.5573	AB	495	39.4161	DE
95.3.91	15.6667	1.1967	BD	13.6667	1.0487	ABC	18.6367	1.5573	AB	340.33	39.4161	AB
95.6.47	15.3333	1.1967	BCD	13	1.0487	AB	19.0567	1.5573	AB	485	39.4161	D
95.6.85	12	1.1967	AC	11	1.0487	A	17.77	1.5573	AB	369.33	39.4161	ABC
95.6.87	15.6667	1.1967	D	15	1.0487	BC	17.7567	1.5573	AB	471	39.4161	CD
95.6.85.4	15.1538	0.5749	BE	13.3846	0.5038	B	18.9454	0.7481	AB	505.46	18.9349	D
95.3.91.4	15.7692	0.5749	D	14.5385	0.5038	BC	20.2792	0.7481	B	578.31	18.9349	E

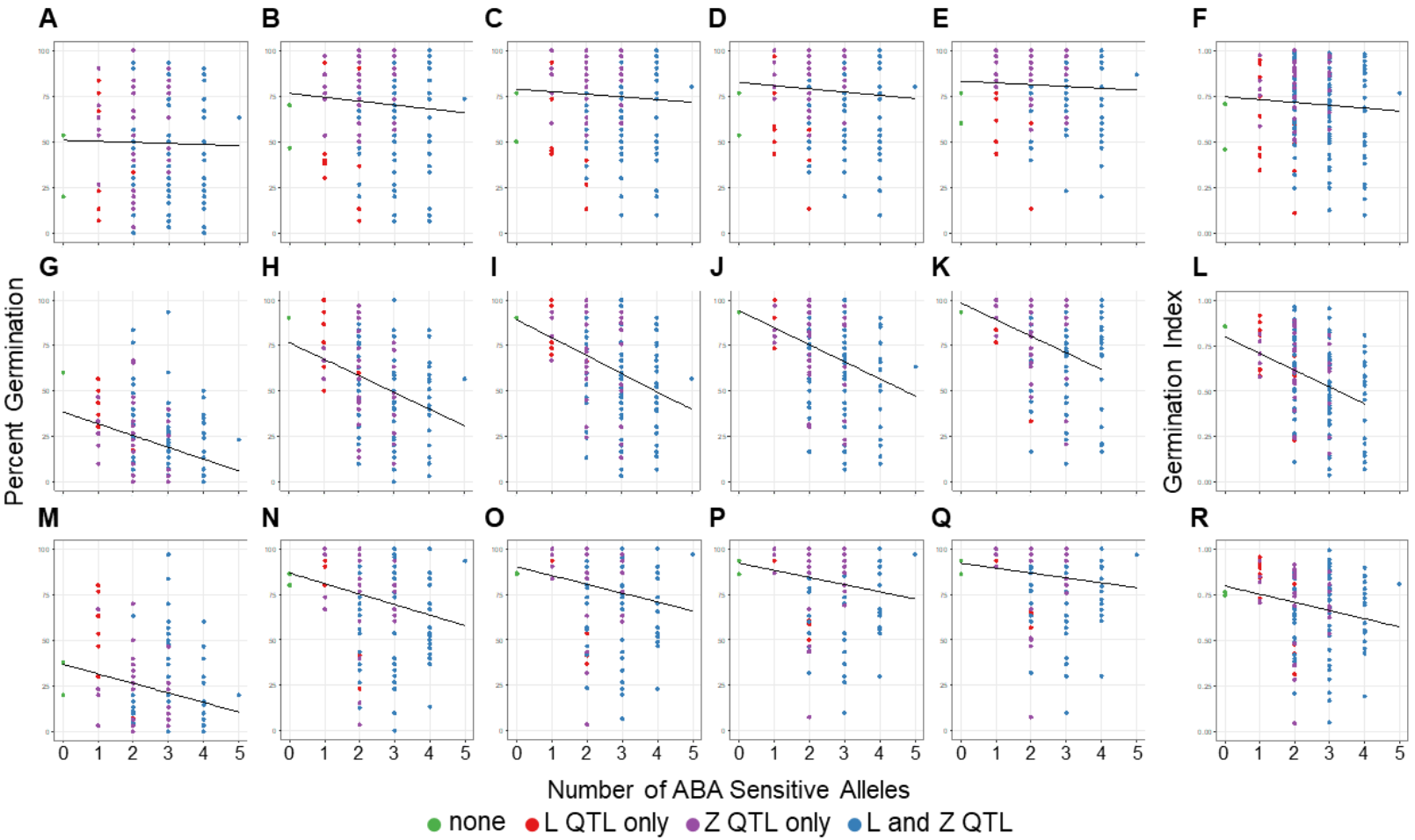

Figure 3.14. Distribution of the total number of ABA sensitive alleles from significant QTL within the Louise x Zak*ERA8* RIL population. RILs have either no (green), only the Louise (red), only the Zak*ERA8* (purple), or both the Louise and Zak*ERA8* (blue) ABA sensitive alleles. For Environment 1 (A-F), Environment 2 (G-L), and Environment 3 (M-R), percent germination is shown after 1 day (A,G,M), 2 days (B,H,N), 3 days (C,I,O), 4 days (D,J,P), and 5 days (E,K,Q) of imbibition on ABA along with the germination index across all 5 imbibition days (F,L,R).

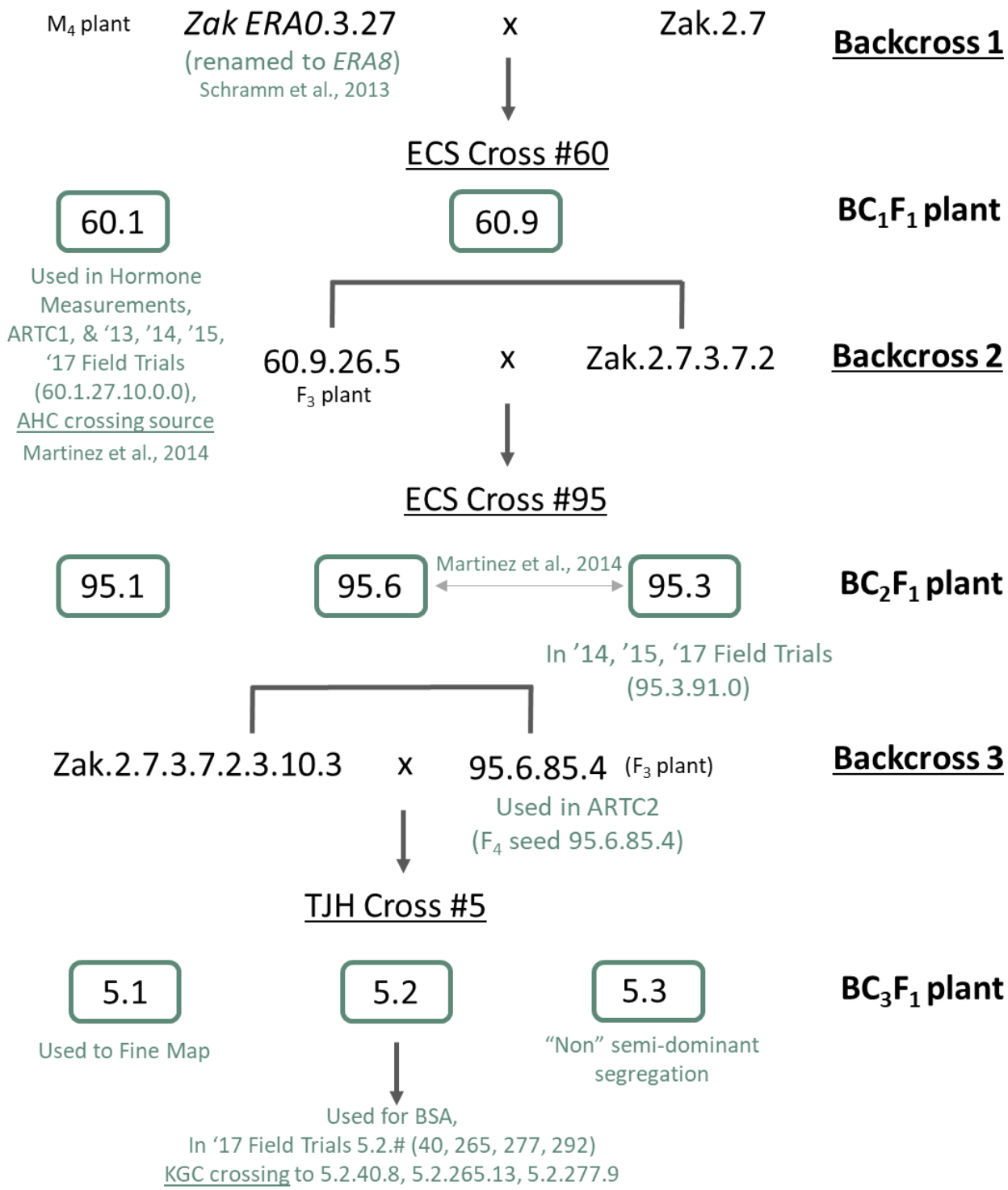

Figure 3.15 – Summary of Zak*ERA8* experiment history, notes, and crosses. Abbreviations: ECS Elizabeth C Schramm; TJH Tracy J Harris; BSA Bulk Segregant Analysis; ARTC after-ripening time course;

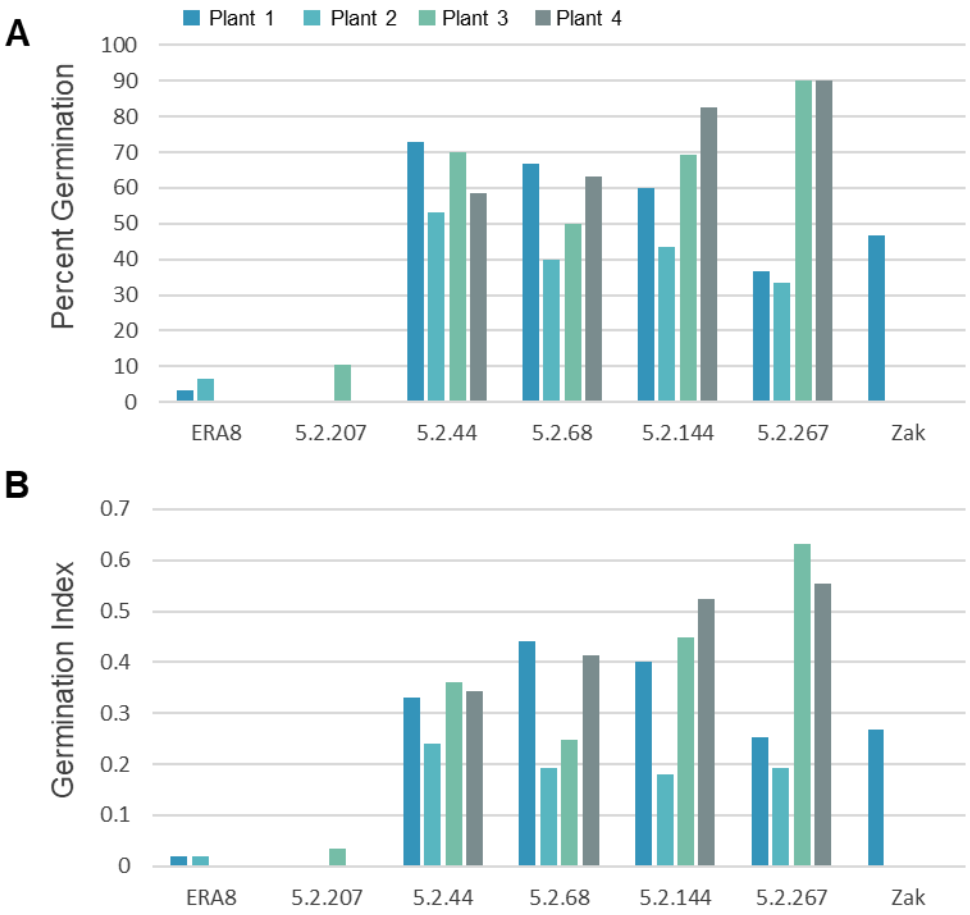

Figure 3.16 - Confirmation of homozygocity on a subset of *ERA8*-like (207) and WT-like (44,68,144, and 267) bulk lines in the BC_3F_4 generation used for BSA on 5uM of ABA. Percent germination is reported after 5 days of imbibition at 30 °C.

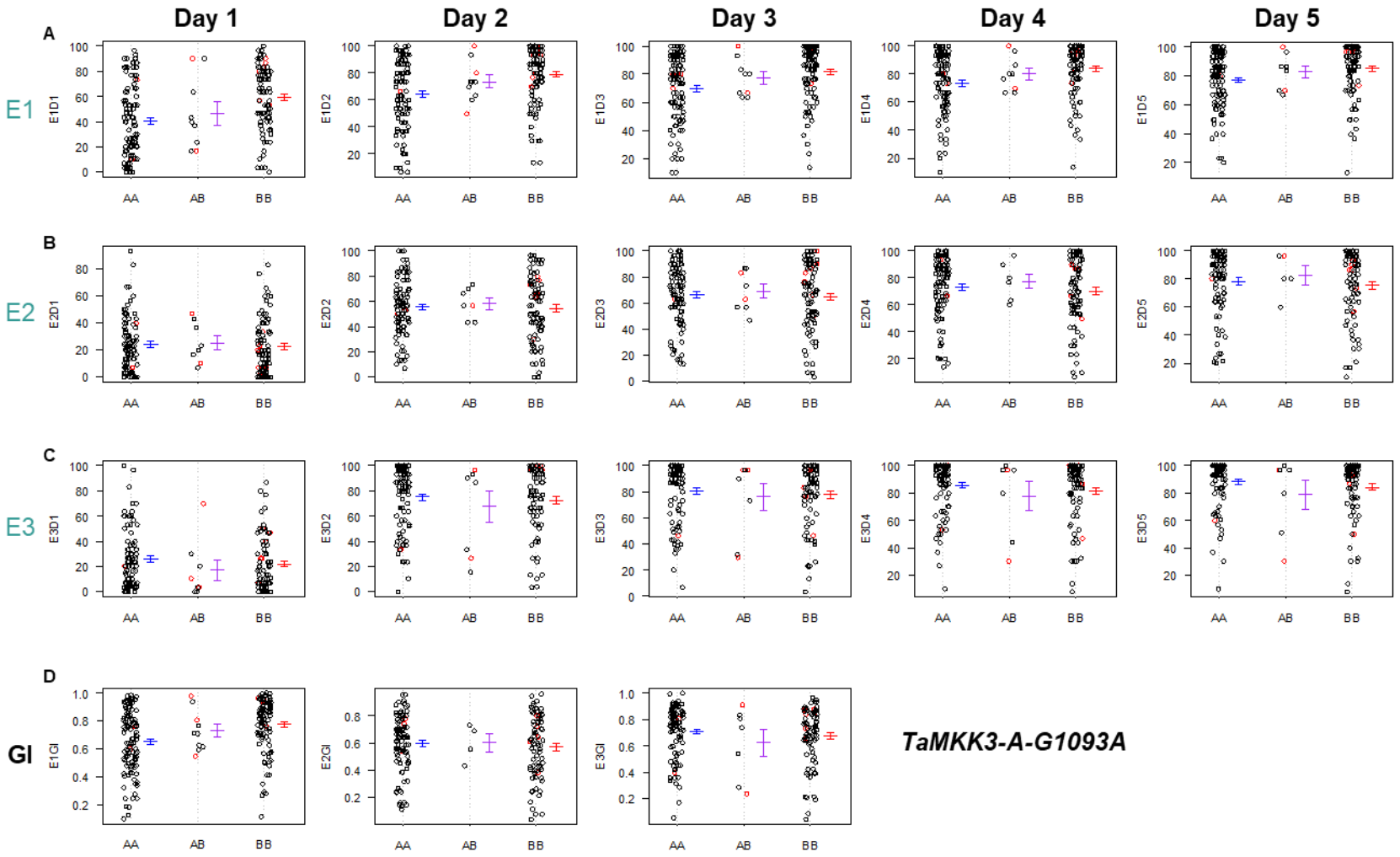

Figure 3.17 – SNP_17 and *MKK3* allelic effects in the Louise/Zak*ERA8* RIL population. Homozygous Louise (AA) and homozygous *ERA8* (BB) alleles are labeled in purple and blue, respectively. Allelic effects of SNP_17 (**A**-**D**) and MKK3 (**E**-**H**) are shown across all three environments E1: Greenhouse grown in 2013 (**A**, **E**); E2: Field grown in 2014 (**B**, **F**); and E3: Field grown in 2015 (**C**, **G**). Percent germination is shown after 1, 2, 3, 4, and 5 days of imbibition and germination index (**D**, **H**) at 30 °C on ABA across 225 RILs. Note that red dots correspond to inferred genotypes from Rqtl (Broman *et al*., 2003).

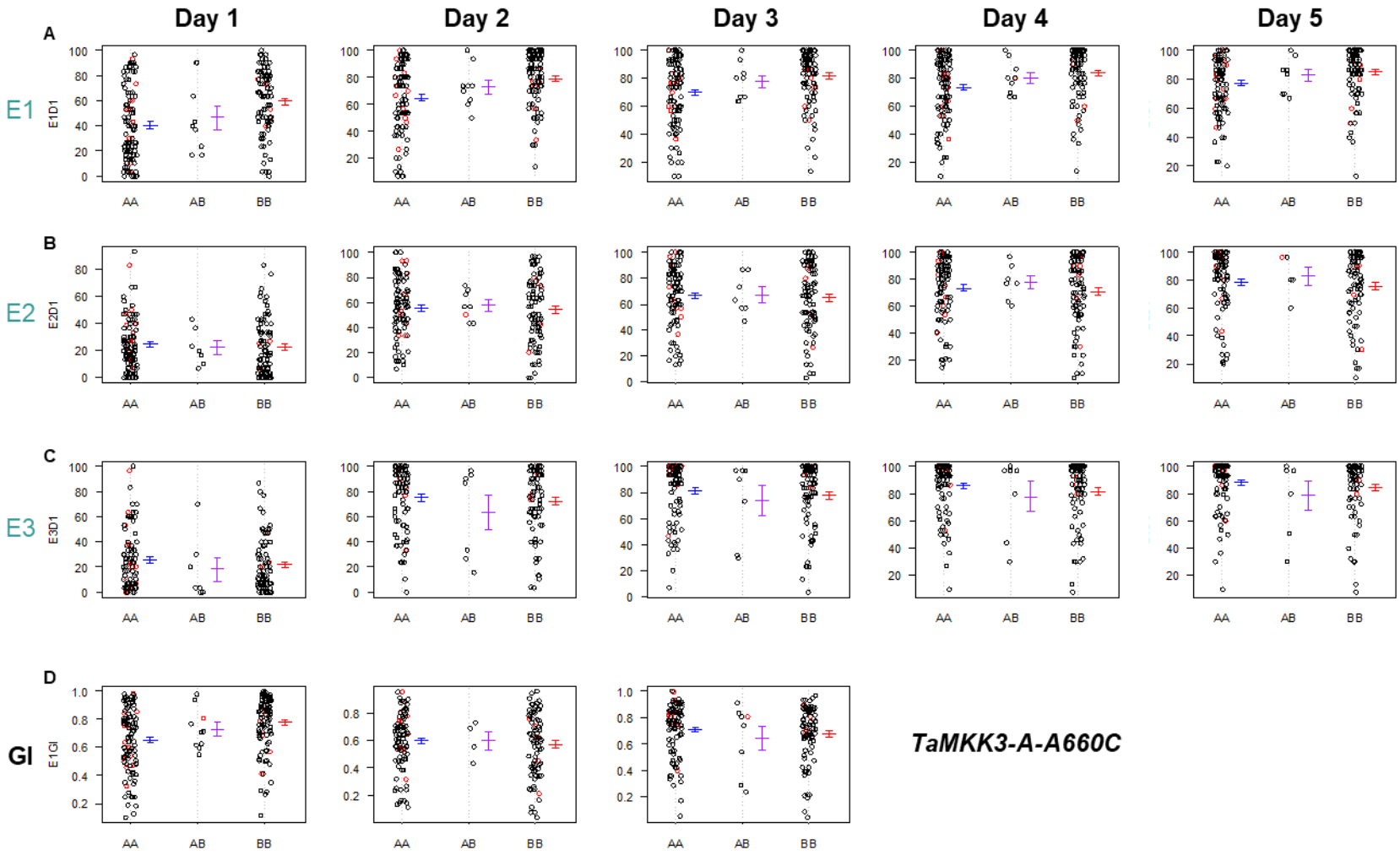

Figure 3.17 – SNP_17 and *MKK3* allelic effects in the Louise/Zak*ERA8* RIL population. Homozygous Louise (AA) and homozygous *ERA8* (BB) alleles are labeled in purple and blue, respectively. Allelic effects of SNP_17 (**A**-**D**) and MKK3 (**E**-**H**) are shown across all three environments E1: Greenhouse grown in 2013 (**A**, **E**); E2: Field grown in 2014 (**B**, **F**); and E3: Field grown in 2015 (**C**, **G**). Percent germination is shown after 1, 2, 3, 4, and 5 days of imbibition and germination index (**D**, **H**) at 30 °C on ABA across 225 RILs. Note that red dots correspond to inferred genotypes from Rqtl (Broman *et al*., 2003).

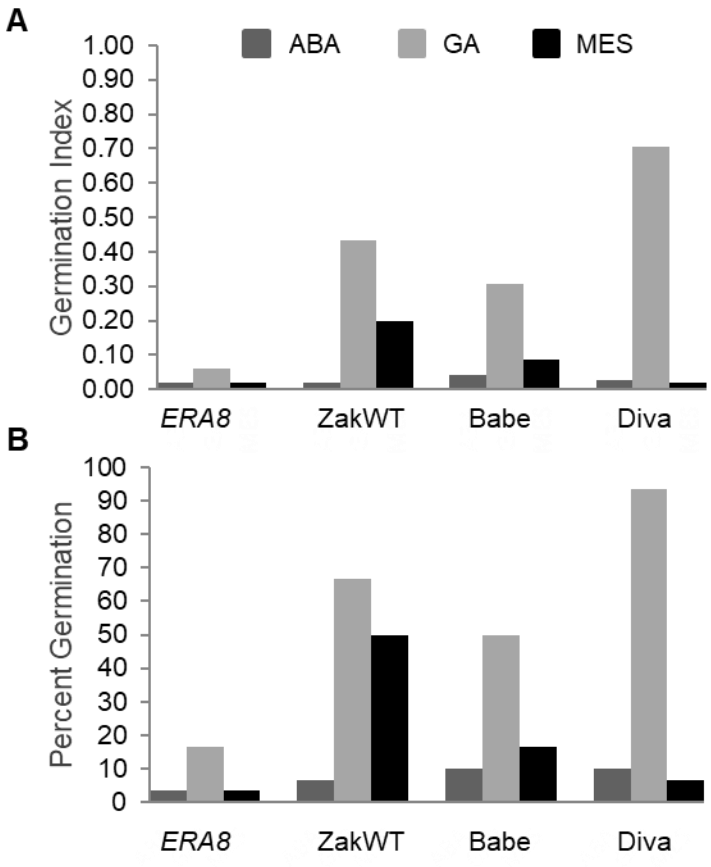

Figure 3.18 – The germination profile of *ERA8*, Zak WT, Babe, and Diva on 5 µM ABA, 10 µM GA and no hormone. **A**) Percent germination after 5 days of imbibition and **B**) germination index is shown. Seeds were after-ripened for 2 weeks at 30 °C.

Table 4.4 - List of environments that the Louise/Alpowa RIL population was grown in between 2009 and 2014.

Year	Population	Pop	Purpose	Location	RAIN	genotypes (NO)	Reps	Data in Agrobase	NOTES	CID in file
2010	Alp/LouiseRIL	L/A		Pullman	Rain	66 Alp/Lou 72Lou/Alp	1	none in Agro		no
2010	Alp/Lou RIL f6	L/A		Pullman	Rain	94	1	none in Agro		no
2011	Lou/Alp RIL	L/A		Prosser	Irr	141	3	several agro and qual traits		yes
2011	Lou/Alp RIL	L/A		Prosser	dry	141	3	several agro and qual traits		no
2011	Lou/Alp RIL	L/A		Pullman	Rain	141	1	several agro and qual traits		no
2012	Lou/Alp RIL HR	L/A		Pullman	Rain	135	3	stripe rust		no
2012	Lou/Alp RIL	L/A		Lind	dry	141	4	several agro and qual traits		no
2012	Lou/Alp RIL	L/A		Lind	Irr	141	4	several agro and qual traits		yes
2012	Lou/Alp RIL	L/A		Pullman	Rain	141	4	several agro and qual traits		no
2012	Lou/Alp RIL F8 Winter	L/A		Pullman	Rain	39	1	several agro and qual traits		no
2013	Lou/Alp RIL	L/A		Pullman	Rain	141	3	agro traits, need qual		yes
2013	Lou/Alp Sel	L/A		Lind	dry	42	5	agro traits, need qual		yes
2013	Lou/Alp Sel	L/A		Lind	Irr	42	5	agro traits, need qual		yes
2014	Lou/Alp Sel	L/A		Lind	dry	35	5	In field		unkown
2014	Lou/Alp Sel	L/A		Lind	Irr	35	5	In field		unkown
2014	Lou/Alp Sel	L/A		Pullman	Rain	35	5	In field		unkown
2010	Avs/Alp Ril	Av/A		Lind	Dry	157	2	none in Agro		-
2010	Avs/Alpowa	Av/A		Pullman	Rain	153	1	none in Agro		-
2011	AVS/ALP Irr Lind	Av/A		Lind	Irr	154	3	several agro and qual traits		-
2011	AVS/ALP dry Lind	Av/A		Lind	dry	154	3	several agro and qual traits		-
2011	AVS/ALP	Av/A		Pullman	Rain	154	3	only 3 traits		-
2011	AVS/ALP RIL PUR	Av/A	Increase	Pullman	Rain	152 + 2 parents	1	none in Agro	Cant' Find in Agrobase	
2010	Alp/LouRILf6-Spring	L/A		Pullman	Rain	138	1	none in Agro	No Idea Where this is in agrobase	
2013	Lou/Alp RIL Winter	L/A		Lind	Rain	15	1	none in Agro	NO TRAITS UPLOADED	
2013	Lou/Alp RIL Winter	L/A		Pullman	Rain	15	1	none in Agro	NO TRAITS UPLOADED	
2009	Alpowa/Louise RIL F5	L/A	Increase	PGF	GH	94	1	none in Agro		
2010	Alp/LouRILf6 -Spring	L/A		PGF	GH		1	none in Agro		
2010	Alp/LouRILf6 -Winter	L/A		PGF	GH		1	none in Agro		
2011	Lou/Alp Winter F7	L/A	Increase	PGF	GH	39	1	none in Agro		
2010	Alp/LouRILf6 -Spring	L/A		Pullman	GH	94	1	none in Agro		
2011	Lou/Alp RIL F7 INC	L/A	Increase	Pullman	Rain	141	3	none in Agro		
2009	Alpowa/Express	A/E	Increase	Pullman	Rain	145	1	none in Agro		
2010	Alpowa/Express	A/E	Increase	PGF	GH	145+Parents	1	none in Agro		
2010	Avs/Alpowa RIL F7	Av/A	Increase	PGF	GH	153	6 subsar	none in Agro		
2010	Lou/Avocet RIL F5	L/Av	Increase	PGF		214	1	none in Agro		
2011	Avs/Alp RIL F8 sel	Av/A	Increase	PGF	GH	14	1	none in Agro		
2011	Avs/Alp RIL F9	Av/A	Increase	PGF	GH	138	1	none in Agro		
2011	Lesley Parents			Endicott	Rain	4	4	none in Agro		
2011	Lesley Parents			Readan	Rain	4	4	none in Agro		
2011	Lesley Parents			Walla Walla	Rain	4	4	none in Agro		
2011	Lesley Parents			Prosser	Dry	4	4			
2011	Lesley Parents			Lind	Dry	4	4			
2012	Lou/Alp RIL	L/A		Lind	Dry	141	4	several agro and qual traits	Is this a duplicate of 2012 Lou ALP Lind Dry	

Table 4.5 - List of the Louise /Alpowa RIL population old names that correspond to the published new names. Names were changed because there was never an Alpowa/Louise cross but the names remained the same throughout the years. When publishing, this mistake was corrected.

Old Name	Old Abbr	Published Name	New Abbr
Alp/Lou-26-0	AL26	Lou/Alp-126-0	LA126
Alp/Lou-30-0	AL30	Lou/Alp-130-0	LA130
Alp/Lou-31-0	AL31	Lou/Alp-131-0	LA131
Alp/Lou-32-0	AL32	Lou/Alp-132-0	LA132
Alp/Lou-33-0	AL33	Lou/Alp-133-0	LA133
Alp/Lou-34-0	AL34	Lou/Alp-134-0	LA134
Alp/Lou-37-0	AL37	Lou/Alp-137-0	LA137
Alp/Lou-38-0	AL38	Lou/Alp-138-0	LA138
Alp/Lou-39-0	AL39	Lou/Alp-139-0	LA139
Alp/Lou-42-0	AL42	Lou/Alp-142-0	LA142
Alp/Lou-43-0	AL43	Lou/Alp-143-0	LA143
Alp/Lou-44-0	AL44	Lou/Alp-144-0	LA144
Alp/Lou-45-0	AL45	Lou/Alp-145-0	LA145
Alp/Lou-46-0	AL46	Lou/Alp-146-0	LA146
Alp/Lou-47-0	AL47	Lou/Alp-147-0	LA147
Alp/Lou-49-0	AL49	Lou/Alp-149-0	LA149
Alp/Lou-51-0	AL51	Lou/Alp-151-0	LA151
Alp/Lou-52-0	AL52	Lou/Alp-152-0	LA152
Alp/Lou-1-0	AL1	Lou/Alp-101-0	LA101
Alp/Lou-3-0	AL3	Lou/Alp-103-0	LA103
Alp/Lou-4-0	AL4	Lou/Alp-104-0	LA104
Alp/Lou-5-0	AL5	Lou/Alp-105-0	LA105
Alp/Lou-6-0	AL6	Lou/Alp-106-0	LA106
Alp/Lou-8-0	AL8	Lou/Alp-108-0	LA108
Alp/Lou-9-0	AL9	Lou/Alp-109-0	LA109
Alp/Lou-10-0	AL10	Lou/Alp-110-0	LA110
Alp/Lou-11-0	AL11	Lou/Alp-111-0	LA111
Alp/Lou-12-0	AL12	Lou/Alp-112-0	LA112
Alp/Lou-13-0	AL13	Lou/Alp-113-0	LA113
Alp/Lou-14-0	AL14	Lou/Alp-114-0	LA114
Alp/Lou-15-0	AL15	Lou/Alp-115-0	LA115
Alp/Lou-16-0	AL16	Lou/Alp-116-0	LA116
Alp/Lou-18-0	AL18	Lou/Alp-118-0	LA118

Old Name	Old Abbr	Published Name	New Abbr
Alp/Lou-19-0	AL19	Lou/Alp-119-0	LA119
Alp/Lou-21-0	AL21	Lou/Alp-121-0	LA121
Alp/Lou-22-0	AL22	Lou/Alp-122-0	LA122
Alp/Lou-24-0	AL24	Lou/Alp-124-0	LA124
Alp/Lou-25-0	AL25	Lou/Alp-125-0	LA125
Alp/Lou-55-0	AL55	Lou/Alp-155-0	LA155
Alp/Lou-56-0	AL56	Lou/Alp-156-0	LA156
Alp/Lou-57-0	AL57	Lou/Alp-157-0	LA157
Alp/Lou-60-0	AL60	Lou/Alp-160-0	LA160
Alp/Lou-61-0	AL61	Lou/Alp-161-0	LA161
Alp/Lou-62-0	AL62	Lou/Alp-162-0	LA162
Alp/Lou-63-0	AL63	Lou/Alp-163-0	LA163
Alp/Lou-64-0	AL64	Lou/Alp-164-0	LA164
Alp/Lou-65-0	AL65	Lou/Alp-165-0	LA165
Alp/Lou-66-0	AL66	Lou/Alp-166-0	LA166
Alp/Lou-67-0	AL67	Lou/Alp-167-0	LA167
Alp/Lou-68-0	AL68	Lou/Alp-168-0	LA168
Alp/Lou-69-0	AL69	Lou/Alp-169-0	LA169
Alp/Lou-70-1	AL70	Lou/Alp-170-1	LA170
Alp/Lou-71-0	AL71	Lou/Alp-171-0	LA171
Alp/Lou-75-0	AL75	Lou/Alp-175-0	LA175
Alp/Lou-77-0	AL77	Lou/Alp-177-0	LA177
Alp/Lou-78-0	AL78	Lou/Alp-178-0	LA178
Alp/Lou-79-0	AL79	Lou/Alp-179-0	LA179
Alp/Lou-80-0	AL80	Lou/Alp-180-0	LA180
Alp/Lou-82-0	AL80	Lou/Alp-182-0	LA180
Alp/Lou-84-0	AL84	Lou/Alp-184-0	LA184
Alp/Lou-85-0	AL85	Lou/Alp-185-0	LA185
Alp/Lou-86-0	AL86	Lou/Alp-186-0	LA186
Alp/Lou-90-0	AL90	Lou/Alp-190-0	LA190
Alp/Lou-91-0	AL91	Lou/Alp-191-0	LA191
Alp/Lou-92-0	AL92	Lou/Alp-192-0	LA192
Lou/Alp-1-0	LA1	Lou/Alp-1-0	LA001

Table 4.5 (cont.) - List of the Louise /Alpowa RIL population old names that correspond to the published new names. Names were changed because there was never an Alpowa/Louise cross but the names remained the same throughout the years. When publishing, this mistake was corrected.

Old Name	Old Abbr	Published Name	New Abbr
Lou/Alp-3-0	LA3	Lou/Alp-3-0	LA003
Lou/Alp-5-0	LA5	Lou/Alp-5-0	LA005
Lou/Alp-7-0	LA7	Lou/Alp-7-0	LA007
Lou/Alp-8-0	LA8	Lou/Alp-8-0	LA008
Lou/Alp-10-0	LA10	Lou/Alp-10-0	LA010
Lou/Alp-12-0	LA12	Lou/Alp-12-0	LA012
Lou/Alp-14-0	LA14	Lou/Alp-14-0	LA014
Lou/Alp-15-0	LA15	Lou/Alp-15-0	LA015
Lou/Alp-16-0	LA16	Lou/Alp-16-0	LA016
Lou/Alp-17-0	LA17	Lou/Alp-17-0	LA017
Lou/Alp-18-0	LA18	Lou/Alp-18-0	LA018
Lou/Alp-20-0	LA20	Lou/Alp-20-0	LA020
Lou/Alp-21-0	LA21	Lou/Alp-21-0	LA021
Lou/Alp-22-0	LA22	Lou/Alp-22-0	LA022
Lou/Alp-23-0	LA23	Lou/Alp-23-0	LA023
Lou/Alp-25-0	LA25	Lou/Alp-25-0	LA025
Lou/Alp-26-0	LA26	Lou/Alp-26-0	LA026
Lou/Alp-27-0	LA27	Lou/Alp-27-0	LA027
Lou/Alp-28-0	LA28	Lou/Alp-28-0	LA028
Lou/Alp-29-0	LA29	Lou/Alp-29-0	LA029
Lou/Alp-31-0	LA31	Lou/Alp-31-0	LA031
Lou/Alp-32-0	LA32	Lou/Alp-32-0	LA032
Lou/Alp-33-0	LA33	Lou/Alp-33-0	LA033
Lou/Alp-34-0	LA34	Lou/Alp-34-0	LA034
Lou/Alp-35-0	LA35	Lou/Alp-35-0	LA035
Lou/Alp-36-0	LA36	Lou/Alp-36-0	LA036
Lou/Alp-37-0	LA37	Lou/Alp-37-0	LA037
Lou/Alp-38-0	LA38	Lou/Alp-38-0	LA038
Lou/Alp-42-0	LA42	Lou/Alp-42-0	LA042
Lou/Alp-43-0	LA43	Lou/Alp-43-0	LA043
Lou/Alp-44-0	LA44	Lou/Alp-44-0	LA044
Lou/Alp-46-0	LA46	Lou/Alp-46-0	LA046
Lou/Alp-47-0	LA47	Lou/Alp-47-0	LA047

Old Name	Old Abbr	Published Name	New Abbr
Lou/Alp-48-0	LA48	Lou/Alp-48-0	LA048
Lou/Alp-49-0	LA49	Lou/Alp-49-0	LA049
Lou/Alp-50-0	LA50	Lou/Alp-50-0	LA050
Lou/Alp-52-0	LA52	Lou/Alp-52-0	LA052
Lou/Alp-53-0	LA53	Lou/Alp-53-0	LA053
Lou/Alp-55-0	LA55	Lou/Alp-55-0	LA055
Lou/Alp-56-0	LA56	Lou/Alp-56-0	LA056
Lou/Alp-58-0	LA58	Lou/Alp-58-0	LA058
Lou/Alp-60-0	LA60	Lou/Alp-60-0	LA060
Lou/Alp-61-0	LA61	Lou/Alp-61-0	LA061
Lou/Alp-62-0	LA62	Lou/Alp-62-0	LA062
Lou/Alp-63-0	LA63	Lou/Alp-63-0	LA063
Lou/Alp-64-0	LA64	Lou/Alp-64-0	LA064
Lou/Alp-65-0	LA65	Lou/Alp-65-0	LA065
Lou/Alp-67-0	LA67	Lou/Alp-67-0	LA067
Lou/Alp-68-0	LA68	Lou/Alp-68-0	LA068
Lou/Alp-69-0	LA69	Lou/Alp-69-0	LA069
Lou/Alp-71-0	LA71	Lou/Alp-71-0	LA071
Lou/Alp-73-0	LA73	Lou/Alp-73-0	LA073
Lou/Alp-74-0	LA74	Lou/Alp-74-0	LA074
Lou/Alp-78-0	LA78	Lou/Alp-78-0	LA078
Lou/Alp-79-0	LA79	Lou/Alp-79-0	LA079
Lou/Alp-80-0	LA80	Lou/Alp-80-0	LA080
Lou/Alp-81-0	LA81	Lou/Alp-81-0	LA081
Lou/Alp-83-0	LA83	Lou/Alp-83-0	LA083
Lou/Alp-84-0	LA84	Lou/Alp-84-0	LA084
Lou/Alp-85-0	LA85	Lou/Alp-85-0	LA085
Lou/Alp-86-0	LA86	Lou/Alp-86-0	LA086
Lou/Alp-88-0	LA88	Lou/Alp-88-0	LA088
Lou/Alp-90-0	LA90	Lou/Alp-90-0	LA090
Lou/Alp-91-0	LA91	Lou/Alp-91-0	LA091
Lou/Alp-92-0	LA92	Lou/Alp-92-0	LA092
Lou/Alp-93-0	LA93	Lou/Alp-93-0	LA093