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RESEARCH

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 α-amylase concentrations. Variation in PHS susceptibility across entries ranged from 0% sprout (fully dormant) to 95% sprout (fully nondormant) after 7 d 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.

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Abbreviations: FN, falling number; PCR, polymerase chain reaction; PHS, preharvest sprouting; QTL, quantitative trait locus; SNP, single nucleotide polymorphism; STS, sequence-tagged site.

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 (*Triticum aestivum* L.) is cultivated and is a major consideration associated with grain quality (Ross and Bettge, 2009). The damage from PHS severely affects 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 up to US$1 billion yr^{-1} (Black et al., 2006, p. 528). These huge losses, paired with a requisite for global food security, highlight the necessity of developing PHS-tolerant varieties.

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., 1973). This has

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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.

Preharvest sprouting is a quantitative trait controlled by many genes, environmental conditions, and the interaction of genotype with environment, which generates high haplotype diversity and PHS 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 (FN) assay 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 PHS 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 and high-humidity periods close to harvest and large temperature fluctuations during grain filling (Jiménez 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; Martinez et al., 2016), 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 Rodríguez 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 PHS have been partially characterized, including *TaPHS1* (Nakamura et al., 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 PHS (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 single nucleotide polymorphism (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. Liu et al. (2013) thoroughly characterized two additional SNP mutations that result in a nonfunctional *TaPHS1* transcript. To further 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 that it alone is a major genetic factor affecting PHS. However, large PHS variation is still observed in populations fixed for *TaPHS1* tolerant alleles, indicating that 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, 2018).

Another locus that has been observed to have significant association with PHS, explaining up to 43% of the observed phenotypic variation, is the 4AL QTL (Torada et al., 2005; Lin et al., 2016). In recent publications, the cause of this association was shown to be a SNP in the *TaMKK3* gene (Shorinola et al., 2016; Torada et al., 2016). *TaMKK3* encodes a mitogen-activated protein kinase kinase 3. Mitogen-activated protein kinase kinase (MKK) proteins play vital roles in signal transduction pathways, and the homology of *TaMKK3* to *MKK* genes in *Arabidopsis* suggests that *TaMKK3* affects dormancy by modifying abscisic acid 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 the 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 PHS 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 one 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 eight experimental lines (Table 1). Planting sites used conventional tillage and were planted on 1 Oct. 2015 and 27 Sept. 2016 at a seeding rate of 90.7 kg ha^{-1}. Plots in Creston consisted of seven rows that were 4.6 m long with 15-cm spacing between rows. Field was fertilized using 9–40–40 N–P–K broadcast and was top-dressed with 75–0–40 N–P–K. Soil tests indicated available N–P–K–S to be 235 kg N ha^{-1}, 32 mg P kg^{-1}, 244 mg K kg^{-1}, and 34 mg S kg^{-1}. Plots in Bozeman had three rows that were 4.6 m long with 30-cm spacing between rows. Soil tests indicated available N–P–K to be 48 kg N ha^{-1}, 17 mg P kg^{-1}, and 232 mg K kg^{-1}. The field was top dressed with 232–0–0 N–P–K.

Weeds were controlled at both locations with a postemergence application of Huskie at 0.8 L ha^{-1} (pyrasulfotole, bromoxynil octanoate, and bromoxynil heptanoate; Bayer CropScience), Axial at 1.2 L ha^{-1} (pinoxaden, Syngenta Crop Protection), NIS at 1 L 378 L^{-1} (polyalkylene modified heptamethyltrisiloxane, and nonionic surfactants; Alligare), and 28% urea ammonium nitrate at 0.4 L ha^{-1} (BASF Canada).

Heading date was recorded as the time when 50% of the total primary tillers in the plot had a fully emerged spike. Physiological maturity 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 4100, Perten Instruments) using 200 seeds for each replicate. Grain protein was determined via near-infrared transmission with an Infratec 1241 grain analyzer (Foss). Head and spike collections for each year differed in that the heads collected at Creston in 2016 were collected directly at physiological maturity, whereas the heads collected from Bozeman in 2017 were collected 1 wk prior to harvest.

Preharvest Sprouting Assessment

Ten heads per plot were collected at physiological maturity (characterized by loss of green color from the peduncle) and tested for each year. The heads were dried for 24 h in a 37°C forced-air oven and then frozen at −20°C until assayed. The

Table 1. Winter wheat genotypes surveyed for their genotype at three major loci that influence preharvest sprouting.

Line or variety	Source	*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 and 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

† *TaPHS1* alleles are distinguished by two single nucleotide polymorphisms (SNPs, G646A and A666T). Resistant genotypes are wild types and contain G/A, whereas susceptible genotypes contain A/T.

‡ *TaMKK3* alleles are distinguished by a single SNP (C660A). Resistant genotypes contain a C, and susceptible genotypes containing an A.

§ *Vp1B3* alleles determined using an sequence-tagged site (STS) marker, as described by Yang et al. (2007).

¶ Denotations of R and S represent alleles recognized as resistant or susceptible, respectively, in previous literature.

awns of each head were trimmed to ~1.27 cm. The heads from all three replications were simultaneously placed into a misting chamber for 7 d and misted for 4 s every 5 min. The chamber was 1.5 m wide × 2.4 m long × 0.6 m tall with a 2.54-cm-diam. polyvinyl chloride (PVC) frame covered in 2-mil-thick clear poly plastic. Two benches (0.76 × 2.13 m) consisting of two layers of 0.63-cm 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 (0.3 gallons min^{-1}) overhead misting nozzles (Dramm Corporation). 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) wired to an Irritrol 700B-.75 Ultra Flow NPT threaded valve with flow control (1.9 cm, Irritrol). The temperature and humidity were monitored with a LASCAR EL-GFX-2 temperature and humidity data logger (Lascar Electronics). After 7 d of misting, the heads were graded using a subjective scale of 1 to 7, where 1 is no visible sprouting structures, 2 is <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 nondormant heads that have sprouted 100% with shoots >7.62 cm (Fig. 1).

Falling Number Determination

The Hagberg FN test (Hagberg 1960) was conducted following the USDA Falling Number directive protocol (USDA Directive 9180.38, 20 May 2013, https://www.gipsa.usda.gov/laws/directives/9180-38.pdf). Sound grain was ground into a whole meal flour using a Perten Laboratory Mill 3100 (Perten Instruments). The samples were mixed thoroughly, and flour moisture was determined using a FOSS Infratec 1241 grain analyzer (Foss A/S). The samples (7.0 ± 0.05 g) were then mixed with 25 mL of water and shaken using a Perten Shake-O-Matic 1095. Falling number measurements were conducted using a Perten FN1000 FN machine. The Perten FN1000 was programmed to compensate for altitude. The FN values were manually adjusted on a 14% grain moisture basis using the following equation, from p. 9 of USDA Directive 9180.38: FN(14%) = [FN(100 − 14)]/(100 − flour moisture content).

Alpha-Amylase Determination

Whole-grain flour α-amylase concentrations were determined using an adapted "micro-assay" version of the AACC International Approved Method 22-02.01 (AACC International, 2001). Grain was ground into whole meal flour using Perten Laboratory Mill 3100 (Perten Instruments), 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 microcentrifuge tube, to which 0.50 mL of 1× extraction buffer solution (pH 5.4) was added and mixed vigorously by vortexing. Reactions were incubated on an Eppendorf thermomixer for 20 min at 40°C with continuous mixing at 500 rpm followed by centrifugation at 13,000*g* for 1 min. The enzyme was assayed within 1 h of extraction as follows: 10-μL aliquots of Ceralpha reagent solution was dispensed into microtiter plates and preincubated at 40°C for 5 min with three aliquots per enzyme extract. After preincubation, 7.5 μL of wheat α-amylase extract was added directly to the bottom of each well at 30-s 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.

TaPHS1, *TaMKK3*, and *Vp1B3* Genotyping

For DNA extraction, seeds were planted in the greenhouse and the leaf tissue was collected at the two-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). Polymerase chain reactions (PCR) contained 0.42 μM of the forward and reverse primers, 0.16 mM each deoxynucleotide, 2 mM $MgCl_2$, 1× GoTaq buffer (Promega), 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

Fig. 1. Preharvest sprout scoring scale. A score of 1 is completely dormant after 7 d of wetting, 2 is <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 nondormant heads that have sprouted 100% and shoots are >5.05 cm.

Veriti 96 well thermocycler (Applied Biosystems Corporation) using the following steps: 1 cycle of 96°C for 5 min; 40 cycles of 96°C for 40 s, 65°C for 30 s, and 72°C for 60 s; and 1 cycle of 72°C for 7 min, with a final hold at 4°C. The PCR products were sequenced using amplification primers (GENEWIZ). Sequence files were analyzed with DNAStar Seqman Pro 2014 bioinformatics software (DNASTAR, 2014). *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 fifth 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 deoxynucleotide, 2 mM $MgCl_2$, 1× GoTaq buffer (Promega), 0.65 U GoTaq G2 Flexi DNA polymerase, and ∼100 ng genomic DNA in a total reaction volume of 25 μL. The reactions were run as described above but with the following thermocycler program: 1 cycle of 94°C for 5 min; 40 cycles of 94°C for 40 s, 61°C for 40 s, and 72°C for 60 s; and 1 cycle of 72°C for 8 min, with a final hold at 4°C. The *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 deoxynucleotide, 2 mM $MgCl_2$, 1× GoTaq buffer (Promega), 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 Corporation) using the following steps: 1 cycle of 96°C for 5 min; 36 cycles of 96°C for 40 s, 61°C for 1 min, and 72°C for 1 min; and 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 2 h 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 that included year, PHS resistance gene, year × 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 were considered random, whereas all other factors were considered fixed effects. The resistant vs. 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 eight 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 PHS.

TaPHS1-3A alleles were determined by direct sequencing of the end of the third exon and into the third intron, wherein laid 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, whereas 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.

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 nine lines containing the resistant 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 FN values that were ∼20 s longer ($P < 0.01$) and an increased kernel diameter of ∼0.1 mm ($P < 0.05$).

Vp1 allelic variation was determined by analysis with the "*Vp1B3*" STS marker developed by Yang et al. (2007). There were nine 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 FNs by up to 5% ($P < 0.05$), 17% higher α-amylase concentrations ($P < 0.05$), and 1% higher grain protein ($P < 0.05$) (Tables 2 and 3) than lines containing the resistant allele.

Agronomic data were 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 showed no correlation with other traits. Alpha-amylase activity was 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 × year. The ANOVA indicated that PHS scores were significantly affected by year, *TaPHS1* genotype (resistant vs. susceptible), and year × *TaPHS1* interaction (Table 4). The other resistance genes, *TaMKK3* and *Vp1B*, were not observed to have any impact on PHS scores.

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 have not been determined in Montana winter wheat germplasm. This was accomplished by growing 13 named varieties and eight experimental lines in triplicate, over 2 yr, and assessing their susceptibility to PHS. Other traits of interest included FN and α-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 α-amylase concentrations of sound grain and PHS susceptibility.

The *TaPHS1* gene contains mutations that both positively (decrease susceptibility) and negatively (increase susceptibility) affect PHS (Nakamura et al., 2011; 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 nondormancy, a moderately high degree of variance was observed within each group (Fig. 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,

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

Allele	PHS score†	Heading date	Physiological maturity	Plant height	Test weight	Yield
		Julian d		cm	kg hL^{-1}	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 nondormant.

‡ Denotations of R and S represent alleles recognized as resistant or susceptible, respectively, in previous literature.

Table 3. *TaPHS1*, *TaMKK3*, and *Vp1B3* allelic means of seed traits averaged over two field seasons.

Allele	Falling number	α-Amylase	Grain protein	Kernel hardness	Kernel weight	Kernel moisture	Kernel diameter
	s	U g^{-1}	g kg^{-1}		mg	%	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

Table 4. Mean squares and *P* values for sources of variation from ANOVA for preharvest 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 × PHS gene	3.72	0.03	1.55	0.18	0.04	0.84

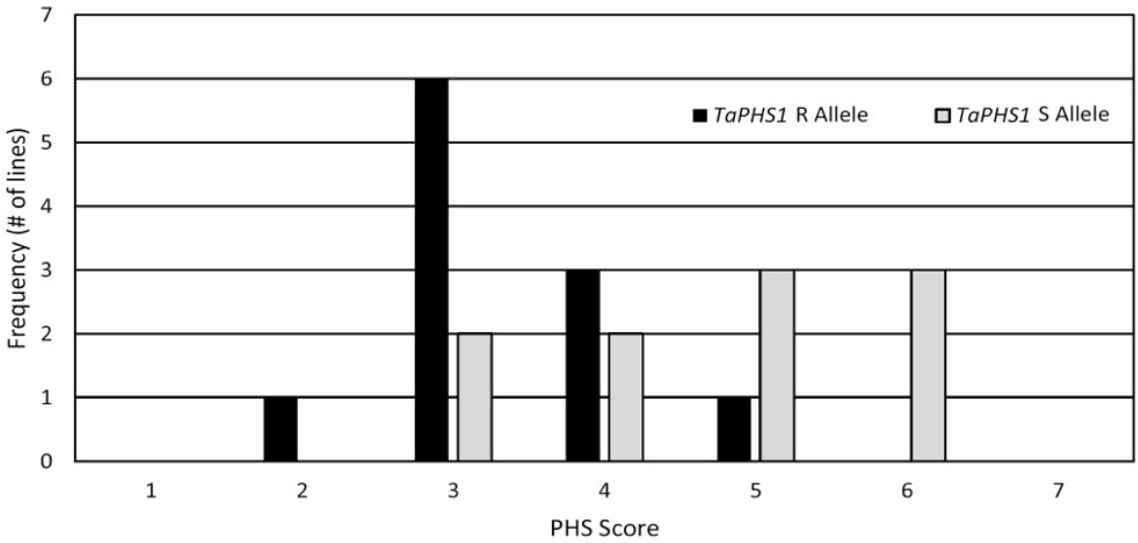

Fig. 2. Frequency distribution of *TaPHS1* allelic variation for preharvest sprout (PHS), measured on a 1-to-7 scale where 1 is completely dormant and 7 represents completely nondormant. *TaPHS1* resistant genotypes are represented with black bars, and *TaPHS1* susceptible genotypes are represented with grey bars.

but in the current study, the effects are much reduced and accounted for only ∼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. The *TaPHS1* resistant allele groups tended to be shorter and had significantly higher protein. This phenomenon is previously unreported in literature regarding *TaPHS1* and is either the result of an unknown mechanism by which *TaPHS1* directly affects protein content or is an artifact of the small sample size. Note that the mean seed size of the TaPHS1 resistant group was smaller on average, and it is likely that the increased protein content is a result of the reduced seed size.

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 (Shorinola et al., 2016; Torada et al., 2016). 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; Liu et al., 2011). This was confirmed by Shorinola et al. (2016) 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 Northern Great Plains germplasm but may be masked by the large effects produced by *TaPHS1* variation. Significant differences were observed between the allelic groups for FN and kernel diameter. For example, the group with the susceptible allele had significantly higher FN and larger kernel diameter, with trends towards lower α-amylase concentrations and higher kernel weight. The larger kernel size of the susceptible allelic group may contribute to a higher FN and lower α-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, 2011). This could lead to less total α-amylase, as α-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 α-amylase suggests either a positive correlation (Evers et al., 1995) or no correlation (Farrell and Kettlewell, 2008), whereas the current findings suggest a strong negative correlation between seed size parameters and α-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 α-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 α-amylase analysis. The reason for using sound grain was to determine if there was any relationship between varietal variation in FN or α-amylase with PHS susceptibility, which was not observed.

Like *TaMKK3*, *TaVp1* has been reported in multiple studies as having association with PHS. In the current study, however, there was no difference in PHS score between the *TaVp1* allelic groups (Yang et al., 2007; 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 FN, α-amylase, and grain protein. These differences could be caused from the small dataset, as they were unreported in previous literature.

Data analysis showed strong effects of year on PHS score, as well as significant effects of *TaPHS1* and year × *TaPHS1* interaction, which may present another reason that the observed effects of *TaPHS1* are not 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 and dry year in comparison with 2016, and heads from 2017 were collected much closer to harvest. The reason we see a reduced effect of *TaPHS1* in 2016 ($P > 0.001$) compared with 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, whereas 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. Although *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 affecting PHS in the region of interest. These data suggest that *TaPHS1* allelic variation could be used in breeding programs to decrease PHS, whereas the effects of *TaMKK3* and *TaVp1* may be masked by *TaPHS1* allelic 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.

Conflict of Interest

The authors declare that there is no conflict of interest.

Table 5. Correlations between agronomic traits and seed traits.

Trait	PHS† score	Falling number	α-Amylase	Heading date	Physiological maturity	Plant height	Grain protein	Test weight	Yield	Kernel hardness	Kernel weight
Falling number	−0.120	1									
α-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***

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

† PHS, preharvest sprouting.

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