GENETICS OF SEED DORMANCY IN WHEAT AND BARLEY

by

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DEDICATION

This document is dedicated to my wife, my sons, and mentors… you have always held me up and helped shaped the person I have become.

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ABSTRACT

Montana producers have many biotic and abiotic stresses to contend with. One of interest to avoid is preharvest sprouting (PHS), which is the precocious germination of grains before harvest. PHS affected grain is discounted at the elevator, resulting in grain being unsuitable for many foods and direct losses to producers. PHS is not widespread every year in Montana but in some years causes large economic loss. Although PHS is a response to environmental cues it is largely controlled by genetics. Genes that control seed dormancy are the most likely candidate genes for PHS resistance and the series of studies presented in this dissertation examine the impact of several genes upon small grain PHS susceptibility. The studies used several methods to assess PHS susceptibility and determine which alleles of individual genes were present. The methods included seed dormancy screening assays, alpha amylase enzyme activity analysis, falling numbers analysis, genotyping by direct sequencing and via use of various markers, RNA-sequencing, and gene expression analysis. The first study served as a PHS susceptibility survey and provides PHS tolerance information on MT grown wheat varieties. This study also found that of the three most reported PHS associated genes (*TaMFT 3A*, *TaMKK3 4A*, and *TaVp1 3B*), only *TaMFT 3A* was associated with PHS in Montana winter wheats, even though the previously reported variation was observed in all three genes. The second study looked at PHS susceptibility among current and historically grown barley varieties. It was found that malt varieties vary greatly in their susceptibility to PHS with the top grown AMBA recommended varieties among the most susceptible. It was also found that a previously unstudied mutation in the *HvMKK3* gene is associated with PHS susceptibility. Interestingly, *HvMKK3* and *HvAlaAT1* affect dormancy at different time points in grain maturity indicating dormancy may be tailored to a specific timeframe (high dormancy at harvest with rapid decay of dormancy after harvest). The final study revisited winter wheat PHS and found that the *TaMFT 3B* and *3D* homeologs are associated with PHS which has not been shown to date.

CHAPTER ONE

INTRODUCTION

Preharvest sprouting (PHS) is a serious economic concern in many crop species, particularly in small grains such as barley (Hordeum *vulgare*) and wheat (Triticum *aestivum*). This phenomenon results when grains are subjected to elevated moisture levels (usually in the form of heavy rain or prolonged excess humidity) around harvest time and begin to sprout. Once sprouted, the grain is no longer suitable for many end-uses. Wheat that has experienced PHS damage generally makes bread that is porous, sticky, and dark or off color, all of which are traits that are quite undesirable. It does not take much PHS damage to have a large negative impact on quality. In the case of barley, PHS damage is a main concern in malt varieties as damaged grains negatively affect the malting process. Additionally, the most widely grown modern malt varieties have little dormancy at harvest and are therefore more susceptible to PHS.

There are three main ways of assessing preharvest sprouting damage in a given sample of grain. The first method is a simple visual evaluation. Grains that have experienced preharvest sprouting can be swollen, cracked, and discolored. Severe PHS damage is indicated by the emergence of roots and/or shoots from the grain. The negative aspect of relying upon the visual inspection to determine degree of PHS damage is that sprouting has been initiated but has not progressed enough to produce any obvious visual signs of damage. The second method that can be used is direct testing for alpha amylase activity. Grains that have experienced PHS damage generally have an increased alpha amylase activity which can be assayed in multiple ways. In the following studies, the Ceralpha method was used to determine the alpha amylase activity of samples. The Ceralpha method consists of an enzymatic assay and spectrophotometer reading

that indicates enzyme activity. The final method is different for wheat and barley but both tests are a measure of flour viscosity. For wheat, the Hagberg Falling Numbers method (FN) is used and is a standard method used to grade bread wheat at the elevator. Low FN directly results in lower prices for the producer. For barley, stirring number tests are used to measure PHS damage. Both of these tests are designed to measure starch degradation caused by initiation of germination. Undegraded flour is generally much thicker when heated and stirred in an aqueous solution.

To date there are few options for prevention of PHS. First, planting can be planned so that normal seasonal rains are avoided during harvest. Although, due to growing season restrictions and the uncertain nature of weather it is not always feasible to adjust planting times to avoid moisture. The second option for prevention is to plant a variety that is PHS resistant. Varieties with high levels of dormancy from physiological maturity through harvest tend to be the most resistant to PHS damage. But selection for high dormancy in this particular timeframe can be challenging as dormancy shortly after harvest is undesirable in some circumstances, such as for direct replanting or for some end-uses such as malting. Thus, investigation of the genes responsible for controlling dormancy is important for creation of varieties that have high dormancy up to harvest but that rapidly release dormancy upon a short after-ripening period.

PHS is largely controlled by genetics yet relatively few genes have been identified that contribute to the large observed variation in the trait. Furthermore, there has been little study on PHS genetics conducted in northwestern great plains germplasms. To help address the dire economic impacts of PHS as well as to investigate the genetics of PHS in Northern Great Plains region germplasms, multiple studies were designed and carried out. Common themes across all

the studies include the genes investigated and methods for evaluation of the PHS trait. Chapter two is a focused review paper looking at the genes affecting dormancy and PHS in winter wheat. Chapter three is a survey of known genes and variation in these genes that are associated with PHS. This was important as little was known about variation in PHS among northern great plains winter wheats. The fourth chapter shifts focus into barley where a large group of named varieties were assessed for PHS and analyzed for allelic variation in known dormancy genes. The fifth chapter expands upon the results of the first chapter looking closely at a double haploid population of winter wheat in which the parents were two of the lines assessed in the first study. This study also expands our understanding of PHS in winter wheat by showing association between *TaMFT* homeologs and PHS, which has not been shown to date.

CHAPTER TWO

REVEALING THE GENETIC MECHANISMS OF PREHARVEST SPROUTING

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

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Contributions: Carried out experiments, interpreted results, drafted the manuscript.

Co-Author: Robert N. Stougaard

Contributions: Helped plan and carry out experiments, interpreted results, edited manuscript.

Co-Author: John M. Martin

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Abstract

Pre-harvest sprouting (PHS) of wheat (Triticum aestivum L.) is an important phenomenon that results in weather dependent reductions in grain yield and quality across the globe. Due to the large annual losses, breeding PHS resistant varieties is of great importance. Many quantitative trait loci have been associated with PHS and a number of specific genes have been proven to impact PHS. *TaPHS1*, *TaMKK3, Tamyb10,* and *TaVp1* have been shown to have a large impact on PHS susceptibility while many other genes such as *TaSdr*, *TaQsd*, and *TaDOG1* have been shown to account for a smaller, but significant, proportions of variation. These advances in understanding the genetics behind PHS are making molecular selection and loci stacking viable methods for affecting this quantitative trait. The current review article serves to provide a brief synthesis of recent advances regarding PHS, as well as provide unique insight into the genetic mechanisms governing PHS in bread wheat.

Preharvest sprouting (PHS) is the precocious germination of grain prior to harvest. This phenomenon occurs when warm and humid or wet conditions are prevalent close to harvest time. Once initiated, the sprouting process progressively damages grain via the production of α-amylase and successive endosperm starch degradation. Observation of PHS can occur almost anywhere wheat is cultivated and is a major consideration associated with grain quality (Ross and Bettge, 2009). The damage from PHS severely impacts yield and end use quality by producing wheat-based products that are porous, sticky, off color, and generally undesirable (Moot and Every, 1990). Worldwide, this has resulted in losses of over 1 billion dollars per year (Bewley et al. 2006). These huge losses paired with a requisite for global food security, highlights the necessity for development of PHS tolerant varieties.

1. Introduction

Pre-harvest sprouting (PHS) in wheat (*Triticum aestivum* L) is the precocious germination of grain before harvest. Once germination is initiated an event cascade leads to the production of starch and protein degradative enzymes which break down endosperm starch and protein to provide energy for germination. Precocious germination is directly associated with reduced yield and product quality [1]. Yield is primarily reduced via decreased test weight attributed to endosperm degradation occurring during the initial stages of germination. Products made with sprouted grain are undesirable because they may be off color, porous, and sticky [2].

PHS occurs in almost every wheat growing region in the world and is responsible for up to $1 billion in annual losses [3]. Considering that wheat accounts for up to 20% of human calorie consumption, these losses directly impact global food security. The large economic impacts paired with human dependence on wheat for calories makes understanding PHS and its underlying genetic factors critical. This review synthesizes recent advances in understanding the genetic factors associated with PHS across the globe.

1.1 Evaluating Preharvest Sprout

Pre-harvest sprouting is most often evaluated using three methods: visual observation, the Hagberg-Perten Falling Numbers (FN) assay, and alpha amylase testing. Visual assessment is simply the visual observation of roots or shoots. If roots and/or shoots are visible the grain has commenced past the initial germination stage and is considered severely damaged and can only be used for animal feed. In the absence of visual signs of germination, the FN assay is used to

determine if sprouting has initiated and is used as a quality factor in grain trading [4]. FN determines if starch degradation has occurred by thoroughly mixing a whole meal flour with water, heating the solution and then letting a stirring paddle drop through the resulting gel. The length of time it takes the paddle to fall is indicative of starch structure. Flour from PHS contaminated grain have a less viscous gel allowing the stir bar to drop faster than in a sound sample. The last evaluation method commonly employed is alpha amylase testing. Alpha amylase is an enzyme involved in starch degradation and is produced during PHS. High flour alpha amylase is often indicative of PHS.

For research purposes it is often necessary to artificially induce PHS because many wheat growing areas do not experience PHS favorable conditions every year. In a field setting, PHS can be induced using overhead irrigation to simulate rainfall near the time of harvest. There are at least two commonly used laboratory methods for evaluating PHS susceptibility: misting chambers with intact wheat heads or a weighted germination index. For either method, heads are generally collected around physiological maturity, dried for 1-2 days at 37°C, and then kept frozen until assayed. For the misting chamber method, the heads are then put into the misting chamber for a pre-determined amount of time (often seven days) and degree of sprouting is assessed using subjective scales or direct measurement of the increase in head area. Methods utilizing area change involve scanning heads before and after sprouting. The misting chamber is the preferred method for screening large numbers of genotypes for breeding and genetic studies. The second major way to assess PHS susceptibility is weighted germination index. In this method, heads are collected at physiological maturity. The seeds are then germinated in a

temperature-controlled chamber and germination over time is recorded [5]. In this method, earlier germinating grains are more heavily weighted than later maturing grains.

1.2 Origins of the Preharvest sprouting problem

Early hunter gatherers had little immediate effect on the seed dormancy of plant populations. No matter the method used to gather seeds, there was always enough seed left in the soil to maintain the genetic variation that was already present [6]. It was with the selective harvest and sowing of seed (farming) that heavy pressure was put on plant populations, inadvertently leading to drastic decreases in seed dormancy [7]. These early farmers (and current plant breeders) selected for uniform germination and reduced dormancy to allow for immediate replanting and synchronous germination after harvest. Immediate and synchronous germination enabled them to plant a crop and know that all the seeds would emerge quickly and uniformly, not weeks or even years apart as is observed in landraces. The ability to plant after harvest has also been very important in modern plant breeding since fall planted wheat varieties need to be sown soon after harvest. Selection for both factors has enabled agricultural productivity increases but has also made PHS a significant threat to production and end-use quality.

2. Environmental and Physiological factors

There are many environmental factors that contribute to PHS but primary among them are rainy or humid conditions close to harvest. The temperature during grain-fill also has a large impact on PHS susceptibility. Cool (~13 ºC) or hot temperatures paired with moisture stress (>30 ºC for >12 days of grain fill) during grain-fill are associated with increased seed dormancy, while moderately elevated temperatures without moisture stress (<5 ºC above daily max

temperature) reduce dormancy around the time of harvest [8-10]. Although environmental conditions can cause PHS, and year to year variation in environment can regulate the effects of alleles contributing to PHS tolerance, genotypic rankings are typically unaffected by environment [11]. This indicates that genotype-based selection could yield increased environment independent PHS tolerance.

There are a number of physiological factors associated with preharvest sprouting. Chief among these are dormancy and ear morphology. Dormancy is a complex quantitative trait and has the largest impact on PHS susceptibility. Wheat seed dormancy rises from grain-fill to just past physiological maturity, and the rate of dormancy decay directly impacts a varieties ability to resist PHS. Cereal dormancy is impacted by environmental and hormonal factors, grain structure, coat chemical composition, and ear morphology [12]. Ear morphology is actually quite important in PHS resistance with both longer awn length and wider awn angles contributing to increased incidence of pre-harvest sprout, most likely due to increased water imbibition [13, 14]. Longer awns with wider angles maximize awn surface area exposure allowing the ear to collect more water droplets.

3. Genetic Factors

The focus of PHS research in recent years has been on identifying individual genes impacting seed dormancy. Multiple studies have identified PHS quantitative trait loci (QTLs) on each of the seven groups of hexaploid wheat chromosomes. However, only a few QTLs have been consistently detected across germplasm groups and in varying environments [14, 15, 16]. As a response to the influx of QTL data multiple studies have identified markers tightly linked to the most significant PHS QTLs. Creation of these tightly linked markers provides the potential

for molecular based selection in breeding programs. However, due to the large number of reported QTLs and genetic variation between breeding programs, the developed markers are often only useful in the region in which the markers were designed [17, 18]. Therefore, it is important for breeders and geneticists to evaluate each PHS linked marker in their germplasm. One way to avoid the need for regional verification of linked markers is to use perfect markers, which detect individual polymorphisms in genes that directly impact PHS. Perfect markers are generally more broadly applicable across breeding programs but more difficult to generate as it requires identification of the causal gene underlying the QTL. Studies on PHS resulting in perfect markers are lacking in the recent literature but are becoming more of a focus [19, 20, 21].

There are a few genes that have been verified across multiple studies to be associated with PHS. While the function of each gene is not fully characterized, recent studies have clearly demonstrated that each impacts PHS (Table 1). The genes that have been most validated include: *Mother of Flowering Locus T* and *Terminal Flower 1* (*Tfl1*) (referred to as *TaMFT/TaPHS1* in wheat), *Mitogen Activated Kinase 3* (TaMKK3), Red seed color genes (*Tamyb10*), and *Viviparous-1* (*TaVp-1*). Due to studies in related (barley, maize, and rice)) or model (*Arabidopsis*) organisms, several other genes have been hypothesized to affect wheat PHS but more studies are needed to define their impacts in wheat on a broad scale; these genes include: *TaSdr*, *TaQSd*, and *TaDOG1*.

3.1. *TaMFT*

TaMFT-3A (sometimes called *TaPHS1*) was identified in 2011 as a candidate gene for the wheat chromosome 3AS PHS QTL: *QPhs.pseru-3A* (Figure 1). This is a major QTL found in multiple studies accounting for up to 58% of PHS phenotypic variation [14]. *TaMFT-3A* was

found to be causal to the 3AS QTL and then an SNP (located at -222) in the promoter region of the gene was shown to upregulate expression and result in increased dormancy, particularly when cool temperatures were experienced during grain fill. Later experiments reported two additional SNP mutations located at the beginning of the third intron (+646/+666) which introduce a premature stop codon resulting in PHS susceptibility [22]. A third allele that contains a 33-bp insertion in the *TaMFT-3A* promoter (-194) was recently described and observed to be PHS associated [23]. The -194 region is a binding site for the APETALA 2 transcription factor suggesting the -194 insertion impacts transcript levels. The same study also provided support regarding the effects of the -222 promoter SNP and the 646/666 SNPs by conducting association and linkage studies and observing haplotype effects. It was concluded that lines that carry favorable alleles at -222 and 646/666 had significantly higher PHS resistance [23].

Table 1. Genes found to be associated with preharvest sprouting in bread wheat in recent literature.

Gene	Predicted Product	Accession number	References
MFT-3A	Phosphatidylethanolamine binding protein	AB571513.1	[7][10][22][23][24]
MKK3-4A	Mitogen activated kinase kinase 3	KT187393.1	[19][20][25][26]
myb10-A,B,D	*C1*/*Pl* family (Myb-type) transcription factor	AB599721.1, AB191459.1, AB191460.1	[29][30][31][32][33]
Vp-1A,B	Viviparous 1 transcription factor	AB047554.1	[34][35][36][37][38][39][40][41]
Sdr-2A,B	Zinc finger protein	KF021988.1, KF021990.1	[44][45][46]
Qsd1	Alanine aminotransferase	LC209618.1	[47][48]
DOG1	Uncharacterized product	AB555729.1	[49][50][51][52][53]

A

B

Mutation position (from start codon)	Effect	Reference
-222 T to C	Causes upregulation of *TaMFT*-3A and increases dormancy.	[10]
-194 to -161 33bp insertion	Causes upregulation of *TaMFT*-3A and increases dormancy and is linked .	[23]
+646 G to A	Causes mis-splicing of the third intron.	[22]
+666 A to T*	Causes a premature stop codon that results in a truncated, non-functional protein product.	[22]

*Mutation only causes a truncated protein product in the presence of the A mutation at +646.

Figure 1. The most prevalent TaMFT mutations and their impact on wheat dormancy. A) TaMFT-3A gene structure and graphical summary of mutations that have been associated with PHS susceptibility or resistance. B) Summary of TaMFT-3A mutation effects and associated references.

Repeated independent observation of the *TaMFT*-3A QTL paired with multiple studies characterizing how *TaMFT* impacts dormancy demonstrate that *TaMFT* is perhaps the most important single gene impacting PHS in modern germplasm. However, some wheat populations are fixed for the *TaMFT* tolerant alleles yet still experience a high level of PHS variation. This indicates that there are alternate genes or gene interactions playing significant roles [17]. It is somewhat surprising that *TaMFT* homeologs on 3B and 3D have not been found to be PHS associated since each homeolog produces a nearly identical protein product and varieties varying in dormancy differ in expression of individual *TaMFT* homeologs (Figure 2A). There are two

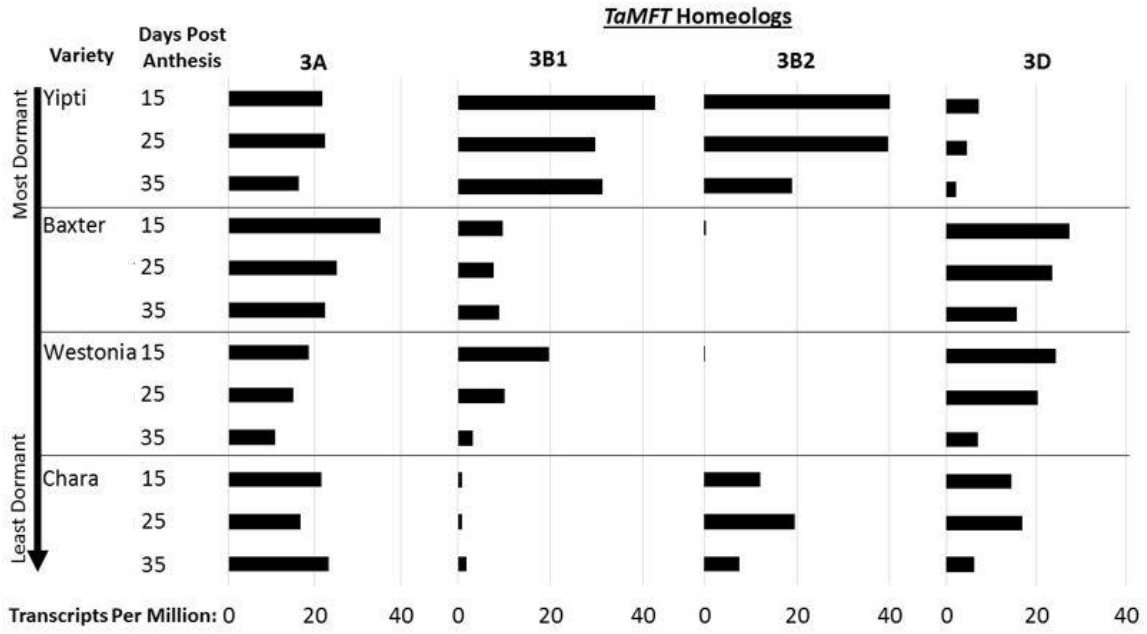
copies of *TaMFT* on chromosome 3B that differ from the single *TaMFT* present on 3A by less than 6 amino acids and one on 3D that differs from 3A by 19 amino acids (reference or unpublished data?). Simultaneous analysis of dormancy and expression data reveals possible correlations of 3B2 with germination index (Figure 2B) (unpublished data). This seems to make sense since the 3B2 homeolog is the only one to encode a protein of the exact length of and highest identity to *TaMFT*-3A. It could be that the homeologs of *TaMFT*-3A are all nonfunctional, but we hypothesize that it is simply the case that allelic variation impacting PHS within the *TaMFT* homeologs on 3B and 3D have yet to be discovered. The exact function or mechanism of *TaMFT* (UniProt accession: G1UE17) in wheat is yet to be fully characterized, but the effects of *TaMFT* have been confirmed in at least two transgenic experiments involving knockout and overexpression which support the hypothesis that *TaMFT* is a positive regulator of ABA sensitivity [10, 22]. The TaMFT protein is part of the super-family of proteins called phosphatidylethanolamine binding proteins (InterPro accession: IPR036610) and members of this family have been observed to play a role in phase transitions, such as turning from vegetative to flowering states (FT genes) [24].

3.2. TaMKK3

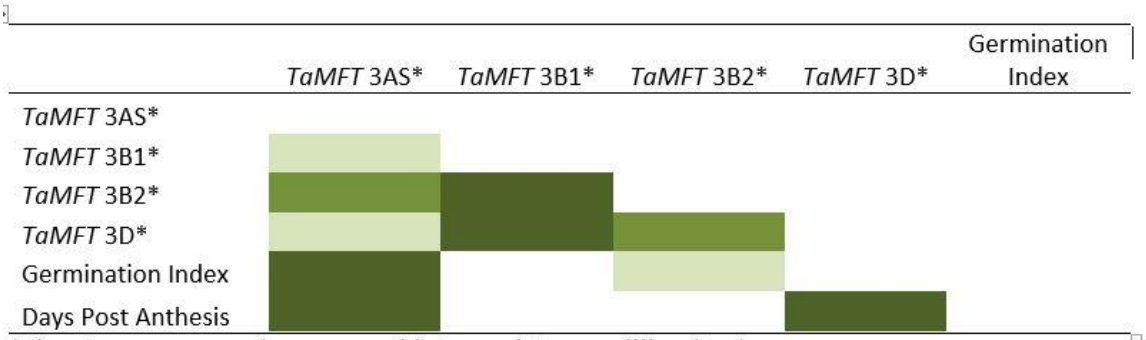
TaMKK3 (mitogen activated kinase kinase 3) is an identified PHS candidate gene underlying a second major effect PHS QTL (sometimes referred to as the *Phs1-4AL* locus) [19]. This QTL has also been observed across multiple studies and accounts for up to 43% of phenotypic variation [25, 26]. The cause of this association is a single SNP (located +660 bp from initiation, excluding introns) in *TaMKK3* that causes a missense mutation (K220N) in the kinase domain [19, 20]. Transformation with the *TaMKK3* susceptible allele caused a large increase in PHS

susceptibility in dormant backgrounds [19]. Complementation experiments in which transgenic populations were sampled at 30, 40, and 50 days after anthesis (DAA) showed that the wild type (WT) control variety "Leader" maintained dormancy past 50 DAA while lines expressing the *TaMKK3* susceptible allele all had significantly higher germination rates [19]. The effects of *TaMKK3* alleles were confirmed in an association panel that consisted of 11 biparental mapping populations as well as a multi-parent advanced generation inter-cross (MAGIC) population developed for the 4AL QTL [27]. The results from this study combined with studies of homologous genes in related and model organisms lend support to *TaMKK3* as the causal gene behind the *Phs1-4AL* QTL. *TaMKK3* likely encodes a mitogen activated protein kinase, kinase 3, which play vital roles in phosphorylation of proteins resulting in signal transduction pathways [28]. The homology of *TaMKK3* to *MKK* genes in Arabidopsis suggests *TaMKK3* affects dormancy by positively modifying ABA responsiveness [19, 28].

A.



B.



* denotes gene expression measured in transcripts per million (tpm)
** N = 14

Figure 2. Expression of specific TaMFT homeologs is correlated with dormancy. A) TaMFT homeolog specific expression of four common Australian lines (Westonia, Chara, Baxter, and Yipti) with well characterized dormancy levels [54]. Yitpi is the most dormant, while Chara is the least dormant with Baxter and Westonia showing intermediate dormancy levels [27]. All expression data was gathered from wheat-expression.com, accessed on 7/12/2018 using the expVIP database [54]. B) Heat-map representing significant correlations between TaMFT homeolog gene expressions (transcripts per million), germination index, and days after anthesis at three maturity points (15, 25, and 35 DAA). No shading (white) represents no significant associations at any of the maturity points. The lightest shade represents significant association (P value <0.05) at one maturity point, the second lightest shade represents significant association at two maturity points, and the darkest shade represents significant association at three maturity points.

3.3. Effects of Grain Color Genes

The seed phenotype most classically associated with PHS resistance is grain color. In general, red wheats are more PHS resistant than white wheats [29]. Red color in wheat is controlled by the grain color genes, *R-A1*, *R-B1*, and *R-D1*, located on the long arm of the group 3 chromosomes [30]. The genes are additive and have pleiotropic effects on PHS. The effects on PHS are hypothesized to be from accumulation of the red pigment precursor, catechin, which inhibits germination [29]. The *R* genes have been detected as QTL for both grain color and seed dormancy [31, 32]. Flavonoid biosynthetic pathway transcription factors (*Tamyb10* genes) are the candidate genes for grain color [29]. Until recently, it has been unclear if *Tamyb10* allelic variation directly impacts PHS resistance or if *Tamyb10* was simply linked to another gene that affects dormancy. To address this question a set of 185 winter wheat accessions and 155 F_6 recombinant inbred lines (derived from a cross between Tutoumai A x Siyang 936) were simultaneously analyzed for both grain color and PHS looking for overlapping QTLs [32]. It was found that grain color genes (GC) accounted for 26% to 44% of PHS phenotypic variation. In a second study, *Tamyb10-B*, *TaMFT* and *Qphs-5AL* were backcrossed into durum wheat and *Tamyb10-B* had the largest effect on PHS resistance [33]. Consistent detection of *R*-gene related QTL in PHS studies lend support to the hypothesis that the *R*-genes encode *Tamyb10* transcription factors and that these genes positively impact PHS resistance.

3.4. *TaVp1*

TaVp1, a wheat homolog of the maize *Viviparous-1* gene [34] and an ortholog of *ABA Insensitive 3* (ABI3), is a fourth PHS associated locus [35-37]. Wheat *Vp1* homeologs are on the long arms

of chromosomes 3A, 3B, and 3D and the genes are hypothesized to encode an embryo specific dormancy related transcription factor [38]. *Vp-1* sequences contain a highly conserved four-domain protein structure [39]. *TaVp1* expression was found to be positively correlated with seed dormancy and impact embryo ABA sensitivity [39]. Interestingly, most *Vp1* mRNAs are mis-spliced and encode deviant translational products and *Vp1* mis-splicing predates evolution of hexaploid wheat [35]. Later studies identified extensive allelic diversity in both *TaVp-1A* and *TaVp-1B* while *TaVp-1D* lacks allelic variation [40, 41]. To date, at least five *Vp-1A* and five *Vp-1B* alleles have been investigated in association with PHS [41, 42, 43]. Recent efforts have highlighted *TaVp-1* as a good candidate for increasing PHS resistance via stacking of *Vp1* alleles conferring higher levels of seed dormancy.

3.5. Additional dormancy target genes

Seed dormancy is an extremely complex trait that could be affected by hundreds of genes. We have thus far discussed genes shown in multiple studies to impact PHS. There are other genes that have been considered target genes for PHS because of their role in dormancy and/or germination in related or model plant species.

TaSdr is an ortholog of the rice seed dormancy related gene *OsSdr4*. Its exact function in wheat is unknown but in rice it is thought to regulate *OsDOG1* and be directly regulated by *OsVp1* [44]. *TaSdr* has been the focus of several recent studies and was found to explain a moderate amount of PHS phenotypic variation in various bread wheat germplasms. A significant association between a *TaSdr-B1* promoter SNP and PHS resistance was found among roughly 800 wheat cultivars from 19 countries [45]. *TaSdr-B1a* (associated with reduced dormancy) was more prevalent in cultivars from Japan, Australia, Argentina, the Middle and Lower Yangtze

Valley Winter Wheat Region and the Southwest Winter Wheat Regions of China. *TaSdr-A1* allelic variation was also recently described and at least one allele was significantly associated with PHS resistance and explained ~7.5% phenotypic variation in germination index [46]. Individually, these alleles present moderate potential increases in PHS resistance, but the effects may be additive by stacking favorable alleles at each loci.

TaQsd1, the wheat homolog of *Qsd1* in barley, encodes an alanine aminotransferase. This gene was recently characterized in barley and found to significantly impact seed dormancy [47]. Following the barley study [47], Onishi et al. [48] set out to characterize wheat *Qsd1* homologs. They found that all wheat *Qsd1* homeologs were embryo-specific in their expression, with *Qsd1-B* being the most like barley *Qsd1* and exhibited allelic variation across four varieties of wheat. The one line containing the B sub-genome variation had a significantly longer seed dormancy period compared to Chinese Spring but more thorough analysis is needed to confirm any association with seed dormancy [48].

The *Delay of Germination 1* (DOG1) gene was shown to affect seed dormancy in *Arabidopsis* and is likely to impact wheat dormancy and PHS [49, 50, 51]. DOG1 is of unknown function but is involved with control of seed maturation and dormancy via ABA-dependent inhibition of *Hypersensitive Germination 1* (a negative regulator of germinating seed ABA responses) [51]. Wheat and barley *DOG1* homologs (*TaDOG1* and *HvDOG1*) were ectopically expressed in *Arabidopsis* resulting in increased dormancy, indicating wheat and barley *DOG1* homologs have a similar function to *AtDOG1* [52]. *DOG1* expression is positively correlated with dormancy in non-dormant to intermediate-dormant lines [53]. *DOG1* expression peaks around 20 DAA but it was the transcript levels at 50 DAA that are most relevant to GI. Analysis

of all *TaDOG1* homeologs in multiple PHS susceptible and resistance cultivars will help determine if *DOG1* plays a role in PHS resistance or if it strictly acts as a regulator of dormancy later in seed development, such as during after-ripening. Further studies will also help determine if *DOG1* is regulated by *Sdr* as was observed in rice [44].

4. Conclusions

There are numerous recent QTL studies that have detected novel loci associated with preharvest sprouting, but this is to be expected as PHS is a very complex trait potentially affected by hundreds of genes and many environmental ques. QTL studies are very dependent upon population structure and experimental design and, although they are quite useful in a regional sense, their global application is somewhat lacking. Perfect markers developed based on causal genes are much more broadly applicable and to date only a few high impact genes have been identified. Global PHS research would greatly benefit from elucidation of genes underlying currently known QTL compared with continual generation of broad region/cross specific QTL. Many high impact genes have been described in recent literature, including: *TaMFT*, *TaMKK3*, *TaVp1*, *Tamyb10*, *TaSdr*, *TaQsdr1*, and *TaDOG1*. Allelic variants of these genes are rarely analyzed in tandem but should be considered so as to not confound results via masking by or linkage to one of these major loci. There is immense room for improvement of PHS in susceptible varieties across the globe. With continued revelations of specific genes, a key challenge will be assessing the impact these genes and their alleles have on agronomic parameters.

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Conflict of interests

The authors declare that they do not have any conflicts of interests.

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CHAPTER THREE

ALLELIC IMPACTS OF TaPHS1, TaMKK3, AND Vp1B3 ON PREHARVEST SPROUTING OF NORTHERN GREAT PLAINS WINTER WHEATS

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Abstract

Preharvest sprouting (PHS) of bread wheat (*Triticum aestivum* L.) is a common problem that can lead to negative economic impacts arising from yield loss and undesirable end-use quality. Twenty-one winter wheats adapted to northwestern Montana were grown over two field seasons and used to assess three loci observed in previous studies to have moderate to large impacts on PHS. The main goal was to validate the usefulness of TaPHS1-3A (a MOTHER OF FLOWERING TIME-like gene), TaMKK3-4A (a mitogen-activated protein kinase kinase 3), and Vp1-1B (Viviparous 1) in breeding for modified dormancy before harvest, as well as to determine their potential relationships to agronomic and seed traits, specifically, falling number and alpha amylase concentrations. Variation in PHS susceptibility across entries ranged from 0 % sprout (fully dormant) to 95 % sprout (fully non-dormant) after 7 days of wetting. Most entries showed an intermediate level of sprouting susceptibility ranging between 10% and 50% sprouted. Alleles previously reported to impact dormancy were found for all three genes but TaPHS1 was the only locus found to be significantly associated with PHS. It is unclear whether variation caused by TaPHS1 may be masking the effects of the other loci but it is evident that TaPHS1 could be used in a breeding program to modify the level of seed dormancy in winter wheat before harvest.

Introduction

Preharvest sprouting is a modern issue arising from the domestication of wheat. Early farmers imposed selection on multiple traits, including determinate growth, large seed size, reduced shattering, higher yield, and decreased seed dormancy. As seed dormancy was quite strong in

progenitor species it was necessary to select for reduced dormancy to achieve uniform and rapid germination of field crops (Harlan et al. 1972). This has inadvertently resulted in increased susceptibility to PHS in modern wheat cultivars (Liu et al. 2015). There is currently a great need to develop varieties with an intermediate degree of dormancy, particularly in regions that experience wet conditions near harvest.

PHS is a quantitative trait controlled by many genes, environmental conditions, and the interaction of genotype with environment which generates high haplotype diversity and preharvest sprouting variability. This can make PHS analysis and selection for PHS resistance challenging (Nakamura et al. 2011). Classically, PHS is measured by use of the Hagberg Falling Number Assay (FN) which detects sprouting damage before it is visible by indirectly measuring α-amylase (Hagberg 1960), or by visual observation of emerging shoots or roots from the kernel. The FN test is not always a perfect indicator of preharvest sprouting damage, as there is varietal variation in FN in the absence of PHS (Johansson 2002). Due to challenges associated with field evaluation, PHS susceptibility is often assessed by artificial means such as full-head wetting treatments or germination index assays performed in controlled conditions.

The primary environmental factors contributing to wheat PHS are wet/high humidity periods close to harvest and large temperature fluctuations during grain filling (Jimenez et al. 2017). Other major factors are genetically imposed and include: endogenous hormone levels (Tuttle et al. 2015), hormone sensitivity (Walker-Simmons 1987, Steinbach et al. 1995, and Martinez et al. 2017), spike morphology (King and Richards 1984), seed coat permeability (Debeaujon et al. 2000), seed color (Lin et al. 2016), and seed dormancy (Reviewed by Mares and Mrva 2014 and Rodriguez et al. 2015). Previous studies have identified quantitative trait loci

(QTLs) significantly affecting PHS on most wheat chromosomes (reviewed by Flintham et al. 2002 and Gao et al. 2013). A relatively small number of QTLs account for much of the genetic variability in sprouting variation, with the major QTLs being located on chromosomes 2B, 3A, and 4A (Graybosch et al. 2013, Cao et al. 2016). Some causal genes underlying significant QTLs have been identified and their effects on preharvest sprouting have been partially characterized, including: TaPHS1 (Nakamura 2011, Liu et al. 2013), TaMKK3 (Torada et al. 2016), TaVP1 (Yang et al. 2007), TaSdr (Zhang et al. 2014) and Tamyb10 (Wang et al. 2016, Lin et al. 2016).

The 3AS QTL has been shown to explain up to 58% of the phenotypic variation for preharvest sprouting (Liu et al. 2008) and has been independently identified in multiple studies as a significant PHS QTL (Liu and Bai 2010, Nakamura et al. 2011, Graybosch et al. 2013, Cao et al. 2016, Lin et al. 2016 and others). Nakamura et al. (2011) established TaPHS1 (formerly termed TaMFT) as a candidate gene for the 3AS PHS QTL and identified a SNP mutation in its promoter that upregulated TaPHS1 expression. To validate the effects of higher TaPHS1 expression they overexpressed TaPHS1 in Chinese Spring wheat embryos and observed an inhibition of germination that was only overcome with the addition of gibberellic acid (GA). Liu et al. (2013) thoroughly characterized two additional SNP mutations that result in a non-functional TaPHS1 transcript. To validate the effects of TaPHS1 they reduced TaPHS1 expression in the highly dormant PHS resistant cultivar Rio Blanco and observed reduced dormancy. The impact on dormancy and sprouting in transgenic studies, paired with the consistent independent identification of this QTL in genetic studies indicate it alone is a major genetic factor affecting PHS. However, large PHS variation is still observed in populations fixed

for TaPHS1 tolerant alleles indicating alternate genes or gene interactions play significant roles (Fakthongphan et al. 2016). TaPHS1 is a part of a large protein super-family called phosphatidylethanolamine binding proteins (InterPro accession: IPR036610). The exact function of TaPHS1 (UniProt accession: G1UE17) is currently unknown, but members of this protein family have been observed to play a role in phase transitions, such as the turning from vegetative to flowering states (UniProt Consortium, 2017).

Another locus that has been observed to have significant association with preharvest sprouting, explaining up to 43% of the observed phenotypic variation, is the 4AL QTL (Torada et al. 2005, Lin et al. 2017). In recent publications, the cause of this association was shown to be a SNP in the TaMKK3 gene (Torada et al. 2016, Shorinola et al. 2017). TaMKK3 encodes a mitogen activated protein kinase kinase 3. MKK proteins play vital roles in signal transduction pathways and the homology of TaMKK3 to MKK genes in Arabidopsis suggests TaMKK3 affects dormancy by modifying ABA responsiveness (Torada et al. 2016).

TaVp1 is a homolog of the Viviparous-1 gene identified in maize in the 1990s by McCarty et al. (1991). This gene is thought to encode a dormancy related transcription factor that plays a large role in determining susceptibility to vivipary or precocious germination prior to separation from the parent plant. There are single copies of this gene in bread wheat located on the long arm of chromosomes 3A, 3B, and 3D. Although, 3D TaVP1 has been shown to lack sequence variation in modern germplasm, variation in the 3A and 3B copies have been associated with PHS susceptibility (Yang et al. 2014). The role of this gene is still somewhat poorly understood in bread wheat but its relationship to PHS has been validated by both QTL and association mapping studies (Yang et al. 2007, Zhou et al. 2017).

There is large variation in PHS susceptibility among wheat germplasm. Governing genetic factors are very region specific making individual evaluation of each region necessary to aid plant breeders in selecting for PHS tolerance. In the current study, the variability of PHS and the contributing genetic components are examined. Specifically, previously published genes that have been shown to explain a large portion of variation in PHS were evaluated to determine allelic and haplotype variability and their contributions to preharvest sprouting in wheat commonly grown in northwestern Montana.

Materials and Methods

Plant Materials and Seed Trait Analysis

Plant materials were grown at the Montana State Northwestern Agricultural Research Station in Creston, MT, as well as the Montana State University Post Farm located in Bozeman, MT. Plant samples came from the 2016/2017 Advanced Yield trials with 21 winter wheat entries (20 hard red and 1 hard white) grown in a randomized complete block design with three replications at each location. Of the 21 lines, there were 13 named varieties and 8 experimental lines (Table 1). Planting sites used conventional tillage and were planted on 1 October, 2015 and September 27, 2016 at a seeding rate of 90.7 kg ha^{-1}. Plots in Creston, MT consisted of 7 rows that were 4.6 m long with 15 cm spacing between rows. Field was fertilized using 9-40-40 NPK broadcast and was top dressed with 75-0-40 NPK. Soil tests indicated available NPKS was 235 kg ha^{-1}N, 32 ppm P, 244 ppm K and 34 ppm S. Plots in Bozeman, MT had 3 rows that were 4.6 m long with 30 cm spacing between rows. Soil tests indicated available NPK to be 48 kg ha^{-1}N, 17 ppm P, and 232 ppm K. The field was top dressed with 232-0-0 NPK.

Weeds were controlled at both locations with a post-emergence application of Huskie 0.8 L ha^{-1} (Pyrasulfotole, Bromoxynil Octanoate, and Bromoxynil Heptanoate, Bayer CropScience, Research Triangle PK, NC), Axial 1.2 L ha^{-1} (Pinoxaden, Syngenta Crop Protection, Greensboro, NC), NIS 1 L/378 L (Polyalkylene Modified Heptamethyltrisiloxane and nonionic surfactants, Alligare, Opelika, AL), and UAN 28% 0.4 L ha^{-1} (urea ammonium nitrate, BASF Canada Inc. Mississauga, ON, Canada).

Heading date was recorded when 50% of the total primary tillers in the plot had a fully emerged spike. Physiological maturity (PM) was recorded at 75% loss of green color from the peduncle for the majority of the plot. Plant height was measured by taking the average of multiple stalks in the center of each plot. Kernel weight, diameter, and hardness were assessed using a single-kernel characterization system (SKCS) (SKCS 4100, Perten Instruments, Springfield, IL). using 200 seeds for each replicate. Grain protein was determined via near-infrared transmission with an Infratec 1241 grain analyzer (Foss, Eden Prairie, MN). Head/spike collections for each year differed in that the heads collected at Creston, MT 2016 were collected directly at PM while the heads collected from Bozeman, MT 2017 were collected one week prior to harvest.

Preharvest Sprouting Assessment

Ten heads per plot were collected at PM (characterized by loss of green color from the peduncle) and tested for each year. The heads were dried for 24 hours in a 37°C forced air oven and then frozen at -20°C until assayed. The awns of each head were trimmed to approximately 1.27 cm. The heads from all three replications were simultaneously placed into a misting chamber for 7 days and misted for 4 seconds every 5 minutes. The chamber was 1.5 m W x 2.4 m L x 0.6 m H

with a 2.54 cm PCV frame covered in 2 mil clear poly plastic. Two benches (0.76m x 2.13m) consisting of two layers of 0.63cm steel mesh were constructed and placed in each side of the chamber to hold the wheat heads vertically. The heads were misted by three Misty-Mist 1.25 mm (0.3gpm) overhead misting nozzles (Dramm Corporation, Manitowoc, WI). The misters were centered and linearly spaced 50.8 cm apart on 0.3 m high risers. The misters were controlled by a Galcon 8056 AC-6S irrigation controller (Galcon USA LTD, San Rafael, CA) wired to an Irritrol 700B-.75 Ultra Flow NPT Threaded Valve with Flow Control, 1.9 cm (Irritrol, Riverside, CA). The temperature and humidity were monitored with a LASCAR EL-GFX-2 Temperature and Humidity Data Logger (Lascar Electronics Inc., Erie, PA). After 7 days of misting, the heads were graded using a subjective scale of 1 to 7 where 1 is no visible sprouting structures, 2 is less than 10% sprouted, 3 is 10% to 24% sprouted, 4 is 25% to 49% sprouted, 5 is 50% to 74% sprouted, 6 is 75% to 99% sprouted, and a score of 7 represents completely non-dormant heads that have sprouted 100% and shoots are over 7.62 cm (See Fig. 1).

Figure 3. Pre-harvest sprouting scoring scale. A score of 1 is completely dormant after seven days of wetting, 2 is less than 25% sprouted, 3 is 25% to 49% sprouted, 5 is 50 to 75% sprouted, 6 is 75% to 99% sprouted, and a score of 7 represents completely non-dormant heads that have sprouted 100% and shoots are over 2 inches.

Falling Number determination

The Hagberg Falling Number test (Hagberg 1960) was conducted following the USDA Falling Number directive protocol (USDA Directive 9180.38, May 20, 2013). Sound grain was ground into a whole meal flour using a Perten Laboratory Mill 3100 (Perten Instruments, Hägersten, Sweden). The samples were mixed thoroughly, and flour moisture determined using a FOSS Infratec 1241 Grain Analyzer (Foss A/S Hillerød, Denmark). The samples (7.0 ± 0.05 g) were then mixed with 25 mL water and shaken using a Perten Shake-O-Matic 1095. FN measurements were conducted using a Perten FN1000 falling number machine. The Perten FN1000 was programmed to compensate for altitude. The falling number values were manually adjusted on a 14% grain moisture basis using the following equation, from page 9 of USDA Directive 9180.38, where FN is falling number: FN(14%) = (FN x (100-14))/(100-flour moisture content).

Alpha amylase determination

Whole grain flour alpha amylase concentrations were determined using an adapted "micro-assay" version of the Ceralpha Method from Megazyme (AACCI Method 22-02.01). Grain was ground into whole meal flour using Perten Laboratory Mill 3100 (Perten Instruments, Hägersten, Sweden), subsampled and frozen at -80 °C until assayed. Alpha amylase was extracted by putting 0.1 g of ground whole grain flour into a 2 mL conical micro-centrifuge tube, to which 0.50 mL of 1x Extraction Buffer solution (pH 5.4) was added and mixed vigorously by vortexing. Reactions were incubated on an Eppendorf thermomixer (Eppendorf Hamburg, Germany) for 20 minutes at 40 °C with continuous mixing at 500 rpm followed by centrifugation

at 13,000 x g for one minute. The enzyme was assayed within 1 hour of extraction as follows: 10 µL aliquots of Ceralpha Reagent Solution was dispensed into microtiter plates and pre-incubated at 40 °C for 5 min with three aliquots per enzyme extract. After pre-incubation, 7.5 µL of wheat alpha-amylase extract was added directly to the bottom of each well at 30 second intervals. The plate was then incubated at 40 °C for exactly 20 min and 0.15 mL of Stopping Reagent was then added to each well. The absorbance of the reactions (including a blank control) was read at 400 nm against 170 µL of distilled water using a Molecular Devices SpectraMax 384 Plus Microplate Reader (Molecular Devices, LLC Sunnyvale, California).

Genotyping for TaPHS1, TaMKK3, and Vp1B3

For DNA extraction, seeds were planted in the greenhouse and leaf tissue collected at the 2-leaf stage. A composite sample consisting of one 2 cm long leaf section from each of five plants was used for each extraction.

TaPHS1 sequence was analyzed by amplification and sequencing of an 863 bp fragment of the gene (from -82 through +782) that captures the causal SNPs (+646 and +666) described by Liu et al. (2013). PCR reactions contained 0.42 µM of the forward and reverse primers, 0.16 mM each dNTP, 2 mM $MgCl_2$, 1x GoTaq Buffer (Promega, Madison, WI) 0.65 U GoTaq G2 Flexi DNA Polymerase, and ~100 ng genomic DNA in a total reaction volume of 25 µL. The PCR reactions were cycled using an Applied Biosystems Veriti 96 well Thermocycler (Applied Biosystems Corp., Foster City, California) using the following steps: 1 cycle of 96 °C for 5 min; 40 cycles of 96 °C for 40 sec, 65 °C for 30 sec, 72 °C for 60 sec; 1 cycle of 72 °C for 7 min, with a final hold at 4 °C. PCR products were sequenced using amplification primers (GENEWIZ, Cambridge, MA). Sequence files were analyzed with DNA Star Seqman Pro 2014 bioinformatics

software (DNASTAR Software Co. Madison, Wisconsin). TaPHS1 sequence from cultivar Chinese Spring wheat (AB571512.1) containing the susceptible TaPHS1 allele was used as the reference for sequence alignment of all amplified TaPHS1 fragments.

TaMKK3 was analyzed by amplifying an 800 bp fragment of the gene using primers AF3 and AR3 as outlined by Torada et al. (2016). This fragment is centered on the 5th exon and includes the C660A SNP associated with PHS susceptibility. The PCR reactions contained 0.42 µM of the forward and reverse primers, 0.16 mM each dNTP, 2 mM $MgCl_2$, 1x GoTaq Buffer (Promega), 0.65 U GoTaq G2 Flexi DNA Polymerase, and ~100ng genomic DNA in a total reaction volume of 25µL. The reactions were run as described above but with the following thermocycler program: 1 cycle of 94 $^{\circ}$C for 5 min; 40 cycles of 94 $^{\circ}$C for 40 sec, 61 $^{\circ}$C for 40 sec, 72 $^{\circ}$C for 60 sec; 1 cycle of 72 $^{\circ}$C for 8 min, and a final hold at 4 $^{\circ}$C . TaMKK3 sequence from wheat cultivar Chinese Spring wheat (LC091368.1), containing the TaMKK3 resistant allele, was used as a reference sequence for alignments.

Vp1-1B was analyzed using a sequence-tagged site (STS) marker (Vp1B3, Yang et al. 2007) which produces fragments of 845 or 569 bp in PHS resistant lines and a fragment of 652 bp in susceptible lines. The PCR reaction contained 0.42 µM of the forward and reverse primers, 0.16 mM each dNTP, 2 mM $MgCl_2$, 1x GoTaq Buffer (Promega, Madison, WI), 0.65 U GoTaq G2 Flexi DNA Polymerase, and ~100 ng genomic DNA in a total reaction volume of 25 µL. The PCR reaction was done using an Applied Biosystems Veriti 96 well Thermocycler (Applied Biosystems Corp. Foster City, CA) using the following steps: 1 cycle of 96 °C for 5 min; 36 cycles of 96 °C for 40 sec, 61 °C for 1 min, 72 °C for 1 min; 1 cycle of 72 °C for 7 min, with a

final hold at 4 °C. The resulting PCR product was analyzed via agarose gel electrophoresis using a 1.5% agarose gel for two hours at 100 V.

Data Analysis

Allelic class means for each of the three PHS resistance genes were compared for each year and combined over years for each response variable by fitting a mixed effects linear model which included year, PHS resistance gene, year by PHS resistance gene, and entries within PHS resistance gene allelic class using the lme4 package (Bates et al., 2015) in R (R Core Team, 2018). The entries within PHS resistance gene allelic class was considered random, while all other factors were considered fixed effects. The resistant versus susceptible allelic class means were compared using a t statistic with the emmeans package in R (Lenth, 2018). Linear correlations among response variables were computed using the entry means averaged over years.

Results

A total of 21 winter wheat varieties consisting of 13 named varieties (commonly grown in Montana) and 8 experimental lines were genotyped to determine allelic variation of TaPHS1-3A, TaMKK3-4A, and VP1-1B (Table 1), as well as assayed to determine susceptibility to preharvest sprouting.

TaPHS1-3A alleles were determined by direct sequencing of the end of the third exon and into the third intron, wherein lay the previously described SNP mutations associated with susceptibility to PHS. There were 10 lines containing the susceptible TaPHS1 allele and 11 lines containing the TaPHS1 resistant allele. The susceptible allele group had an average PHS score of

4.30 ± 0.35, while the resistant allele group had an average PHS score of 2.90 ± 0.23. Significant differences between the two allelic groups were observed for PHS scores, plant height, and grain protein (Tables 2 and 3). TaPHS1 susceptible allele lines tended to have higher PHS scores ($P<0.01$) and were 4 cm taller ($P=0.01$) than resistant allele counterparts.

Table 1. Winter wheat genotypes surveyed for their genotype at three major loci that impact pre-harvest sprouting.

Line/Variety	**Pedigree**	**TaPHS1**[†]	**TaMKK3**[‡]	**Vp1B3**[§]
Bearpaw	Montana, 2011	S	R	S
BZ9W09-2212	WestBred experimental line (solid stem)	S	R	S
Loma	Yellowstone//MTS0112/MTS0125	S	R	S
Keldin	WestBred, 2011	S	S	R
MT1348	PI572290/BigSky	S	S	R
MT1471	Yellowstone/NuDakota	S	S	R
MTW1491	MT08189//MT08187/(MTW08166, WB3768 sib)	S	S	R
Northern	Montana, 2015	S	S	R
Yellowstone	Montana 2005	S	S	R
MT1488	MTR00118/MT0241//CDC Falcon	S	S	S
Brawl CLP	Colorado Wheat Res. Fdn., 2011	R	S	S
SY Sunrise	Syngenta (AgriPro), 2015	R	S	S
Decade	Montana/North Dakota, 2010	R	R	S
SY Monument	Syngenta (AgriPro), 2014	R	R	S
SY Wolf	Syngenta (AgriPro), 2010	R	R	S
BZ9W09-2075	WestBred experimental line	R	S	R
Warhorse	Montana, 2013	R	S	R
WB4623CLP	WestBred, 2014	R	S	R
Judee	Montana, 2011	R	R	R
WB4614	WestBred, 2013	R	R	R
WB-Quake	WestBred, 2011	R	R	R

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TaMKK3-4A alleles were determined by direct sequencing of a portion of the gene encompassing the fifth exon, which is the location of the previously described SNP mutation that has been associated with PHS susceptibility (Torada et al. 2016). There were 12 lines containing the susceptible TaMKK3 allele and 9 lines containing the R allele. The susceptible group had a mean PHS score of 3.63 ± 0.19 and the resistant group had a mean PHS score of 3.46 ± 0.22. There were no significant differences in PHS scores between the two TaMKK3 allelic groups

(Tables 2 and 3). Lines containing the TaMKK3 susceptible allele group had falling number that were about 20 seconds longer ($P<0.01$) and an increased kernel diameter of approximately 0.1mm ($P<0.05$).

VP1 allelic variation was determined by analysis with the "Vp1B3" STS marker developed by Yang et al. 2007. There were 9 lines containing the Vp1B3 susceptible allele and 12 lines containing the Vp1B3 resistant allele. The mean PHS score of the susceptible allele group was 3.64 ± 0.22 and the resistant allele group had a mean PHS score of 3.50 ± 0.19. There were no significant differences in PHS score between the two Vp1B3 groups. Lines that had the Vp1B3 susceptible allele tended to have lower falling numbers by up to 5% ($P<0.05$), 17% higher alpha amylase concentrations ($P<0.05$), and 1% higher grain protein ($P<0.05$) (Tables 2 and 3) when compared to lines containing the resistant allele.

Table 2. TaPHS1, TaMKK3, and Vp1B3 allelic means for agronomic traits averaged over two field trials.

	PHS Score[†]	**Heading Date (Julian Day)**	**Physiological Maturity (Julian Day)**	**Plant Height (cm)**	**Test Weight (kg hl^{-1})**	**Yield (kg ha^{-1})**
TaPHS1 S (10)	4.3 ± 0.35	155.1 ± 0.2	201.4 ± 4.3	100.2 ± 1.4	72.3 ± 1.4	6913.3 ± 599.8
TaPHS1 R (11)	2.9 ± 0.23	154.5 ± 0.7	200.7 ± 3.6	95.8 ± 1.1	74.0 ± 1.4	5547.4 ± 574.3
P value	<0.01	0.28	0.19	0.01	0.12	0.06
TaMKK3 S (12)	3.63 ± 0.19	154.4 ± 0.7	201.3 ± 0.3	99.2 ± 1.0	75.7 ± 2.2	6664.7 ± 280.44
TaMKK3 R (9)	3.46 ± 0.22	155.3 ± 0.8	200.6 ± 0.4	96.1 ± 1.1	73.6 ± 2.3	5575.1 ± 316.75
P value	0.37	0.30	0.13	0.11	0.35	0.11
VP1B3 S (9)	3.64 ± 0.22	154.3 ± 0.8	200.6 ± 0.4	95.4 ± 1.1	72.7 ± 2.3	5679.3 ± 322.1
VP1B3 R (12)	3.50 ± 0.19	155.2 ± 0.7	201.3 ± 0.3	99.1 ± 1.0	76.5 ± 2.2	6586.6 ± 285.1
P value	0.39	0.39	0.12	0.13	0.10	0.16

[†]Preharvest sprout measured on a 1 to 7 scale where 1 is completely dormant and 7 represents completely non-dormant.

Table 3. TaPHS1, TaMKK3, and Vp1B3 Allelic Means of Seed Traits averaged over two field seasons.

	Falling Number (s)	Alpha Amylase (U g^{-1})	Grain Protein (g kg^{-1})	Kernel Hardness	Kernel Weight (mg)	Kernel Moisture (%)	Kernel Diameter (mm)
TaPHS1 S (10)	363.5 ± 4.8	0.069 ± 0.005	125.0 ± 2.9	72.4 ± 1.1	31.8 ± 1.2	9.1 ± 0.1	2.7 ± 0.04
TaPHS1 R (11)	356.0 ± 5.8	0.068 ± 0.005	132.8 ± 1.8	72.1 ± 1.6	29.1 ± 1.4	8.9 ± 0.1	2.6 ± 0.06
P value	0.17	0.44	0.02	0.43	0.08	0.08	0.22
TaMKK3 S (12)	368.9 ± 3.8	0.066 ± 0.003	127.0 ± 2.0	71.8 ± 1.6	32.0 ± 0.9	9.0 ± 0.1	2.7 ± 0.03
TaMKK3 R (9)	347.1 ± 4.4	0.071 ± 0.003	132.0 ± 2.0	72.7 ± 1.8	28.4 ± 1.0	9.0 ± 0.1	2.6 ± 0.03
P value	0.002	0.44	0.18	0.62	0.06	0.91	0.04
VP1B3 S (9)	351.1 ± 4.5	0.076 ± 0.003	133.0 ± 2.0	71.5 ± 1.8	29.3 ± 1.0	8.9 ± 0.2	2.6 ± 0.03
VP1B3 R (12)	365.9 ± 3.9	0.063 ± 0.002	126.0 ± 2.0	72.8 ± 1.6	31.3 ± 0.9	9.0 ± 0.1	2.7 ± 0.03
P value	0.05	0.04	0.05	0.56	0.33	0.37	0.26

Agronomic data was collected to determine relationships between agronomic traits, seed traits and PHS susceptibility. Correlations were observed between PHS score and physiological maturity, yield, and kernel diameter. Lines with higher PHS scores tended to reach maturity later, had higher kernel weight and were associated with higher yield (all $P<0.05$). Falling number on showed no correlation with other traits. Alpha amylase concentrations were negatively correlated with heading date, kernel weight, test weight and yield.

Data analysis using a mixed effects linear model was performed in an effort to detect significant associations between PHS, resistance gene, year, and resistance gene by year. The analysis of variance indicated PHS scores were significantly affected by year, TaPHS1 genotype (R vs S) and year x TaPHS1 interaction (Table 4). The other resistance genes, TaMKK3 and Vp1B were not observed to have any impact on PHS scores.

Table 4. Mean squares and P values for sources of variation from analysis of variance for pre-harvest sprout score (PHS) reflecting allelic differences for three PHS resistance genes (TaPHS1, TaMKK3, and Vp1B3) for 21 winter wheat genotypes.

	TaPHS1-3A		**TaMKK3-4A**		**Vp1-B1**	
Source	Mean square	P value	Mean square	P value	Mean square	P value
Year	27.82	<0.01	30.19	<0.01	27.98	<0.01
PHS gene	7.78	<0.01	0.08	0.75	0.06	0.79
year x PHS gene	3.72	0.03	1.55	0.18	0.04	0.84

Discussion

The goal of this study was to determine allelic diversity of three major loci (TaPHS1-3A, TaMKK3-4A, and Vp1-3B) and assess potential of each loci to be used as a breeding tool to modify dormancy prior to harvest. Mutations associated with PHS in these loci have been widely observed. However, the presence of variation and the contributions to PHS susceptibility has not been determined in Montana winter wheat germplasm. This was accomplished by growing 13 named varieties and 8 experimental lines in triplicate, over two years, and assessing their susceptibility to preharvest sprouting. Other traits of interest included falling number and alpha amylase concentrations in the absence of sprouting, as both parameters are associated with PHS damage but citable literature could not be found that investigated the relationship between FN or alpha amylase concentrations of sound grain and PHS susceptibility.

The TaPHS1 gene contains mutations that both positively (decrease susceptibility) and negatively (increase susceptibility) impact PHS (Nakamura et al. 2011 and Liu et al. 2013). In the current study it was observed that TaPHS1 allelic groups were significantly different in sprouting tolerances and observations were consistent with the literature. Despite the trends toward dormancy or non-dormancy, a moderately high degree of variance was observed within each group (Figure 2). The group containing the resistant allele (n=11) tended to be more dormant with a range in PHS scores from 1.4 to 4.2. The susceptible group (n=10) tended to be

less dormant with a range of PHS scores from 2.6 to 5.9. TaPHS1 has been shown to account for up to 58% of PHS phenotypic variation in some populations, but in the current study the effects are much reduced and accounted for only about 18% of the observed variation. The reduced impact in northwestern germplasm could be due to the action of other genes, the growing environment or that the full effect of this gene was not observed due to the low number of entries. TaPHS1 R groups tended to be shorter and had significantly higher protein. This phenomenon is previously unreported in literature regarding TaPHS1 and has resulted by an unknown mechanism or is an artifact of the small sample size.

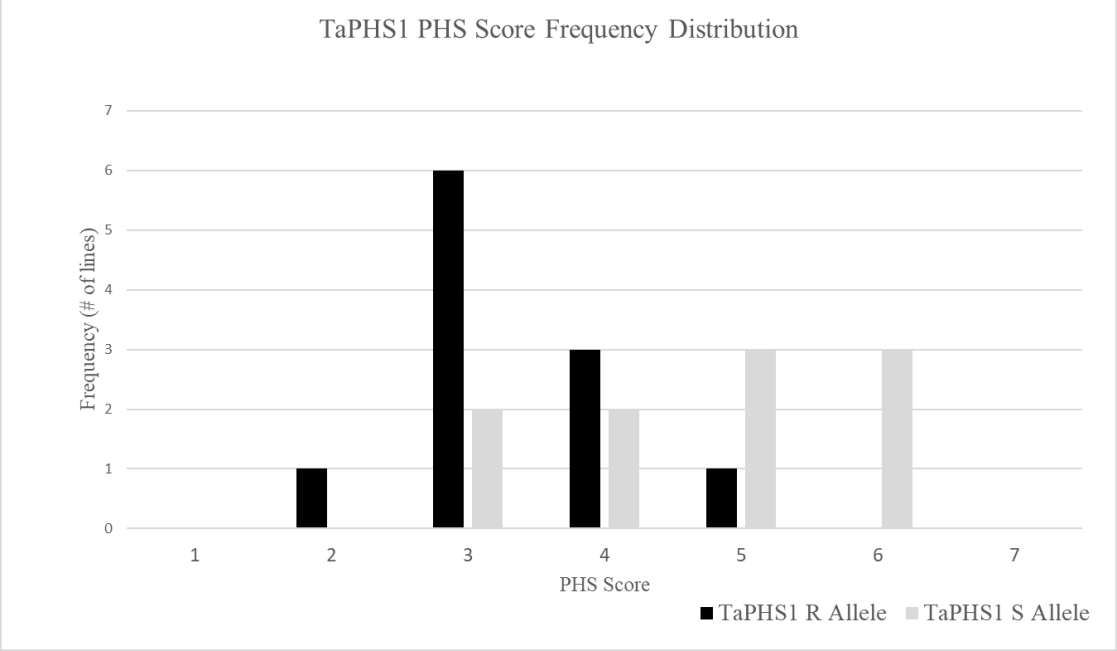

Figure 4. Frequency distribution of TaPHS1 allelic variation for preharvest sprout, measured on a 1 to 7 scale where 1 is completely dormant and 7 represents completely non-dormant. TaPHS1 resistant genotypes represented with black bar and TaPHS1 susceptible genotypes represented with grey bars.

Two other genes, TaMKK3 and TaVP1, were selected for investigation due to literature that indicates these two loci can have a large impact on PHS susceptibility (Torada et al. 2016, Shorinola et al. 2017). Torada et al. (2016) found that TaMKK3 is the causal gene underlying the

large effect PHS 4AL QTL previously described in multiple QTL studies (Kato et al., 2001, Mares and Mrva 2001, Mares et al., 2005, Torada et al., 2005, Chen et al., 2008, Ogbonnaya et al., 2008, and Liu et al., 2011). This was confirmed by Shorinola et al. (2017) via fine mapping of the 4AL QTL region. In the current study, no observable differences between TaMKK3 allelic groups were detected for PHS score. TaMKK3-4A variation may still be playing a role in PHS susceptibility in Norther Great Plains germplasm but may be masked by the large effects produced by TaPHS1 variation. Significant differences were observed between the allelic groups for falling number and kernel diameter. For example, the group with the susceptible allele had significantly higher falling number and larger kernel diameter with trends towards lower alpha amylase concentrations and higher kernel weight. The larger kernel size of the susceptible allelic group may contribute to a higher FN and lower alpha amylase levels because it is likely that the scutellum of larger grains accounts for a smaller percentage of the total volume when compared with smaller kernels (Nik et al., 2011). This could lead to less total alpha amylase, as alpha amylase is initially produced in the scutellum, and higher FN possibly due to the greater starch content in a sample of large kernels relative to a comparably sized sample of small kernels (Ranki and Sopanen, 1984). Previous literature regarding seed size, FN and alpha amylase suggests either a positive correlation (Evers et al., 1994) or no correlation (Farrell and Kettlewell, 2008), while the current findings suggest a strong negative correlation between seed size parameters and alpha amylase concentration across all entries (Table 5). Alternatively, the observed differences may be due to chance considering the small sample size representing each allelic group and the lack of previous literature identifying an association between TaMKK3 alleles and FN values. Falling number and alpha amylase are highly correlated if grain has

suffered sprout damage and the lack of correlation in the current study is most likely due to the use of sound grain for the FN and alpha amylase analysis. The reason for using sound grain was to determine if there was any relationship between varietal variation in FN or alpha amylase with PHS susceptibility, which was not observed.

Table 5. Correlations between agronomic traits and seed traits.

	PHS Score	Falling Number	Alpha Amylase	Heading Date	Physiological Maturity	Plant Height	Grain Protein	Test Weight	Yield	Kernel Hardness	Kernel Weight
Falling number	-0.120	1									
Alpha Amylase	-0.105	-0.140	1								
Heading Date	0.111	0.039	-0.462*	1							
Physiological Maturity	0.472 *	0.286	-0.304	-0.087	1						
Height	0.272	0.215	-0.341	-0.103	0.531*	1					
Protein	-0.416	-0.028	0.348	-0.008	-0.703**	-0.714***	1				
Test weight	0.378	0.041	-0.819***	0.539*	0.377	0.474*	-0.573	1			
Yield	0.482 *	-0.009	-0.731***	0.309	0.396	0.645**	-0.655***	0.921***	1		
Kernel Hardness	-0.066	0.397	-0.070	0.401	-0.144	-0.095	0.433	0.004	-0.118	1	
Kernel weight	0.435 *	0.221	-0.594**	0.025	0.498*	0.543*	-0.623**	0.689***	0.795***	-0.199	1
Kernel Diameter	0.232	0.340	-0.583*	-0.002	0.307	0.487*	-0.466*	0.625**	0.705***	-0.111	0.931***

*, **, and *** denote significance at 0.05, 0.01, and 0.001, respectively)

Like TaMKK3, TaVP1 has been reported in multiple studies as having association with PHS. Yet, in the current study, there was no difference in PHS score between the TaVP1 allelic groups (Yang et al. 2007, Feng et al. 2015, and Zhou et al. 2017). Again, this could be due to masking by the more powerful effects of TaPHS1. Recent evidence also suggests that there are many haplotypes of TaVP1 that may have associations with PHS. Therefore, perhaps the Vp1B3 marker is not as indicative when used as the sole marker for PHS resistance associated with TaVP1. The Vp1B3 marker did produce groups that showed differences in falling number, alpha amylase, and grain protein. These differences could be caused from the small dataset as they have been unreported in previous literature.

Data analysis showed strong effects from year on PHS score, as well as significant effect from TaPHS1 and year x TaPHS1 interaction which may present another reason the observed effects of TaPHS1 may not be as strong in the current study. The effects of year on PHS score

are most likely due to three things: 2017 planting was in a different part of the state, 2017 was a very hot/dry year in comparison to 2016, and heads from 2017 were collected much closer to harvest. The reason we see reduced effect of TaPHS1 from 2016 ($P>0.001$) to that of TaPHS1 in 2017 ($P>0.05$) is also due to the disparate collection time as it has been shown that TaPHS1 expression is inversely correlated with time since physiological maturity. Therefore, a later collection would logically result in a reduced impact. There were significant effects from TaPHS1 on PHS score regardless of year, while there was no effect on PHS score observed for the other two genes, TaMKK3 or Vp1B, in either year or combination thereof.

Conclusion

This study evaluated the association of three loci (TaPHS1-3A, TaMKK3-4A, and TaVP1-B3) previously reported to have major impacts on preharvest sprout susceptibility and analyzed their relationships to agronomic and seed quality traits in commonly grown and experimental northwestern Montana germplasm. Results indicate that there is allelic variation within all three genes and TaPHS1-3A has the largest impact of the three loci on PHS susceptibility. While TaMKK3 and TaVp1 have been shown to have association with PHS in other populations around the world, there was no significant evidence suggesting they are impacting PHS in the region of interest. This data suggests TaPHS1 variation could be used in breeding programs to decrease PHS while the effects of TaMKK3 and TaVp1 may be masked by TaPHS1 variation. If TaMKK3 and TaVp1 are indeed masked, then more research in populations fixed for TaPHS1 may be needed to validate effects in the germplasm that was studied.

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CHAPTER FOUR

MUTATIONS IN THE *HvMKK3* AND *HvAlaAT1* GENES AFFECT BARLEY PRE-HARVEST SPROUTING AND AFTER-RIPENED SEED DORMANCY

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

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Contributions: Carried out experiments, interpreted results, wrote manuscript.

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Contributions: Conducted gene sequencing, provided experimental planning and study design support.

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Abstract

Preharvest sprouting (PHS) is a natural phenomenon that negatively impacts various crops across the globe when late season rainfall causes seeds to germinate prior to harvest. Prevention of PHS in small grains such as barley and wheat is of particular importance due to the large acreage devoted to them. Barley (*Hordeum vulgare*) is the fourth most grown cereal crop and is often susceptible to PHS damage, which limits the regions in which barley can be grown without heavy losses. PHS damage could be mitigated by incorporation of genetic alleles that impart desired levels of dormancy at specific times in grain maturity, ideally high dormancy at harvest with a fast loss of dormancy upon after-ripening. Toward this goal, 114 barley varieties were assessed for dormancy at physiological maturity and in after-ripened grains. Three genes previously associated with dormancy in barley or wheat (*HvAlaAT1*, *HvMKK3*, and *HvMFT*) were sequenced from all lines and assessed for allelic diversity. The resulting alleles were assessed for association with dormancy at each of the maturity time points. In total, seven missense mutations were discovered across the three genes. It was observed that a single missense mutation in *HvAlaAT1* (L214F) is associated with loss of dormancy in after-ripened grain while a single missense mutation in *HvMKK3* (E165Q) is associated with a large loss of dormancy starting around physiological maturity. This study suggests that different genes may be associated with dormancy at different time points in grain maturity, which would allow for selective breeding of specific dormancy time periods.

Mutations in the *HvMKK3* and *HvAlaAT1* Genes Affect Barley Pre-Harvest Sprouting and After-Ripened Seed Dormancy

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Abbreviations: AlaAT1, Alanine aminotransferase 1; MFT, mother of FT and TFL1; MKK3, mitogen activated kinase kinase 3; QTL, quantitative trait loci; PM, physiological maturity; PHS, preharvest sprouting;

INTRODUCTION

Preharvest sprouting (PHS) is a phenomenon that negatively affects many crops. PHS of cereal grains is of interest due to the large acreage devoted to them paired with global dependence on cereals for food and feed uses. Cereal grain PHS occurs when maturing seeds encounter prolonged periods of moisture close to harvest and begin to germinate prior to harvest. The germination process activates starch and protein degrading enzymes which reduces end-use quality, particularly in malting barley (*Hordeum vulgare*) (Briggs *et al,.* 1981). Barley, the fourth most produced cereal worldwide, is one of the crops most susceptible to PHS damage and thus experiences some of the greatest losses, especially in growing areas that are subject to

significant rainfall close to harvest. Barley seed damaged by PHS may be unsuitable for all but feed uses which financially impacts both producers and end-users, making the study of PHS in barley of economic importance.

Susceptibility to PHS can be mitigated by selecting for grain that remains dormant prior to harvest. Generally, wild type grains are dormant as they approach physiological maturity, and the dormancy begins to wane as the plants approach harvest maturity with accelerating reductions of dormancy occurring during after ripening (Rodriguez *et al.,* 2015). Cereal grain dormancy is a quantitative trait affected by multiple pathways and environment interactions preceding grain maturity, making reliable analysis and selection challenging (Nakamura *et al.,* 2011; Rodríguez *et al.,* 2015; and Jiménez *et al.,* 2017). In the case of malting or winter growth habit barley, too much dormancy can be undesirable as it increases required storage times prior to use in malting or for fall planting. An ideal cultivar in terms of dormancy would remain dormant till harvest and the dormancy would then diminish quickly to allow for synchronous germination for use in malting or for replanting.

Quantitative trait loci (QTL) for seed dormancy have been detected on all seven barley chromosomes, although only two Quantitative Seed Dormancy 1 (Qsd1) and Quantitative Seed Dormancy 2 (Qsd2) are recognized as major QTL validated across multiple studies. Qsd1 is located near the centromere on the short arm of chromosome 5; while Qsd2 is also located on chromosome 5, but is near the telomere region on the long arm (Buraas and Skinnes 1984, Ullrich *et al.,* 1992; Takeda 1996; Han *et al.,* 1996; Li *et al.,* 2003; Prada *et al.,* 2004; Hori *et al.,* 2007; Sato *et al.,* 2009 and 2016; Gong *et al.,* 2014; and Nakamura *et al.,* 2016). The causal gene underlying the Qsd1 seed dormancy QTL is an alanine aminotransferase gene (*HvAlaAT1*)

mutation (Sato *et al.,* 2016). Similarly, the Qsd2 QTL was associated with a missense mutation in a mitogen activated kinase kinase 3 gene (*HvMKK3*) through fine-mapping (Nakamura *et al.,* 2016). Orthologs of these genes in bread wheat (*Triticum aestivum*) were associated with preharvest sprouting susceptibility (Onishi *et al.,* 2017; Shorinola *et al.,* 2017). A third gene, Mother of flowering time gene (*TaMFT*; also known as *TaPHS1*), also impacts PHS variation in wheat (Nakamura *et al.,* 2011; Liu *et al.,* 2013) and multiple other species. However, variation in the barley MFT ortholog has not yet been associated with seed dormancy.

The Qsd1 loci association with dormancy has been attributed to a L214F amino acid substitution in the *AlaAT1* gene. Interestingly, there are five alanine amino transferase genes in barley and each is located on a different chromosome, but to date these homologous *AlaAT* genes have not been associated with PHS (International Barley Genome Sequencing Consortium 2012). The exact mechanism that allows *HvAlaAT1* to affect dormancy is yet to be elucidated.

The Qsd2 locus on chromosome 5H is the second major locus consistently associated with dormancy variation across barley QTL experiments (Nakamura *et al.,* 2016). Near isogenic lines were developed and the QTL was fine mapped to a 7 kb region that spanned introns 2 to 10 of the *HvMKK3* gene and four non-synonymous SNPs were observed between barleys that varied in dormancy. Only one amino acid mutation, A260T, was found to be significantly associated with dormancy (Nakamura *et al.,* 2016). The A260T mutation is in the kinase domain and is an evolutionarily conserved residue in MKK3 proteins from green algae to seed plants. *MKK3* is a component of a mitogen-activated protein kinase cascade. These pathways serve as signaling modules that convey a large variety of extracellular signals and elicit an intracellular response, which allows for environmental sensing (reviewed by Colcombet and Krysan 2018). *MKK3*

affects seed dormancy in rice (*Oriza sativa*) and wheat (Torada *et al*., 2016; Shorinola *et al.,* 2017; Mao *et al.* 2019).

The *MFT* gene encodes a seed specific phosphatidylethanolamine binding protein shown to impact seed dormancy in many plant species, including: *Arabidopsis thaliana*, *Dimocarpus longan*, *Glycine max*, *Pyrus pyrifolia*, *Oryza sativa*, and *Triticum aestivum* (Xi *et al.,* 2010, Nakamura *et al.,* 2011; Liu *et al.,* 2013; Wang *et al.,* 2013; Li *et al.,* 2014; Chen *et al.,* 2018; Mao *et al.* 2019). MFT sequence variation explains up to 58% of wheat seed dormancy variation (Liu *et al.*, 2013). However, there are few studies of *MFT* in barley, making it a logical target of investigation. Rice *MFT* is partially regulated by *MKK3* via the *MKKK62-MKK3-MAPK7/14* module (Mao *et al.,* 2019). Knockouts of *MKK3* lead to increased MFT expression which in turn increases seed dormancy. Likewise, *MKK3* overexpression leads to decreased *MFT* expression which decreases seed dormancy.

The first goal of this study was to assess barley dormancy at physiological maturity and in after-ripened grain of a collection of barley varieties originating from the U.S., Canada, or Europe. The second goal was to evaluate whether the *HvAlaAT1*, *HvMKK3*, and *HvMFT* genes which were previously reported to impact wheat and barley seed dormancy are associated with dormancy at different points in barley seed maturation. By identifying alleles that affect dormancy at different points in maturity, it may be possible to breed barley that is highly dormant till harvest, but in which dormancy rapidly decays during a short after-ripening period. Breeding for dormancy at harvest time would eliminate economic loss due to PHS, but not have a negative effect on malt quality.

MATERIALS AND METHODS

Plant Materials, Agronomic and Seed Trait Analysis

Plant materials consisted of 114 barley varieties that were grown during the 2017 and 2018 field seasons at the Montana State University Post Farm near Bozeman, MT (Supplemental Table 1). All entries were grown in single 1 m rows spaced 30 cm apart in a randomized complete block design with three replications in each year. Irrigation consisted of 5 cm applied one week prior to and one week after heading.

Agronomic traits measured consisted of: 1) days to heading, 2) days to physiological maturity, 3) grain protein, and 4) seed weight. Days to heading was recorded as the number of days from planting to when 50% of primary heads had completely emerged from the boot. Days to physiological maturity was the number of days from planting to loss of 75% of green color from the head. Seed trait analysis was performed on only two replications from each growing season. An Infratec 1241 grain analyzer (Foss, Eden Prairie, MN) was used to determine grain protein percentage and grain moisture content by near-infrared transmission. Individual kernel weight was determined as the average individual seed weight of a 200 seed subsample.

Preharvest Sprout Susceptibility Assessment

Preharvest sprouting assessments were conducted by first collecting 5 heads from each plot at physiological maturity (Zadok scale 88-90) and drying heads in a forced air-drying oven at 37 °C for 24 hrs. The heads were then kept -20 °C until assayed. To assay PHS susceptibility, intact heads were removed from the freezer and positioned vertically inside a misting chamber. The misting chamber was constructed and sprouting susceptibility measurements were recorded as

described by Vetch *et al.,* (2019) using a 1 through 7 visible scale where 1 is no signs of sprouting structures, 2 is less than 10% sprouted, 3 is 10% to 24% sprouted, 4 is 25% to 49% sprouted, 5 is 50% to 74% sprouted, 6 is 75% to 99% sprouted, and a score of 7 represents completely non-dormant heads that have sprouted 100% and shoots are over 7.62 cm.

After-ripened Dormancy Assessment

Germination index tests on after-ripened seed were conducted in 2017 and 2018 using seed obtained after harvest. Harvested mature seed was immediately stored in a walk-in cold storage room at 4 °C for twelve weeks prior to performing germination index. For each plot from two field replications, 25 to 50 after-ripened seeds were placed in 90 mm glass Petri-dishes lined with two layers of Whatman no. 5 filter paper and 4 ml of distilled water, and then incubated at 20 °C in the dark for 7 days. Additionally, 2-mL of distilled water was added on day 2 or 3 to prevent drying. Germination index was evaluated visually by protrusion of the radicle or coleoptile (≥3 mm). The number of germinated grains was recorded daily and used to calculate a weighted germination index using the method of Walker-Simmons, 1988 as follows:

$$\text{Germination Index} = \frac{((7 \text{ x } n1) + (6 \text{ x } n2) \ldots + (1 \text{ x } n7))}{\textbf{\textit{Total days x Total Embryos}}}$$

where $n1$, $n2$ … $n7$ are numbers of grains germinated on day 1 to 7 and 7 to 1 are the weighted values given to grains that germinated on the first, second and subsequent days. The maximum germination index is given a value of 1.0.

Genotyping

Barley (*Hordeum vulgare*) seeds were grown at the Cereal Crops Research Units greenhouse in Madison, WI. One week old leaf tissue was collected from each seedling and flash frozen in

liquid nitrogen. Samples were stored at -80°C until used for DNA extractions. Frozen samples were crushed into fine powder using a "Mini-Beadbeater" (Biospec, Bartlesville, OK). Briefly, tubes containing frozen leaf tissue were placed in a MiniBeadBeater96 and homogenized for exactly 45 sec using 3 x 3.2 mm stainless steel beads. DNeasy Plant mini kits (QIAGEN, Germantown, MD) were used to isolate DNA from each sample. DNA samples were eluted with AE Buffer according to the manufacturer's handbook. Sample quality and quantity was determined using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). DNA samples with an OD260-OD280 absorbance ratio between 1.8 and 2.0 were used for further analysis.

All amplification reactions were performed in either PCR plates or individual PCR tubes. A 25-µl reaction per sample was prepared according to the manufacturer's instructions (Takara Ex Taq; Takara, Mountain View, CA). All products were separated using 1% agarose gels and stained with SyBr Safe DNA Stain (Thermo Fisher Scientific) and visualized on a gel documentation system.

HvAlaAT1

Amplification reactions were performed using the following PCR cycle: initial denaturation for 2 min at 98°C, followed by 30 cycles each with 15 sec at 98°C, 30 sec at 59°C and 2 min at 72°C, with a final extension of 5 min at 72°C. The exonic regions were sequenced in two parts (part 1: exons 13 and 14 and part 2: exons9, 10, and 11, respectively) (Supplemental Tables 2 and 3).

HvMKK3

Nested PCR reactions with flanking primers sets, followed by internal primers were used to obtain the *MKK3* amplicon as described by Nakumura *et al*., 2016. The amplicon was then used

to sequence the exonic regions of the *MKK3* gene in six parts using specific forward and a reverse primer sets (Supplemental Tables 2 and 3).

HvMFT

HvMFT amplicons were generated using an initial PCR followed by band excision and re-amplification. In each case, the PCR cycle involved an initial denaturation for 4 min at 94°C, followed by 30 cycles each with 30 sec at 94°C, 30 sec at 64°C and 45 sec at 72°C, with a final extension of 7 min at 72°C. The NucleoSpin-Gel and PCR Clean-Up (Macherey-Nagel, Bethlehem, PA) kit was used to purify the PCR products. The amplicon was sequenced in two parts including the four *HvMFT* exons, (Supplemental Tables 2 and 3).

Amplicons were labeled with BigDye (Thermo Fisher Scientific) according to manufacturer's instructions and submitted to the UW Biotechnology Center for DNA cleanup and direct amplicon sequencing. Sequencing reads were manually edited and trimmed for quality and assembled using DNAstar Sequence annotation and alignment software (DNASTAR, Madison, WI). Sequence variation within each gene and among lines was assessed using the MegaAlign Sequence alignment software (DNASTAR).

Conservation of Amino Acids Assessment

Conservation of amino acids at each mutation site was determined by using the Gene Tree function paired with the WASABI viewer in the ENSEMBL database (Kersey *et al.,* 2018). The WASABI viewer aligns the sequence of interest to homologous sequences from other species.

Data Analysis

The Craig Venter Instititutes: Protein Variation Effect Analyzer (PROVEAN) was used to predict the severity of amino acid mutations observed in each gene sequence (Choi and Chan 2015). The R progromaning language was used via R Studio to compute an analysis of variance for each trait using a linear model for a randomized complete block design by year and combined over years using the lm function in R (R Core Team 2018). Entry means were obtained for each year and combined over years. The presence of any interaction between year and each gene of interest (HvAlat1, HvMKK3 or row type class) for each trait was obtained by fitting a model with the lm function in R that included year, gene of interest, entry within gene of interest and a year by gene of interest interaction using the year x entry means. We then compared allelic class means for each gene and the two- vs six-row classes for all traits using a two independent sample t test assuming unequal allelic class variances. Correlations among agronomic and seed related traits were computed using the entry means combined over years. T tests and correlations were performed using Microsoft Excel 2016.

RESULTS

A total of 114 modern and historical U.S., Canadian, and European barley varieties were grown in replicated trials for two field seasons. The genotypes consisted of both two-row (86) and six-row (27) varieties that fell into four market classes: feed, forage, malt, and malt barley varieties approved for malting by the American Malting Barley Association. All varieties were assessed for multiple agronomic traits, seed traits, and dormancy at physiological maturity and in 12 week after ripened grain. All exons from genes *HvMKK3*, and *HvMFT* and the majority of exons from *HvAlaAT1* were directly sequenced to assess allelic diversity for association with dormancy at

both grain maturity points. Seven missense mutations were observed across the three genes (Table 1). We identified one mutation in *HvAlaAT1,* four in *HvMKK3*, and two in *HvMFT* that had not previously been reported for a total of two *HvAlaAt1*, five *HvMKK3* and two *HvMFT* alleles.

Table 1. Observed alleles for the genes: AlaAT1, MKK3, and MFT

Gene	Allele	No. Varieties	Mutation(s)			
AlaAT1			L214F			
	a	23	L			
	b	90	F			
PROVEAN score†			1.94			
MKK3			E165Q	L232V	R350G	N383D
	a	14	Q	L	G	D
	b	59	E	L	R	N
	c	1	E	L	G	N
	d	38	E	L	G	D
	e	2	E	V	R	N
PROVEAN score†			-2.98†	2.15	-0.20	0.32
MFT			A33T	V88E		
	a	1	T	V		
	b	1	A	E		
PROVEAN score†			0.83	-5.69†		

† PROVEAN scores of less than -2.5 are considered “deleterious”.

Variation in depth of seed dormancy at both physiological maturity and in after-ripened grain was observed for all market classes (Figure 1). Significant differences were observed between two- and six-row types for most traits for both years and when averaged over both years (Table 2). Two-row varieties headed about three days later and reached physiological maturity about three days ($P<0.001$) later than six-row varieties. Six rowed varieties had longer grain-fill in 2018 but not in 2017. The two-rowed types also had a slightly higher average germination index in after ripened grain (0.86 vs 0.77averaged over both years; $P<0.01$)), and a higher average

1000 kernel weight (*P*<0.001). Two rowed varieties had higher grain protein than six rowed varieties (P<0.01) in 2017 but in 2018. No differences were observed, for sprout score at physiological maturity.

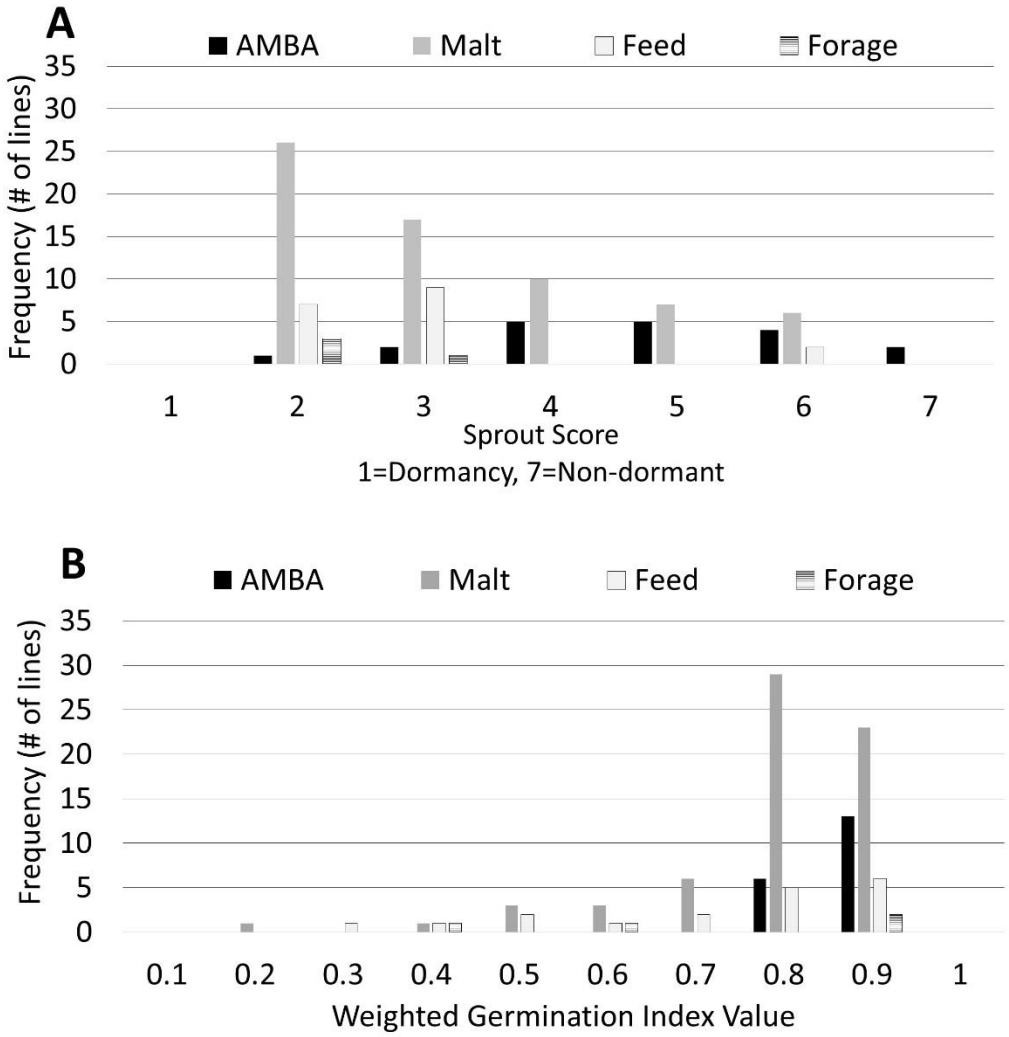

Figure 1. A) Market class sprout score frequencies at physiological maturity. A sprout score of 1 is completely dormant after seven days of wetting, 2 is less than 10% sprouted, 3 is 10% to 24% sprouted, 4 is 25% to 49% sprouted, 5 is 50 to 74% sprouted, 6 is 75% to 99% sprouted, and a score of 7 represents completely non-dormant heads that have sprouted 100% and shoots are over 2 inches. **B)** Weighted germination index values of after-ripened grains. Market class was only determined for 108 of the 114 varieties included in the experiment. There were 19

American Malting Barley Association (AMBA) varieties, 66 general malt varieties, 18 feed varieties, and 4 forage varieties.

A single missense mutation (L214F), previously reported by Sato *et al.,* (2016), was observed in the *AlaAT1* gene. There were 23 varieties carrying the wild type (WT, as described by Sato *et al.,* 2016) allele (L) and 90 carrying the mutant allele (F). The *AlaAT1* WT varieties, averaged over both years headed and reached physiological maturity three days earlier ($P < 0.001$). Grain fill was longer for two row varieties than six row varieties, but was greater in 2018 (P=0.04) than in 2017. The WT varieties tended to be more dormant at physiological maturity with that difference being greater in 2018 than 2017, and were significantly more dormant when after-ripened with an average germination index of 0.64 for WT compared to 0.88 in varieties carrying the *AlaAT1* mutant allele ($P < 0.001$). Again, this difference was greater in 2018 than 2017. This mutation is considered a neutral mutation when analyzed using Protein Variation Effect Analyzer (PROVEAN) prediction software (Choi and Chan 2015).

Four missense mutations were observed in the *MKK3* gene: E165Q, L232V, R350G, and N383D. The N260T mutation previously reported to be associated with dormancy by Nakamura *et al.,* (2016) was not detected in this study. Of the four mutations, only E165Q was associated with dormancy and was rated as "deleterious" using PROVEAN (Choi and Chan 2015). Additionally, E165Q is the only mutation in a region that is highly conserved across plant species (Figure 2). For the varieties investigated in our variety survey 100 carried the WT allele containing a glutamate at position 165, while 14 varieties carried the glutamine residue. The WT varieties were much more dormant at physiological maturity with an average sprout score of 2.5 vs 5.2 in the mutants, ($P <0.001$) and more dormant than the mutants when after-ripened (0.82

vs 0.93, $P < 0.001$) averaged over both years. Varieties containing the WT allele also were higher in seed protein ($P < 0.001$).

Two missense mutations were detected in the *HvMFT* gene (A33T and V88E) but each mutation was only observed in a single line. The A33T mutation is predicted to be neutral and is in a position that is not highly conserved (Choi and Chan 2015). The V88E mutation is predicted to be deleterious and lies at a position that is highly conserved across multiple plant species. Due to the low small sample size the *HvMFT* mutations were not able to be analyzed further.

Table 2. Two vs. Six row, *AlaAT1*, and *MKK3* allelic means for agronomic and seed traits.

Allele	No. varieties	Heading Date	Physiological Maturity	Grain Fill Period	Physiological Maturity Sprout Score	After-ripened Germination Index	Grain Protein	Kernel Weight
		--------- Julian Day ---------		No. of Days	1 : 7		%	g
Two-Row	86	186.5 ± 0.3***	218.2 ± 0.2***	31.7 ± 0.1*	2.9 ± 0.2	0.86 ± 0.06*	13.8 ± 1.1	43.9 ± 1.2***
Six-Row	27	183.2 ± 0.4	215.9 ± 0.1	32.7 ± 0.1	2.7 ± 0.2	0.77 ± 0.12	13.5 ± 1.15	39.6 ± 0.9
Row type x year interaction		<0.001	0.213	<0.001	0.548	0.048	<0.001	<0.001
AlaAT1 214 L	23	183.9 ± 0.5***	216.2 ± 0.2**	32.3 ± 0.1	2.4 ± 0.2	0.64 ± 0.12***	13.7 ± 1.1	44.2 ± 1.6
AlaAT1 214 F	90	186.2 ± 0.3	218.1 ± 0.2	31.8 ± 0.1	3.0 ± 0.2	0.88 ± 0.03	13.8 ± 1.2	42.5 ± 1.2
AlaAT x year interaction		0.806	0.073	0.126	0.014	<0.001	<0.001	0.068
MKK3 165 E	100	185.8 ± 0.3	217.8 ± 0.2	32.0 ± 0.1	2.5 ± 0.2***	0.82 ± 0.09***	13.9 ± 1.2	42.6 ± 1.4
MKK3 165 Q	14	185.5 ± 0.7	216.8 ± 0.2	31.2 ± 0.2	5.2 ± 0.1	0.93 ± 0.01	13.2 ± 0.7	44.1 ± 0.5
MKK3 x year interaction		0.343	0.083	0.468	<0.001	0.028	0.071	0.282

*, **, and *** denote significant difference between allele class means at 0.05, 0.01, and 0.001, respectively. A sprout score of 1 is completely dormant after seven days of wetting, 2 is less than 10% sprouted, 3 is 10% to 24% sprouted, 4 is 25% to 49% sprouted, 5 is 50 to 74% sprouted, 6 is 75% to 99% sprouted, and a score of 7 represents completely non-dormant heads that have sprouted 100% and shoots are over 5.1 cm. By year interactions are expressed as P values.

Protein	Species	Ensembl Sequence ID	*H. vulgare* Amino Acid Position														
						L214F											
AlaAT1			**211**	**212**	**213**	**214**	**215**	**216**	**217**								
	Hordeum vulgare	HORVU5Hr1G062990	D	G	I	L	C	P	L								
	Triticum dicoccoides	TRIDC5BG036550	D	G	I	L	C	P	L								
	Triticum aestivum	TraesCS5B02G214700	D	G	I	L	C	P	L								
	Oryza Sativa Indica	BGIOSGA032846	D	G	I	L	**C**	P	L								
	Sorghum bicolor	SORBI_3002G211300	D	G	I	L	**S**	P	L								
	Zea mays	Zm00001d020590	D	G	I	L	**S**	P	L								
						E165Q								L232V			
MKK3			**162**	**163**	**164**	**165**	**166**	**167**	**168**	...	**229**	**230**	**231**	**232**	**233**	**234**	**235**
	Hordeum vulgare	HORVU5Hr1G125290	I	A	L	E	Y	M	D		D	F	G	V	S	A	G
	Triticum turgidum	TRITD4Av1G206810	I	A	L	E	Y	M	D		D	F	G	V	S	A	G
	Triticum aestivum	TraesCS5D02G549600	I	A	L	E	Y	M	D		D	F	G	V	S	A	G
	Oryza Sativa Indica	BGIOSGA021253	I	A	L	E	Y	M	D		D	F	G	V	S	A	G
	Sorghum bicolor	SORBI_3007G105100	I	A	L	E	Y	M	D		D	F	G	V	S	**T**	G
	Zea mays	Zm00001d013510	I	A	L	E	Y	M	D		D	F	G	V	S	A	G
	Vitis vinifera	VIT_14s0066g00670	I	A	L	E	Y	M	D		D	F	G	**I**	S	A	G
						R350G								N383D			
			347	**348**	**349**	**350**	**351**	**352**	**353**	...	**380**	**381**	**382**	**383**	**384**	**385**	**386**
	Hordeum vulgare	HORVU5Hr1G125290	E	E	T	G	V	D	L		L	L	F	D	G	S	E
	Triticum turgidum	TRITD4Av1G206810	E	**Q**	T	G	V	D	L		L	L	F	**N**	G	S	E
	Triticum aestivum	TraesCS5D02G549600	E	**Q**	T	G	V	D	L		L	L	F	**N**	G	S	E
	Oryza Sativa Indica	BGIOSGA021253	E	**N**	T	**T**	V	D	L		L	L	F	**N**	G	T	D
	Sorghum bicolor	SORBI_3007G105100	**A**	**G**	T	**E**	V	D	L		L	L	F	**N**	G	S	D
	Zea mays	Zm00001d013510	**A**	**G**	T	**E**	V	D	L		L	L	F	**N**	G	P	D
	Vitis vinifera	VIT_14s0066g00670	E	**H**	**A**	**T**	V	D	L		L	L	F	D	G	S	D
						A33T								V88E			
MFT			**30**	**31**	**32**	**33**	**34**	**35**	**36**	...	**85**	**86**	**87**	**88**	**89**	**90**	**91**
	Hordeum vulgare	HORVU3Hr1G001000	A	Y	G	A	R	D	L		H	W	I	V	V	N	I
	Triticum dicoccoides	TRIDC3AG002240	A	Y	G	A	R	D	L		H	W	I	V	V	N	I
	Triticum aestivum	TraesCS3A02G006600	A	Y	G	A	R	D	L		H	W	I	V	V	N	I
	Oryza Sativa Indica	BGIOSGA007632	A	Y	G	**D**	R	D	I		H	W	I	V	V	N	I
	Sorghum bicolor	SORBI_3003G098800	A	Y	G	**P**	**K**	D	**I**		H	W	I	V	**T**	N	I
	Zea mays	Zm00001d008446	A	Y	G	**P**	**K**	D	**I**		H	W	I	V	**I**	N	I
	Vitis vinifera	VIT_17s0000g02630	Y	Y	G	**A**	**K**	H	**V**		H	W	I	V	**A**	**D**	I

Figure 2. Conservation of amino acid residues across plant species for missense mutations detected in *HvAlaAT1*, *HvMKK3*, and *HvMFT* genes. Amino acids in AlaAT1 at position 214 and in MKK3 at position 165 are highly conserved across species while the other mutation sites detected in the study are not as highly conserved.

The entry means were combined over the two growing seasons and correlations were analyzed among agronomic and kernel traits (Table 3). Heading date was observed to have a significant positive correlation with physiological maturity date ($P < 0.001$), after-ripened germination index ($P < 0.01$), protein percentage ($P < 0.05$), and was negatively correlated with grainfill time

($P < 0.001$). Physiological maturity date was positively correlated with grain fill time ($P < 0.05$) and after ripened germination index ($P < 0.05$). Physiological maturity sprout score was positively correlated with after-ripened germination index ($P < 0.001$) and kernel weight ($P < 0.05$) but negatively correlated with grain protein ($P < 0.01$).

Table 3. Correlations between agronomic and seed traits from a survey of 114 spring barley varieties.

	Heading Date	**Physiological Maturity**	**Grain Fill Period**	**Physiological Maturity Sprout Score**	**After-ripened Germination Index**	**Protein**
Physiological Maturity	0.77***					
Grain Fill Period	-0.44***	0.23*				
Physiological Maturity Sprout Score	-0.07	-0.06	0.02			
After-ripened Germination Index	0.29**	0.24*	-0.11	0.46***		
Grain Protein	0.20*	0.07	-0.20	-0.33**	-0.06	
Kernel Weight	-0.02	-0.02	0.01	0.22*	-0.08	-0.07

*, **, and *** denote significance at 0.05, 0.01, and 0.001, respectively.

DISCUSSION

Mitigation of PHS is a top concern to many barley producers, particularly those who are growing malt barley. The aims of this study were to survey modern and historical barley varieties for PHS susceptibility and determine whether genes reported to impact seed dormancy were segregating among the varieties. To assess the selected genes for their impact upon dormancy we sequenced the coding region of *HvAlaAT1*, *HvMKK3*, and *HvMFT*. These genes were chosen due to their association with barley or wheat seed dormancy (reviewed by Nakamura *et al.,* 2018 and Vetch *et al.,* 2019). While the previous studies have reported the existence of various alleles of these

genes, the frequency and impact of each allele among barley varieties originating in the U.S., Canada, and Europe has not been reported. To address this, 114 modern and historical barley varieties were grown in two field seasons and assessed for dormancy at two maturity points. We then assessed whether allelic variation in *HvAtaAT1*, *HvMFT*, or *HvMKK3* were associated with dormancy, agronomic and seed traits. Allelic means were averaged over two years but gene x year interaction was also assessed and while there were significant effects from year, the rank order remainded the same, only the magnitude of the effect changed.

The *HvAlaAT1* gene (also called *Qsd1*) has not been associated with dormancy in other plant species but seems to have a strong effect on dormancy in after-ripened barley grains and a much smaller effect at physiological maturity (Sato *et al.,* 2016). This was supported by the results of the current study in which the L214F mutation is associated with a 27% increase in germination index in after-ripened grain with no significant change in PHS score. The results are also consistent with the hypothesis of Sato *et al.,* (2016) that this mutation was likely selected for by early malt barley breeders as many top malting varieties carry the mutation. It is currently unknown how *HvAlaAT1* affects grain dormancy. Three varieties in this study carried the WT *AlaAT1* allele and the mutant *MKK3*, and all had high sprout scores at physiological maturity and germinated almost 100% in the germination index analysis indicating mutations in *MKK3* may be having hypostatic or epistatic interactions which mask or mitigate the effects of the WT *AlaAT1* allele.

The second gene investigated was *HvMKK3*. *MKK3* has diverse roles in plant stimuli response and has been associated with seed dormancy in multiple plant species (Nakamura *et al.,* 2016; Torada *et al.,* 2016; Jagodzik *et al.,* 2018; Mao *et al.,* 2019). This gene was recently proposed as

a high-effect gene underlying the major seed dormancy QTL Qsd2 and affects dormancy via the *MKKK62-MKK3-MAPK7/14* MAPK module described by Mao *et al.,* (2019). Increased dormancy was associated with a kinase domain mutation of asparagine to threonine in the 260th amino acid (N260T)(Nakamura *et al.,* 2016). The N260T mutation was not observed in the current study. Rather, a mutation from glutamic acid (E) to glutamine (Q) at the 165th amino acid position (E165Q) was observed in 14 of the studied varieties and was associated with a large decrease in dormancy at physiological maturity and in after-ripened seeds. Similarly to the N260T mutation, E165Q lies in the kinase domain but the effect is in the opposite direction. Seven of the ten least dormant varieties contain the E165Q mutation, with six of those being current AMBA recommended malting varieties (Supplemental Table 1.). This mutation was also associated with lower grain protein. However, the association of the E165Q mutation with low protein is likely due to co-selection.

The final gene investigated was *HvMFT.* In other plant species, *MFT* is a known regulator of dormancy around harvest time. Few studies have specifically targeted *HvMFT* in barley in relation to dormancy. Two missense mutations were observed but only in a single line each, which did not allow for meaningful association with agronomic and seed dormancy traits. We hypothesize that the V88E mutation, which is rated as highly deleterious by PROVEAN, would have a strong negative effect on dormancy due to its location within the gene and high conservation across other species.

In addition to looking at allelic differences, the differences between two- and six-row varieties as well as variation within a market class were also examined. No difference in dormancy was observed between two- and six-row types at physiological maturity but there was a significant

difference in after-ripened dormancy. The difference in after-ripened dormancy may have resulted from a few extremely dormant lines paired with a small number of six-rowed entries. Two-row barleys had significantly higher kernel weight which agrees with previous literature that demonstrates distinct differences in seed size/1000-kernel weight between the two head types (Boyd *et al*., 1971). With regards to market class it was hypothesized that malt varieties would be vastly less dormant at both time points due to selection for little dormancy, as is preferred by malting companies. This was perhaps somewhat true in the case of AMBA recommended varieties which had an average PM sprout score of 4.3, but when looking at malt varieties in general there was large variation in dormancy with the majority being semi-dormant around physiological maturity (avg PM sprout score of 2.7) (Supplemental Table 4). It was also hypothesized that due to little selection for reduced dormancy, forage varieties would be more dormant than other market classes. The forage class was underrepresented in the study with only 4 entries, although they did trend towards being more dormant when after-ripened with the lowest average germination index of 0.7. Many lines with moderate to high dormancy do not contain the dormancy alleles investigated in this study and will require additional experimentation to determine the sources of dormancy.

CONCLUSION

The current survey study demonstrates that the *HvAlaAT1*is strongly associated with dormancy in after-ripened grain and *HvMKK3* is strongly associated with dormancy at physiological maturity which may indicate that some genes impact dormancy at different points in grain maturity. Combinations of multiple genes carrying desired dormancy alleles may enable breeding for specific periods of dormancy. Producers would benefit from moderate to high

dormancy around harvest time while maltsters would prefer dormancy to be gone upon arrival at their facilities. There was no defined genetic structure to the population which makes it difficult to determine linkage or causation. To confirm the observations, recombinant inbred lines and isogenic lines containing the mutations of interest are being developed.

SUPPLEMENTAL MATERIAL

Supplementary data has been provided that includes row type, market class, sprout score, after-ripened germination score, and allele(s) detected for all lines in the study (Supplementary Table 1) as well as tables listing the amplification and sequencing primers (Supplementary Tables 2 & 3) and mean agronomic and seed traits by market class (Supplementary Table 4).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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CHAPTER FIVE

TAMFT HOMEOLOGS ARE ASSOCIATED WITH PREHARVEST SPROUTING WINTER WHEAT.

Contribution of Authors and Co-Authors

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Contributions: Helped plan and carried out experiments, interpreted results, drafted manuscript.

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Contributions: Provided germplasm, project guidance, and edited manuscript.

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Contributions: Project guidance, statistical analysis support, and edited manuscript.

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TAMFT HOMEOLOGS ARE ASSOCIATED WITH PREHARVEST SPROUTING IN WINTER WHEAT.

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Abstract

The phenomenon of preharvest sprouting (PHS) negatively affects wheat production and end-use in many areas. Investigating the genetics that control PHS resistance may result in increased control of seed dormancy. Multiple genes involved in the development of seed dormancy are associated with PHS. In this study the *TaMFT* (3A, 3B1, 3D), *TaMKK3*_4A, and *TaVP1*_3B genes were assessed for association with PHS in a double haploid hard red winter wheat population derived from a BCF1 cross between the varieties Loma and Warhorse. PHS was associated with all three *TaMFT* homeologs (P value < 0.01). No association between PHS resistance and *TaMKK3*_4A or *TaVp1*_3B variation was observed, though Loma and Warhorse vary for mutations previously reported to be PHS associated. To date, *TaMFT*_3A has been shown to have the largest impact on PHS resistance and this study highlights the potential impact of *MFT* hoemologs on dormancy and PHS resistance. This knowledge can potentially be used to tailor the level of dormancy of wheat varieties near harvest time.

Introduction

Preharvest sprouting (PHS) in wheat (Triticum *aestivum*) is when grain begins to germinate before harvest. PHS occurs occasionally in many wheat growing regions worldwide and is responsible for large economic losses to producers annually. The germination process damages grain and makes it unsuitable for most end-uses. Products made from flour prepared from PHS damaged grain are often porous, sticky, and are darker in color. Wheat also provides more than 20% of global human calorie intake and thus is important to global food security (FAOSTAT 2011). The economic impacts paired with the need for stable food production highlight the value in identifying mechanisms that control PHS to allow selection of varieties that are PHS resistant.

Environmental conditions play a primary role in the occurrence of PHS, not only does prolonged exposure to moisture sometime between physiological maturity and harvest trigger PHS but temperature during plant development (prior to grain maturity) has been demonstrated to impact PHS susceptibility (Biddulph *et al.,* 2007; Nakamura *et al.,* 2011; Jiminez *et al.,* 2017). Wheat varieties vary greatly in PHS under similar conditions (Vetch *et al.,* 2019a). This variation can largely be attributed to the genetic mechanisms that control dormancy (Biddulph *et al.,* 2007 and Nakamura 2018).

Preharvest sprouting is a complex, quantitative trait (reviewed by Mares and Mrva 2014, Nakamura 2018, and Vetch e*t al.,* 2019b). Multiple genetic studies focused on PHS in wheat have been completed in recent years and quantitative trait loci (QTL) have been identified on each wheat chromosome. However, many QTL are not repeatable across different populations and environments (Nakamura 2018). Relatively few of the identified QTL have been studied in detail. The QTL QPhs.ocs-3A.1 (also known as *QPhs.pseru-3AS)* on chromosome 3AS has

been consistently identified in many QTL studies and has the largest reported impact, explaining up to 58% of PHS variation (Mori *et al.,* 2005; Liu *et al.,* 2008; Lin *et al.,* 2016; Shao *et al.,* 2018). A second QTL on the long arm of chromosome 4A called Qphs.ocs.1 (also referred to as: Phs1 and Phs-A1) explains up to 43% of PHS variation and has also been consistently identified in multiple studies (Torada *et al.,* 2005; Lin *et al.,* 2017).

TaMFT (also referred to as *TaPHS1*) has been identified as the causal gene underlying the frequently observed QPhs.ocs-3A.1 QTL (Nakamura *et al.,* 2011 and Liu *et al.,* 2013). MFT belongs to a family of proteins known as phosphatidylethanolamine-binding proteins. These proteins have been identified in most plants and are generally involved in phase transitions, such as the transition from vegetative to reproductive phases (Karlgren *et al.,* 2011). *TaMFT* homologs have been studied in multiple plant species and are observed to have a major role in seed dormancy via modulation of hormone responses (Footitt *et al.,* 2017; Liu *et al.,* 2020; Chen *et al.,* 2018).

In wheat, multiple *TaMFT_3A* mutations have been associated with either PHS resistance or susceptibility. Nakamura *et al.,* (2011) identified a single nucleotide polymorphism (SNP) present in the *TaMFT_3A* promoter region (-222) that was associated with upregulated gene expression, resulting in increased PHS resistance. Interestingly, the effects of this mutation were most strongly observed when the plants experienced cool temperatures during grain fill. Liu *et al.,* (2013) found two additional *TaMFT_3A* SNPs in the third intron that reduce PHS resistance. The first SNP (+646) causes mis-splicing of the third exon. The second SNP (+666) introduces a premature stop codon. Together, these SNPs cause production of *TaMFT_3A* transcripts that are truncated and protein products that are either non- or partially functional. The most recent

mutation to be discovered is a 33-bp insertion which is also located in the promoter region of the gene (-194) (Jiang *et al.,* 2018). The location of this 33-bp insertion is suspected to be the binding site of the APETALA 2 transcription factor and is also thought to reduce*TaMFT_3A* expression levels.

Wheat is a hexaploid crop and around 70% of all genes are present and expressed from each sub-genome (Ramírez-González *et al.,* 2018). There are three known TaMFT_3A homeologs, two on chromosome 3B and one on 3D, but, the 3B and 3D homeologs have not been associated with PHS resistance or susceptibility. The two copies of MFT on chromosome 3B are tightly linked and approximately 875 kb apart. According to the most recent wheat genome build (IWGSC Refseq v1.0) accessed in the Ensembl plants database on 6/17/2020 (https://plants.ensembl.org), TaMFT_3A is 96.0% identical to homeolog 3B1 (TraesCS3B02G010100), 90.9% similar to homeolog 3B2 (TraesCS3B02G007400) and 96.6% identical to homeolog 3D (TraesCS3D02G004100) (Bolser *et al.,* 2016).

The second most reported QTL affecting PHS in wheat is the Qphs.ocs.1 4AL QTL (Chen *et al.,* 2008; Torada *et al.,* 2008; Graybosch *et al.,* 2013; Albrecht *et al.,* 2015; Barrero *et al.,* 2015). Recent efforts at fine mapping the QTL have concluded the casual gene is *Mitogen Activated Kinase Kinase 3 (TaMKK3)* (Torada *et al.,* 2016 and Shorinola *et al.,* 2017). *TaMKK3* was first identified as a PHS candidate gene in 2016 and was observed to be a major regulator of dormancy in mature seeds due to a single coding region SNP (N220K) in the kinase domain that reduces dormancy (Torada *et al.,* 2016). A similarly placed mutation (N260T) in the kinase domain of the barley *MKK3* is associated with increased seed dormancy (Nakamura *et al.,* 2016). Recent genome wide association studies have also observed associations between PHS

and *TaMKK3* via linked markers (Lin *et al.,* 2018; Sydenham and Barnard, 2018) but some studies have seen no correlation between *TaMKK3* allelic variation and PHS (Vetch *et al.,* 2019a).

Viviparous-1 (*Vp1*) (homologous to *ABI3*, *Abscisic Insensitive 3*) is another gene that has been identified as impacting PHS. *Vp1*, first discovered in maize, encodes a transcription factor that plays a major regulatory role in anthocyanin synthesis, seed dormancy, and desiccation by altering seed response to ABA (reviewed by McCarty, 1995). In wheat, levels of *TaVp1* transcripts are directly associated with ABA sensitivity levels which impact germination (Nakamura and Toyama 2001). *TaVp1* has been mapped to the long arm of chromosome 3 and mutations in each homeolog have been demonstrated to impact PHS (Bailey *et al.,* 1999; Osa *et al.,* 2003; Yang *et al.,* 2007; Chang *et al.,* 2010; Ren *et al.,* 2015.)

To explore factors affecting PHS in Northern Great Plains winter wheat populations and confirm previous findings, the current study examined the individual and combined effects of *TaMFT_3A*, *TaMKK3* and *TaVp1B3* alleles in a double haploid population of Northern Great Plains, hard red, winter wheat.

Materials and Methods

Plant Materials and Agronomic/seed characteristics

A hard red winter wheat biparental population consisting of 162 double haploid lines (DHL) and two parent lines 'Loma' (PI 6805) (Bruckner *et al.,* 2017) and 'Warhorse' (PI 67015) (Berg *et al.,* 2014), was used for this study. The population was a BC_1 population in which the double haploids were generated by first crossing Loma x Warhorse followed by a single backcross to Loma. All plant materials were grown without irrigation at the Montana State

University Post Farm near Bozeman, MT during the 2016/2017 and 2017/2018 field seasons. For the 2016/2017 field season a single replication of all 164 lines (162 DHL plus the two parents) were planted on October 10th of 2016 in 0.9 m head rows with 0.3 m row spacing. For the 2017/2018 field season the same 164 lines were planted on October 9th,2017 in three replications of a randomized complete block design using 0.9 m rows with 0.3 m row spacing. For 2017, there was 44 cm of precipitation during the growing season and the available N-P-K was: 48 kg/ha, 17ppm, and 332ppm respectively. An additional 260 kg/ha – 0 ppm – 0 ppm (N-P-K) was applied at seeding. For 2018, there was 54 cm of precipitation and the available N-P-K was: 67 kg/ha, 14 ppm, and 306 ppm respectively. An additional 235 kg/ha -35 ppm -10 ppm -20 ppm (N-P-K-S) was applied at seeding. An extra three replications of 3 plants per row (1458 total plants) were planted in late January 2018 in plastic cones (1 plant per cone) with sunshine mix soil and placed in a greenhouse. Upon setting their third leaf, the plants were vernalized at 4 °C with a 10 hour photoperiod for 8 weeks and then transplanted into the field on April 27th, 2018. The three replications of transplant samples were planted in a randomized complete block design using single 0.6 m rows with three plants per row. The plants were spaced 20.3 cm apart with 0.3 m row spacing. All replications contained randomly planted, standardized checks (varieties Loma and Warhorse).

Agronomic traits

Days to heading was measured as when 50% of the heads were completely emerged. Days to physiological maturity was estimated as the days from planting when approximately 80% of the peduncle lost its green color. A single-kernel characterization system (SKCS 4100, Perten Instruments, Springfield, IL) was used to determine kernel diameter, hardness, moisture,

and weight from 200 seeds harvested from each row. Protein percentage was measured by near-infrared transmission using an Infratec 1241 grain analyzer (Foss, Eden Prairie, MN).

Preharvest sprouting assessment

Preharvest sprouting assessments were conducted by snapping five primary heads from each plot at physiological maturity, which was characterized by loss of approximately 80% of green color from the peduncle. Upon collection, heads were stored for 24 hours in a 37 °C forced air oven and then frozen at -20 °C until assayed. A 1.5 m W x 4.8 m L x 0.6 m H misting chamber was constructed of 2.54 cm dia. PVC pipe and covered with 2 mil clear polyethylene plastic sheeting inside a greenhouse with 16-hour light periods and average day and night temperatures of 22 °C and 18 °C. The top and south end of the chamber was shaded to prevent overheating. Heads were placed into the chamber vertically and misted from above for 10 seconds every 5 minutes to maintain constant saturation and high humidity. The misting system consisted of six Misty-Mist 1.25 mm (0.3gpm) overhead misting nozzles (Dramm Corporation, Manitowoc, WI) mounted on 0.3 m risers that were centered in the chamber and linearly spaced every 50.8 cm. An Irritrol 700B-.75 Ultra Flow NPT Threaded Valve with Flow Control, 1.9 cm (Irritrol, Riverside, CA) paired with a Galcon 8056 AC-6S irrigation controller (Galcon USA LTD, San Rafael, CA) controlled the flow rate and misting times. Heads were held vertically by placing them within four 0.76 m x 2.13 m benches that had two layers of 0.63 cm steel mesh that was spaced 7.5 cm apart such that the bottom of the cut stem was held in the lower mesh and the head rested in the upper mesh.

After 7 days of misting the heads were scored using a 1 to 7 scoring system (Vetch *et al.,* 2019a) where 1 is completely dormant (showing no visible sprouting structures), 2 is less than

10% sprouted, 3 is 10% to 24% sprouted, 4 is 25% to 49% sprouted, 5 is 50% to 74% sprouted, 6 is 75% to 99% sprouted, and a score of 7 represents completely non-dormant heads where all seeds have germinated and shoots are over 7.62 cm

Genotyping

DNA was extracted from each DHL and the parent varieties by planting five seeds of each in a greenhouse and collecting a composite sample containing one 2 cm leaf segment from each of at least five plants. DNA was then extracted using a protocol adapted from the one described by Riede and Anderson (1996). *TaMFT_3A,* and *TaMKK3* were genotyped by directly sequencing the causal mutations associated with PHS susceptibility. An 863 bp fragment of *TaMFT_3A*, containing the causal SNPs (+646 and +666), was amplified and sequenced as described by Liu *et al.,* (2013). An 800 bp fragment of *TaMKK3*, containing the single causal SNP (C660A), was amplified and sequenced as described by Torada *et al.,* (2016).

TaVp1 alleles were assessed using a previously developed STS marker (*Vp1B3*) that produces one of three possible bands: an 845 or 569 bp band associated with PHS tolerance or a 652 bp band associated with PHS susceptibility (Yang *et al.,* 2007). PCR conditions and equipment for each reaction were as described by Vetch *et al.,* (2019a).

MFT 3B1 alleles were assessed by identification of a region of the gene that is polymorphic between the two parental varieties. A ~2kb fragment of *TaMFT*_3B1 was amplified using forward primer 5' CTCCTGAATTTGATGGTATGC 3' and reverse primer 5' CGGGATTAAGTATTGTAGCTCG 3'. The amplicon was sequenced using the reverse primer. The PCR reaction was as follows: 1 cycle of 96 °C for 5 minutes, 40 cycles of 96°C for 40 seconds, 60°C for 30 seconds and 72°C for 2.5 minutes, and 1 cycle of 72°C for 7 minutes and

hold at 4°C until taken out of machine. This yielded an approximately 650 bp fragment covering the end of the third intron and half of the 4th exon which contained a single deletion in warhorse (compared to Chinese spring) and multiple missense mutations in Loma (see supplemental figure 1 for sequence data).

MFT 3D was assessed using an STS marker (MFT3D4) that was observed while attempting to obtain gene sequence. PCR was performed using forward primer 5' TGTTCGAGCAGAAGACGC 3' and reverse primer 5' TTGCCTTGGCGTAGCG 3'. The PCR reaction parameters were as follows: 1 cycle of 96°C for 5 minutes, 40 cycles 96°C for 5 minutes, 56°C for 30 seconds, 72°C for 1 min and 1 cycle of 72°C for 7 minutes followed by hold at 4°C until taken out of machine. The PCR produces a differential banding pattern with Warhorse producing three bands (approximately 500, 300, and 200 bp) and Loma producing two bands (200 and 300bp, see Figure 1).

Please see the associated correction to declare an extreme error in this chapter. https://scholarworks.montana.edu/xmlui/handle/1/16742

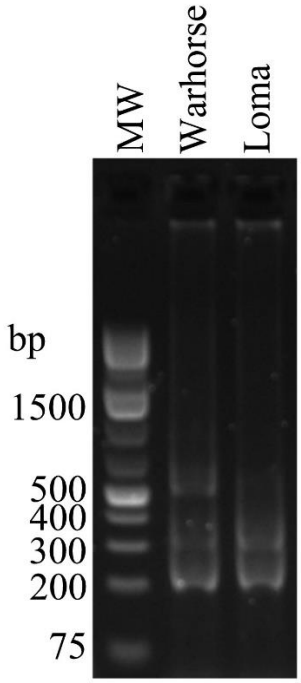

Figure 1. MFT3D4 marker. Warhorse produce three bands of approximately 200 bp, 300bp, and 600bp. Loma produced two bands of approximately 200 bp and 300-bp.

RNA-Sequencing

Whole heads were harvested from each parent at 25 days after flowering summer 2018. The heads were cut and immediately placed in liquid nitrogen and then at stored at -80 °C. The heads were threshed by hand in liquid nitrogen. Seeds were removed from the liquid nitrogen 3 to 5 at a time and allowed to thaw just enough to separate the embryo from the rest of the grain. The embryos were immediately placed back on liquid nitrogen. Frozen embryo tissue was homogenized by adding two 3 mm glass balls to the frozen embryos in a 2 ml microcentrifuge tube and processing them in a Mini Beadbeater-96 (Biospec Products Bartlesville, OK) for one minute. RNA was extracted using a QIAGEN RNeasy Plant Mini Kit (Hilden, Germany) according to the manufacturer instructions. Purified RNA was sent to GENEWIZ (GENEWIZ Inc., NJ) for sequencing. Approximately 30 to 50 million paired-end, 150bp, reads were obtained for each sample.

Gene expression analysis

Targeted gene expression analysis was conducted on three biological replications using ArrayStar v.15 (DNAstar, Madison, WI). Target gene sequences were manually uploaded in FASTA format (see table 3 for Refseq v1.0 gene identification). The following Qseq advanced options were adjusted from default settings: 1) Read match was set to 100% 2) Minimize mers (may degrade results) was unselected, all other settings were default. Statistical comparison between the average expression levels for each genotype were generated within Arraystar using a student's ttest. Fold change between genotypes was calculated within Arraystar with average

Warhorse expression as the control and average Loma expression as the experiment. All expression values were normalized to Cyclophilin A (CYP18-2, AY456122) expression.

Statistical analysis

An analysis of variance was computed for each trait for the two 2018 trials using a model for a randomized complete block design. Entry means were obtrained. Overall entry means were obtained by averaging the entry means from the 2018 trials and the 2017 trial. Allelic class means for TaMFT (3A, 3B, 3D), TaMKK3, and TaVp1 were computed with a t statistic with unequal variances using the t.test function in R (R Core Team, 2018).

Results

A total of 162 hard red winter wheat lines derived from Loma//Warhorse BC_1F_1 and parent lines were planted in a single replication in one year and in two replicated trials in a second year. Agronomic data and seed traits such as heading date, physiological maturity, grain protein, kernel hardness, moisture, and diameter were assessed for each replicate. All lines were also assessed for PHS susceptibility. Entries were then genotyped for *TaMFT_3A, TaMFT_3B1* and *TaMKK3* by direct sequencing of portions of the genes. *TaMFT_3D* and *TaVp1_3B* genes were genotyped using the STS markers MFT3D4 (developed in this study) and Vp1B3 (previously developed by Yang *et al.* 2007). Parental lines contain different alleles for all five genes, varied in protein content, and varied greatly in their susceptibility to PHS, from 1.1 to 4.5 according to the PHS scale (Vetch *et al.,* 2019a).

Table 1: Parental Genotypes and Trait Means (± Standard Error)

Parent Variety	*TaMFT Allele†*			*TaMKK3* Allele†	*Vp1B3* Allele†	Heading Date	Physiological Maturity	Sprout Score	Protein	1000 Seed Weight
						----------(Julian Day)-----				
	3A	*3B1*	*3D*	*4A*	*3B*		-----	1-7	%	g
Loma	S	S	S	R	S	170.0 ± 0.4	215.5 ± 0.4	4.5 ± 0.4***	13.5 ± 0.1**	34.4 ± 0.4
Warhorse	R	R	R	S	R	169.9 ± 0.2	214.9 ± 0.5	1.1 ± 0.1	12.8 ± 0.2	35.2 ± 0.5

*,**,*** denote significance of P value < 0.05, 0.01, and 0.001 respectively.
† Alleles are designated as R for resistant or S for susceptible according to this or previous studies by Liu *et al.,* 2013; Torada *et al.,* 2016 and Yang *et al.,* 2007.
Sprout scoring scale (1-7): A score of 1 is completely dormant after seven days of wetting, 2 is less than 25% sprouted, 3 is 25% to 49% sprouted, 5 is 50 to 75% sprouted, 6 is 75% to 99% sprouted, and a score of 7 represents completely non-dormant heads that have sprouted 100% and shoots are over 2 inches.

Genotyping for *TaMFT_3A* in the DHL progeny identified 53 lines carrying the resistant allele (G at position +646 combined with A at position +666) and 109 lines carrying the susceptible allele (A at position +646 and T at position +666) as described by Liu *et al.,* (2013). The allelic groups differed significantly (P value < 0.001) for heading date and PHS score. Lines containing the R allele had an average PHS score of 2.9 ± 0.1 while lines containing the S allele had an average sprouting score of 3.7 ± 0.1 indicating lines that contain the R allele have reduced susceptibility to PHS at physiological maturity. *TaMFT_3B1* polymorphisms were detected between the parental lines. In comparison to Chinese spring, the Warhorse allele contained a 17 bp deletion in the third intron, 180 bp before the start of the fourth exon. Loma had multiple missense mutations in the portion of exon 4 captured by sequencing (see supplemental figure 1). There were 23 lines in the DHL population carrying the Warhorse allele and they had an average sprouting score of 2.7±0.2 while 126 lines carried the Loma allele and had a mean sprout score of 3.6±0.1. The Loma allele contained multiple mutations relative to Warhorse and Chinese Spring sequences and had a significantly higher mean sprout compared to lines carrying the

Warhorse allele. Because of this, it was decided that the Loma allele was the susceptible allele and the Warhorse allele was resistant.

TaMFT_3D was genotyped using the MFT3D4 marker developed in this study, which produces a differential banding pattern in Loma vs Warhorse. There is no sequence data for this marker. Running this marker on the double haploid population identified 34 lines carrying the Warhorse allele and 126 lines with the Loma allele. The Warhorse allele type mean sprout score was significantly different from that of Loma (P value < 0.001) with sprout scores of 2.7 ± 0.2 and 3.6 ± 0.1 respectively. Warhorse allele was labeled as the resistant allele while the Loma allele was labeled as susceptible. The *MFT* 3D alleles also differed significantly for 1000 kernel weight (P value < 0.05) with the Warhorse allele group having a mean weight of 31.6±0.4g and Loma types having a mean wheat of 32.6±0.2.

TaMKK3 sequencing showed that 99 lines contained the resistant allele (carrying nucleotide C at position +660) and 61 lines contained the susceptible allele (carrying nucleotide A at position +660) as described by Torada *et al.,* (2016), genotype was not acquired for 2 lines resulting in an n of 160.

It was observed through Vp1 genotyping with the Vp1B3 that 24 lines carried the resistant allele (845bp or 569bp repeat) and 136 lines carried the susceptible allele (652bp repeat) as described by Yang *et al.,* (2007). The Vp1 alleles were associated with heading date (P value < 0.01) and kernel weight (P value < 0.05). Lines that carried the resistant allele had a slightly shorter heading time and very slightly reduced grain weight.

Table 2. Allelic means for agronomic and seed traits of entire population (± standard error)

Allele	No. lines	Heading Date	Physiological Maturity	PHS Score	Protein	1000 Kernel Weight
		----------Julian Day----------				
		-		(1:7)	%	
TaMFT_3A R	53	169.9 ± 0.06***	213.9 ± 0.1	2.9 ± 0.1***	13.09 ± 0.1	32.6 ± 0.3
TaMFT_3A S	109	169.6 ± 0.04	214.0 ± 0.1.	3.7 ± 0.1	13.03 ± 0.1	32.3 ± 0.2
TaMFT_3B1 R	23	169.6 ± 0.12	214.3 ± 0.2*	2.7 ± 0.2***	12.88±0.1*	31.1±0.4***
TaMFT_3B1 S	126	169.8 ± 0.04	213.9 ± 0.1	3.6 ± 0.1	13.05±0.04	32.6±0.2
TaMFT_3D R	34	169.7 ± 0.1	214.2 ± 0.1	2.7 ± 0.2***	13.08 ± 0.4	31.6 ± 0.4*
TaMFT_3D S	127	169.8 ± 0.04	214.0 ± 0.1	3.6 ± 0.1	13.04 ± 0.2	32.6 ± 0.2
TaMKK3 R	99	169.7 ± 0.0*	214.1 ± 0.1	3.5 ± 0.1	13.07 ± 0.1	32.3 ± 0.2
TaMKK3 S	61	169.8 ± 0.1	213.9 ± 0.1	3.4 ± 0.1	13.02 ± 0.1	32.5 ± 0.3
Vp1B3 R	24	169.5 ± 0.1**	214.1 ± 0.2	3.6 ± 0.1	12.9 ± 0.1	31.8 ± 0.5*
Vp1B3 S	136	169.8 ± 0.0	214.0 ± 0.1	3.4 ± 0.1	13.1 ± 0.1	32.4 ± 0.2
Range		167 - 170	212 - 216	1.0 - 6.2	12.0 - 14.8	27.6 - 40.0

*, **, *** denote significance of Pvalue < 0.05, 0.01, and 0.001 respectively.

RNA-sequencing was conducted on the parental line embryos at 25 days after flowering. Six dormancy genes: *AlaAT1*, *DOG1*, *MFT*, *MKK3*, *Sdr*, and *Vp1*, and all associated homeologs were targeted for RNAseq analysis (Table 3.). *TaMFT_3A* (TraesCS3A02G006600) was down regulated over 9 fold in the susceptible genotype Loma vs the resistant genotype Warhorse, *TaMFT_3B1* (TraesCS3B02G010100) was significantly upregulated 1.7 fold (P value<0.05), *TaMFT_3B2* (TraesCS3B02G007400) was downregulated 3354 fold (P value<0.01), and *TaMFT_3D* was downregulated about 6 fold (P value<0.001). No significant differences were observed in expression of dormancy genes other than the *MFT* genes.

Table 3. RNA sequencing analysis of embryos from Loma and Warhorse varieties at 25 days after flowering

Name	Ensembl Transcript ID (Refseq 1.0)*	Warhorse - linear total RPKM	Loma - linear total RPKM	Fold change
AlaAT1_5A	TraesCS5A02G216200.1	75719 ± 11773	54064 ± 13442	-
AlaAT1_5B	TraesCS5B02G214700.1	75337 ± 10478	60790 ± 14082	-
AlaAT1_5D	TraesCS5D02G224200.1	132475 ± 18018	106826 ± 21779	-
DOG1_3A	TraesCS3A02G306200.1	1885 ± 219	1578 ± 410	-
DOG1_3B	TraesCS3B02G330400.1	5188 ± 456	5323 ± 840	-
DOG1_3D	TraesCS3D02G295800.1	6168 ± 1154	5598 ± 935	-
MFT_3A	TraesCS3A02G006600.1	33413 ± 7807	3495 ± 965	9.559 down**
MFT_3B1	TraesCS3B02G010100.1	22965 ± 2871	41066 ± 4220	1.788 up*
MFT_3B2	TraesCS3B02G007400.1	57555 ± 4026	ND	down**
MFT_3D	TraesCS3D02G004100.1	89164 ± 6111	14106 ± 1586	6.320 down***
MKK3_4A	BAU88551.1 (NCBI ID)	1823 ± 178	1698 ± 569	-
MKK3_5B	TraesCS5B02G565100.6	2117 ± 280	1514 ± 432	-
MKK3_5D	TraesCS5D02G549600.1	1891 ± 283	1963 ± 566	-
TaSdr_A	TraesCS2A02G191400.1	17033 ± 1324	14042 ± 2924	-
TaSdr_B1	TraesCS2B02G215300.1	15036 ± 561	12100 ± 2150	-
TaSdr_D	TraesCS2D02G196200.1	9842 ± 3260	13300 ± 2463	-
Vp1_3A	TraesCS3A02G417300.1	8878 ± 719	8188 ± 1125	-
Vp1_3B	TraesCS3B02G452200.1	19006 ± 2404	15840 ± 2382	-
Vp1_3D	TraesCS3D02G412800.1	15933 ± 2876	13844 ± 1603	-
Actin	TraesCS1A02G274400	43170 ± 2639	42921 ± 6303	-
CYP18-2	AY456122 (NCBI ID)	453413 ± 0	453413 ± 0	-

*Genome assembly IWGSC GCA_900519105.1 accessed on 10/15/2019

Discussion

The aim of this study was to investigate the effects of three genes on preharvest sprouting susceptibility in a double haploid population of winter wheat. The three genes were chosen based on their observed effects on dormancy from previous studies (Liu *et al.,* 2013; Torada *et al.,* 2016 and; Vetch *et al.,* 2019a and; Yang *et al.,* 2007). These three genes all act to promote seed dormancy, and have a complex regulation that includes multiple genes (Figure 2.) The effects of *TaMFT_3A*, *TaMKK3_4A*, and *TaVp1_3B* on the dormancy of Northern Great Plains winter

wheats has not been well documented in comparison to populations or varieties originating from other regions (Ren *et al*., 2015; Shao *et al.,* 2018; Yang *et al.,* 2007.). Vetch *et al.,* (2019a) found that allelic variation in *TaMFT_3A* was associated with dormancy around physiological maturity but only accounted for 18% of the observed variation while, contrary to previous studies, variation in *TaMKK3* and *TaVp1* were not associated with dormancy. The lack of observed association in *TaMKK3* and *TaVp1* variation with dormancy led to the current study in which a bi-parental recombinant inbred population was used and TaMFT_3A homeologs (3B1 and 3D) were also assessed to determine their association with susceptibility to PHS.

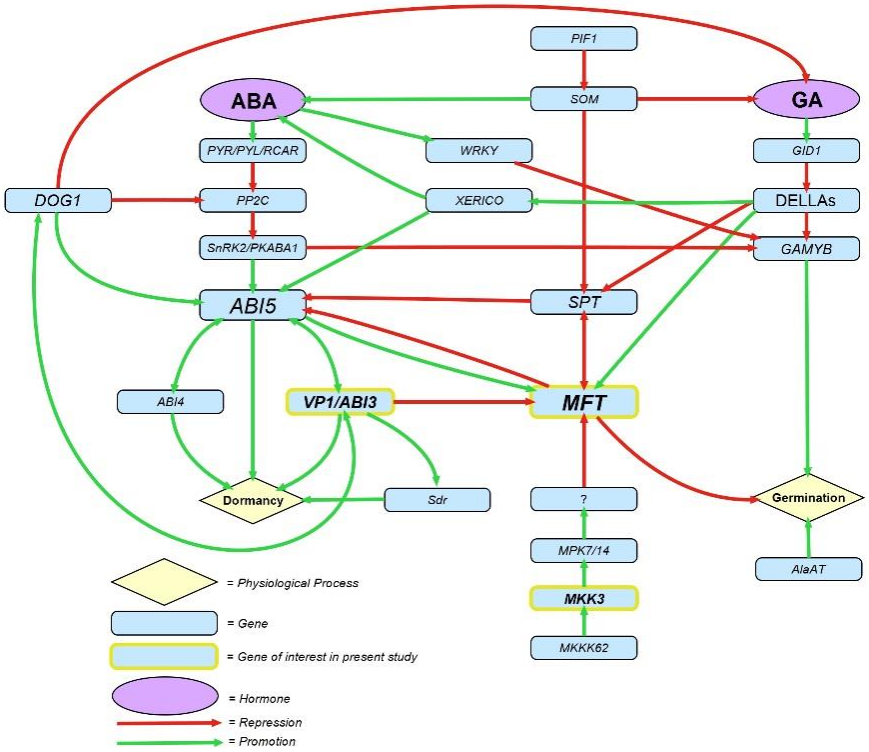

Figure 2. Theoretical genetic regulation of dormancy.

The population investigated consisted of 162 double haploid lines generated from a PHS susceptible variety (Loma) and a resistant variety (Warhorse) both of which have been recently

released and are adapted to Montana, USA. The population was non-conventional in that Loma was crossed to Warhorse and the resulting F1 was backcrossed to Loma before generation of the double haploids. The 162 double haploids displayed unexpected segregation ratios for for *TaMFT 3A, TaMFT 3B1, TaMKK3* and *TaVP1* indicating mutations may infer some fitness cost in general, specifically during the generation of double haploids, or there may be a preferential crossover from certain chromosomes, see supplemental table 1 for chi square results.

The *TaMFT_3A* mis-splicing mutation and downstream nonsense mutation, described by Liu *et al.,* (2013) was observed in 109 of the 162 lines and was significantly associated with PHS susceptibility (P value < 0.001), explaining approximately 7.5% of the observed phenotypic variation. This effect was expected due to the extreme nature of the mutations and previously documented impact. Although the expected trend was observed for *TaMFT_3A* susceptible lines, the magnitude of the effect was expected to be higher based on observations in previous studies, where a much larger degree of variation is explained by variation in the *MFT_3A* gene. *TaMKK3*_4A and *TaVp1_3B* alleles were not observed to have any association with PHS susceptibility which was contrary to what we hypothesized, contrary to previous literature, but in agreeance with our previous study Vetch *et al.* (2019a). The lower than expected impacts of *MFT_3A* variation paired with the undetectable impacts of *MKK3_4A* and *Vp1_3B* variation indicated other loci may be attributed with much of the observed variation.

To determine if and to what extent dormancy genes and associated homeologs are expressed in the two parent lines, embryos were isolated from three replications of field grown parent lines at 25 days post anthesis. RNA was extracted and a targeted RNAseq analysis we conducted.*MFT*_3A and all homeologs were detected to be significantly up or down regulated in

the susceptible parent (Loma) vs the resistant parent (Warhorse). At present, wheat *MFT*_3A homeologs and their independent impacts on dormancy have not been well characterized. Other dormancy genes and gene homoeologs investigated in the RNAseq analysis included *AlaAT1*, *DOG1*, *MKK3*, *Sdr*, and *Vp1*. Expression of these other target genes or their homeologs were not significantly different between the two parents at 25 days after anthesis. This data cannot be used to rule out the potential impacts of the other genes but does support the observations that *MFT* is associated with differences in dormancy while *MKK3* and *Vp1* were not and may indicate that *MFT*_3A homeologs are playing an important, undocumented role in seed dormancy.

Two markers were developed (MFT3B1 and MFT3D4) to track allelic variation that is hypothesized to be associated with dormancy in the DHL population. As noted earlier, there were multiple polymorphisms between the parents in the MFT3B1 sequence we obtained with the Loma allele seeming to have the higher number of and most drastic changes to sequence. This could indicate that the Loma copy of 3B1 is nonfunctional or only partially functional. Additionally, there is a fourth homeolog of *TaMFT*, the 3B2 copy. This gene lies approximately 875kb away from 3B1 and is assumed to be linked. None of the homeologs have previously been shown to affect PHS or seed dormancy and as can be seen in this study, each homeolog appears to be associated with a one-point change in the sprout scoring scale. This effect appears to be additive when the three homeologs are assessed simultaneously (Supplemental table 2), but further investigation is needed for confirmation. This new-found association between *MFT* homeologs and PHS tolerance can potentially be used in breeding programs to tailor the amount of dormancy wanted in a specific variety near harvest time.

Conclusion

Variation was detected in all three genes investigated in this study (*TaMFT_(3A, 3B, 3D)*, *TaMKK3*, and *TaVp1*), however, only variation in *TaMFT_3A, TaMFT_3B, and TaMFT_3D* was significantly associated with PHS scores (*P* value <0.001) (Table 2). Little work has been done to analyze the impacts of MFT homeologs or even to identify variation. In this study, variation in all MFT homeologs was detected and observed to be consistently associated with sprouting score values and may provide more control of dormancy or non-dormancy in breeding programs.

There was no observed association between *TaMKK3* or *Vp1B3* with PHS scores and no observed additive effects. This is contrary to many studies that see large impact from *TaMKK3* but in agreeance with our previous study: "Allelic Impacts of *TaMFT_3A*, *TaMKK3*, and *Vp1B3* on Pre-harvest Sprouting of Northern Great Plains Winter Wheats" where alleles from all three genes were compared in a diverse set of winter wheat varieties adapted to Montana.

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CHAPTER SIX

CONCLUSION

To date, little information was available about the preharvest sprouting (PHS) susceptibility of winter wheat and barley cultivars commonly grown in the Norther Great Plains (NGP) region of the US. Additionally, little was known about the allelic variation in genes known to be associated with PHS susceptibility in other regions of the world. The studies presented in this dissertation sought to shed light on these topics so that better decisions can be made by producers and so that breeders have options for addressing the negative impacts of PHS.

These studies show that there is large variation in PHS susceptibility of common varieties of winter wheats and barley's (of different market classes). This is good in that there is much potential for classical breeding to improve PHS resistance and there are agronomically adapted sources of that resistance. The studies also uncovered some of the allelic variation present in the NGP elite germplasms.

In winter wheat the genes *TaMFT*, *TaMKK3*, and *TaVp1* are among the most reported to be associated with PHS susceptibility. Previously published variation in all three genes was observed in the NGP germplasms but only mutations in *TaMFT 3A* were associated with dormancy. The large reported impacts of *TaMFT* paired with some review of public data lead to the investigation of *TaMFT* homeologs, of which there are three, two copies on chromosome 3B and one on 3D. Until now, there are no known studies that specifically investigate *MFT* homeolog variation and it was found that *MFT* 3B and 3D markers appear to be strongly associated with PHS in a biparental population

derived from the elite NGP winter wheat varieties Loma and Warhorse. We also found that variation at each loci was associated with almost equal effects on PHS susceptibility. This provides a strong tool to breed for resistance to PHS yet needs to be more heavily investigated for potential negative impacts or linkage drag issues.

In barley, the genes *HvMKK3* and *HvAlaAT1* have been the most reported as being associated with dormancy (which is generally the trait associated with PHS susceptibility). The presented studies found that these genes affect dormancy at different time points in grain maturity. This is very exciting in that it indicates that dormancy may be under control of different genes at different points in grain maturity and that breeders may be able to breed for a specific window of dormancy by fine tuning which dormancy alleles are present. It was also observed that many common varieties vary for a previously undescribed mutation in MKK3 that is strongly associated with PHS susceptibility around physiological maturity. This mutation will provide an additional tool for breeding to induce more or less dormancy around harvest time.

This research has demonstrated that PHS is a problem that can be addressed through breeding of the correct genotypes. By breeding for small grains that are more resistant to PHS economic loss to producers can be mitigated.

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