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ORIGINAL RESEARCH

# *TAMFT-3A* and *TAMFT-3B2* homeologs are associated with wheat preharvest sprouting

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**Abstract**

The phenomenon of preharvest sprouting (PHS), caused by rain after physiological maturity and prior to harvest, negatively affects wheat (*Triticum aestivum* L.) production and end use. 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*, *3B2*, *3D*), *TaMKK3-4A*, and *TaVP1-3B* genes were assessed for association with PHS in a double-haploid line (DHL) hard red winter wheat population derived from a $BC_1$ cross between the cultivars Loma and Warhorse, where Loma was the recurrent and PHS susceptible parent. The 162 $BC_1$ DHL lines were grown over two field seasons and PHS susceptibility was assessed by measuring PHS resistance in physiologically mature heads. The PHS variation was associated with the *TaMFT-A* and the *B2* homeolog with Loma carrying mutant forms of each gene. No sequence variation between Loma and Warhorse was detected in the exons of the *TaMFT-B1* and *D* homeologs. No association between PHS resistance and *TaMKK3-4A* or *TaVp1-3B* variation was observed, though Loma and Warhorse vary for *TaMKK3-4A* and *TaVp1-3B* mutations reported to be PHS associated. Previous research has shown *TaMFT-3A* as having a large impact on PHS resistance. In the current study, the *TaMFT-3A* and *TaMFT-3B2* alleles each explained 14% of observed PHS variation. Markers for both *TaMFT-3A* and *TaMFT-3B2* should be used in selecting for increased wheat dormancy and PHS resistance.

## 1 | INTRODUCTION

Wheat (*Triticum aestivum* L.) preharvest sprouting (PHS) happens when grains begin to germinate before harvest. Preharvest sprouting occurs periodically in many wheat growing regions worldwide and is responsible for large economic losses each year. 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 darker in color. Wheat provides >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

**Abbreviations:** DHL, double-haploid line; PCR, polymerase chain reaction; PHS, preharvest sprouting; QTL, quantitative trait loci; RNA-seq, RNA sequencing; SNP, single-nucleotide polymorphism.

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control PHS to allow selection of PHS-resistant cultivars (Briggle, 1980).

Environmental conditions play a primary role in the occurrence of PHS, not only does prolonged exposure to moisture between physiological maturity (when grain fill is complete and >80% of the peduncle on the plant has browned) 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; Jiménez et al., 2017). Montana-grown winter wheat cultivars 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; Nakamura, 2018).

Preharvest sprouting is a complex, quantitative trait (reviewed by Mares & Mrva, 2014; Nakamura, 2018; Vetch et al., 2019b). Preharvest sprouting quantitative trait loci (QTL) have been identified on each wheat chromosome. However, many QTL are not repeatable across different populations and environments (Nakamura, 2018). Few PHS 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 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 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; Liu et al., 2013). *TaMFT* 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; Lei 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) associated with upregulated gene expression and increased PHS resistance. 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 SNPs in the *TaMFT-3A* third intron that reduce PHS resistance. The first SNP (+646) causes missplicing of the third exon. The second SNP (+666) introduces a premature stop codon. Together, these SNPs cause production of *TaMFT-3A* proteins 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 *TaMFT-3A* (−194) and result in reduced *TaMFT-3A* expression (Jiang et al., 2018).

**Core Ideas**

- *TaMFT-A1* is known as a major gene impacting preharvest sprout susceptibility.
- *TaMFT-B2* allelic variation also impacts sprout.
- Selection for functional alleles of *TaMFT-A1* and *TaMFT-B2* would decrease preharvest sprout and increase seed dormancy.

Wheat is a hexaploid crop and ∼70% of all genes are present and expressed from each subgenome (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 previously associated with PHS. The two *TaMFT* copies on chromosome 3B are tightly linked ∼875 kb apart. According to the wheat genome build (IWGSC Refseq v1.0) accessed in the Ensembl plants database on 17 June 2020 (https://plants.ensembl.org), *TaMFT-3A* is 96.0% identical to the *TaMFT-3B1* homeolog, 90.9% similar to the *TaMFT-3B2* homeolog, and 96.6% identical to the *TaMFT-3D* homeolog (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 3 (TaMKK3)* (Torada et al., 2016; Shorinola et al., 2017). *TaMKK3* was observed to be a major regulator of mature seed dormancy seeds because of 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 & Barnard, 2018).

*Viviparous-1* (*Vp1*) (homologous to *ABI3*, *Abscisic Insensitive 3*) is another gene that has been identified as impacting PHS. The *Vp1* gene, 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, which impacts germination (Nakamura & Toyama, 2001). *TaVp1* is on the long arm of Group

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3 chromosomes 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 and test whether the *TaMFT* homeologs impact PHS, the current study examined the individual and combined effects of *TaMFT-3A*, *TaMFT-3B2*, *TaMKK3*, and *TaVp1B3* alleles in a $BC_1$ double-haploid hard red winter wheat population.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant materials and agronomic and seed characteristics

A hard red winter wheat $BC_1$ 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 from which the DHLs were generated by first crossing Loma × Warhorse followed by a single backcross to Loma. Given the nature of $BC_1$ populations, expected segregation ratios for the genes of interest would be 1:3 in the DHL population. Although this segregation ratio is not ideal, the population was chosen because it was available and under no selection pressure. 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) was planted on 10 Oct. 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 9 Oct. 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 $ha^{-1}$, 17 mg $kg^{-1}$, and 332 mg $kg^{-1}$, respectively. An additional 260 kg $ha^{-1}$ of N was applied at seeding. For 2018, there was 54 cm of precipitation, and the available N-P-K was 67 kg $ha^{-1}$, 14 mg $kg^{-1}$, and 306 mg $kg^{-1}$, respectively. An additional 235 kg $ha^{-1}$ of N was applied at seeding. An extra three replications of three plants per row (1,458 total plants) were planted in late January 2018 in plastic cones (one plant per cone) filled with Sunshine Mix #1 (SunGrow) and placed in a greenhouse. Upon setting their third leaf, the plants were vernalized at 4 ˚C with a 10-h photoperiod for 8 wk and then transplanted into the field on 27 Apr. 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 (cultivars Loma and Warhorse).

### 2.2 | Agronomic traits

Days to heading was measured as when 50% of the primary heads were completely emerged. Days to physiological maturity was estimated as the days from planting when ∼80% of the peduncle lost its green color. A single-kernel characterization system (SKCS 4100, Perten Instruments) was used to determine kernel weight from 200 seeds harvested from each row. Protein percentage was measured by near-infrared transmission using an Infratec 1241 grain analyzer (Foss). Heading date and kernel weight were not obtained from the 2017 trial.

### 2.3 | Preharvest sprouting assessment

Preharvest sprouting assessments were conducted by harvesting five primary heads from each plot at physiological maturity. Heads were collected into paper bags and placed for 24 h in a 37 ˚C forced-air oven and then frozen at −20 ˚C until assays were started in a mist chamber. The misting chamber (Vetch et al., 2019a) was constructed of 2.54-cm diam. PVC pipe to be 1.5 m wide by 4.8 m long by 0.6 m high and was covered with 2 mm clear polyethylene plastic sheeting inside a greenhouse with a 16-h photoperiod and average day and night temperatures of 22 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 s every 5 min to maintain constant saturation and high humidity. The misting system consisted of six Misty-Mist 1.25-mm (0.3 gpm) overhead misting nozzles (Dramm Corporation) 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 (Irritrol) paired with a Galcon 8056 AC-6S irrigation controller (Galcon USA LTD) controlled the flow rate and misting times. Heads were held vertically by placing them within four 0.76 m width by 2.13 m length benches that had two layers of 0.63 cm steel mesh 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 d 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 <10% sprouted, 3 is 10–24% sprouted, 4 is 25–49% sprouted, 5 is 50–74% sprouted, 6 is 75–99% sprouted, and a score of 7 represents completely nondormant heads where all seeds have germinated and shoots are >7.62 cm.

### 2.4 | Genotyping

The DNA was extracted from each DHL and the parent cultivars by planting five seeds of each in a greenhouse and collecting a composite sample containing one 2-cm leaf

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segment from each of at least three plants. The DNA was then extracted using a protocol adapted from the one described by Riede and Anderson (1996). *TaMFT-3A* and *TaMKK3* were genotyped by polymerase chain reaction (PCR) amplification and direct sequencing of the PHS-associated mutations varying between Loma and Warhorse. 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 sequence-tagged site 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). The PCR conditions and equipment for each reaction were as described by Vetch et al. (2019a).

Screening for *TaMFT-3B1* polymorphisms between Loma and Warhorse was assessed with two sets of primers that targeted the exons of the gene. A 777-bp fragment of *TaMFT-3B1* was amplified using forward primer 5′ CATATATACACGCAGGCGT 3′ and reverse primer 5′ CTTATTGGGAGAGTGCAATG 3′, which amplified the first three exons. The amplicon was sequenced using the forward primer. The PCR amplification reaction was as follows: one cycle of 96 ˚C for 5 min, 40 cycles of 96 ˚C for 30 s, 61 ˚C for 30 s and 72 ˚C for 60 s, and one cycle of 72 ˚C for 7 min with a final hold at 4 ˚C. A 718-bp fragment of *TaMFT-3B1* was amplified using forward primer 5′ CTTTGTAGGAGTATGTGGTTC 3′ and reverse primer 5′ TGGGTTCGAGTCATGAAAAT 3′, which amplified the last exon. The amplicon was sequenced using the forward primer. The PCR amplification reaction was as follows: one cycle of 96 ˚C for 5 min, 40 cycles of 96 ˚C for 30 s, 59 ˚C for 30 s and 72 ˚C for 60 s, and one cycle of 72 ˚C for 7 min with a final hold at 4 ˚C.

Polymorphism for *TaMFT-3D* alleles between Loma and Warhorse was assessed with a nested amplification using a *TaMFT-3D* genome-specific set of primers that amplified the entire gene followed by two internal sets of primers that targeted the exon regions. A 4,326-bp fragment of *TaMFT-3D* was amplified using forward primer 5′ CTTTGATTTTGGTGTGAGTTTC 3′ and reverse primer 5′ GCAGAAATATAAGGAAAGGC 3′, which amplified the entire gene. The PCR amplification reaction was as follows: one cycle of 96 ˚C for 5 min, 40 cycles of 96 ˚C for 30 s, 59 ˚C for 30 s and 72 ˚C for 5 min, and one cycle of 72 ˚C for 7 min with a final hold at 4 ˚C. Dilutions of the full gene amplification were prepared at 1:100 and used as the DNA for nested reactions. The first three exons of *TaMFT-3D* were amplified in a ∼890-bp fragment using forward primer 5′ ACGTAAGCCATATATACACCCG 3′ and reverse primer 5′ ATAAAATCGAATGGGACCC 3′. The amplicon was sequenced using both the forward and reverse primers. The PCR amplification reaction was as follows: one cycle of 96 ˚C for 5 min, 40 cycles of 96 ˚C for 30 s, 59 ˚C for 30 s and 72 ˚C for 60 s, and one cycle of 72 ˚C for 7 min with a final hold at 4 ˚C. The last exon of *TaMFT-3D* was amplified in a 697-bp fragment using forward primer 5′ GCATGAACGATGGATCATAT 3′ and reverse primer 5′ GATCATGCAAAGTGGGTA 3′. The amplicon was sequenced using both the forward and reverse primers. The PCR amplification reaction was as follows: one cycle of 96 ˚C for 5 min, 40 cycles of 96 ˚C for 30 s, 61 ˚C for 30 s and 72 ˚C for 60 s, and one cycle of 72 ˚C for 7 min with a final hold at 4 ˚C.

A marker to distinguish *TaMFT-3B2* allelic variation between Loma and Warhorse was developed using data from a whole-exome capture of the lines Warhorse and 'Yellowstone'. The Yellowstone line is a parent to the Loma line. Sequence reads from the Warhorse and Yellowstone exome data were aligned to *TaMFT-3B2* sequence taken from the IWGSC Refseq v2.1, using Lasergene software SeqMan NGen. A 10- and 4-bp insertion was identified in Yellowstone in the first intron (+312 & +318), indicating a newly discovered allele *TaMFT-3B2b*. Primers were designed to discriminate based upon this insertion and used to screen for polymorphism between Loma and Warhorse for *TaMFT-3B2*. A 347-bp fragment of *TaMFT-3B2* was amplified using forward primer 5′ GGAACTTGATGGTGATTTTC 3′ and reverse primer 5′ TAGTTGCATCTGTTCCACC 3′. The forward primer ends two base pairs into the 10-bp insert found in the *TaMFT-3B2b* allele, creating a band only when this allele is present (Figure 1.) The PCR amplification reaction was as follows: one cycle of 96 ˚C for 5 min, 40 cycles of 96 ˚C for 30 s, 58 ˚C for 30 s and 72 ˚C for 60 s, and one cycle of 72 ˚C for 7 min with a final hold at 4 ˚C. This PCR reaction included *Puroindoline b* (*PinB*) primers to verify DNA integrity in plants found to be negative for the *TaMFT-3B2b* allele. Primers for *PinB* gene were first described in Swan et al. (2006) consisting of forward primer PB5 5′-ATGAAGACCTTATTCCTCCTA-3′ and reverse primer CAT3.4 5′-GGCACGAATAGAGGCTATATCA-3′. One band of 469 bp is produced by the *PinB* primers when the *TaMFT-3B2b* allele is not present, as observed in Warhorse. An additional band at ∼347 bp is produced when the *TaMFT-3B2b* allele is present, as observed in Loma (Figure 2.) The amplicon was sequenced using both the forward and reverse primers.

## 2.5 | RNA sequencing

Whole heads were harvested from each parent at 25 d after flowering in summer 2018. The heads were cut and immediately placed in liquid nitrogen and stored at −80 ˚C. The heads were threshed by hand in liquid nitrogen. Seeds were

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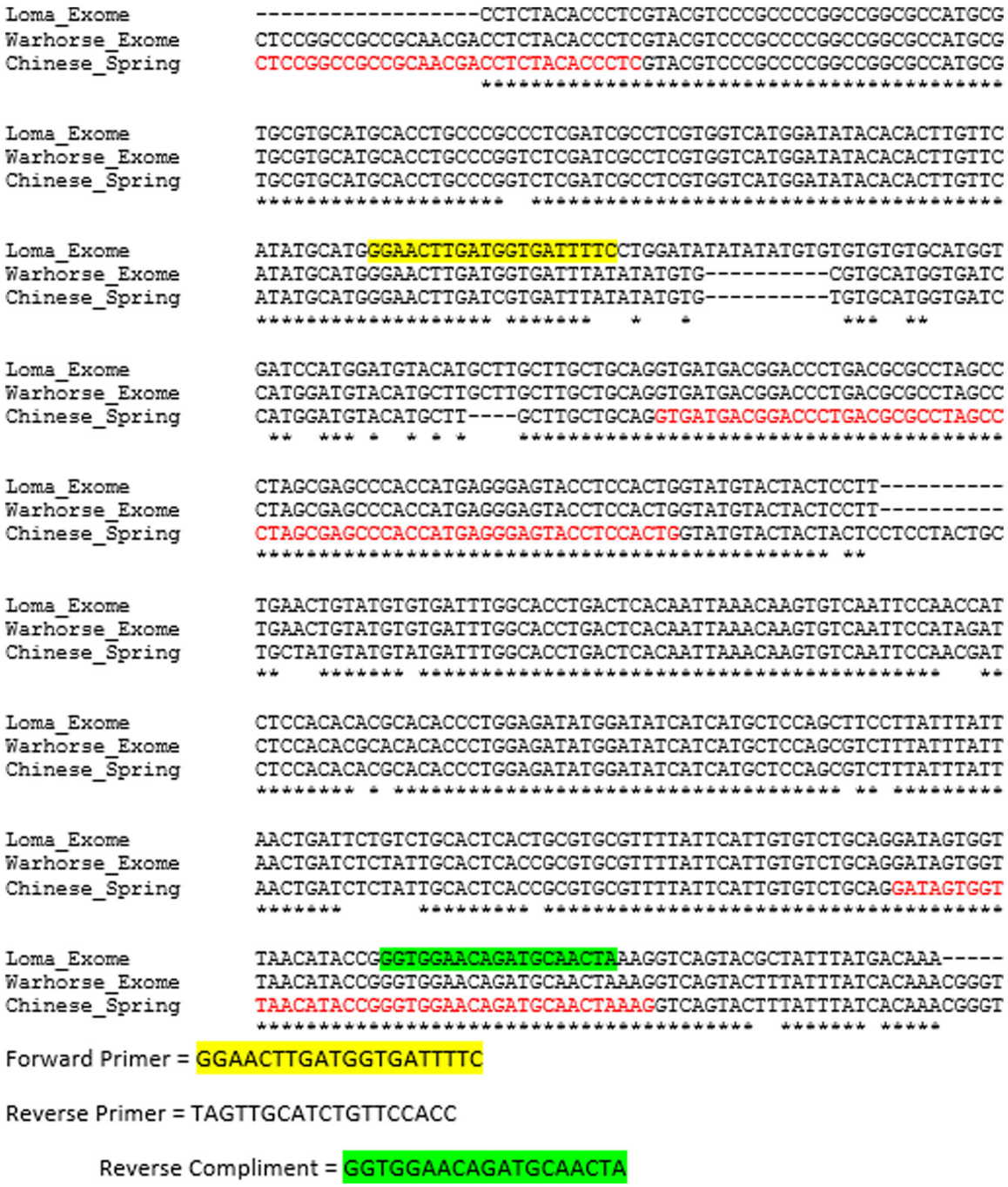


**FIGURE 1** Alignment between 'Loma', 'Warhorse', and 'Chinese Spring' of *MFT-3B2* across the first three exons (depicted in red). Forward primer will only anneal in presence of *MFT-3B2b* allele found in Loma

removed from the liquid nitrogen three to five at a time and allowed to thaw just enough to isolate the embryos. The embryos were immediately placed back on liquid nitrogen. Frozen embryo tissue was homogenized by adding two 3-mm glass beads (Thermo Fisher Scientific) to the frozen embryos in a 2-ml microcentrifuge tube and processing them in a Mini Beadbeater-96 (Biospec Products) for 1 min. RNA was extracted using a Qiagen RNeasy Plant Mini Kit. Purified RNA was sent to GENEWIZ (Cambridge, MA) for sequencing. Approximately 30–50 million paired-end 150-bp sequence reads were obtained for each sample.

## 2.6 | Gene expression analysis

Targeted gene expression analysis was conducted on three biological replications using ArrayStar v15 and v17 (DNAstar). Target gene sequences were manually uploaded in

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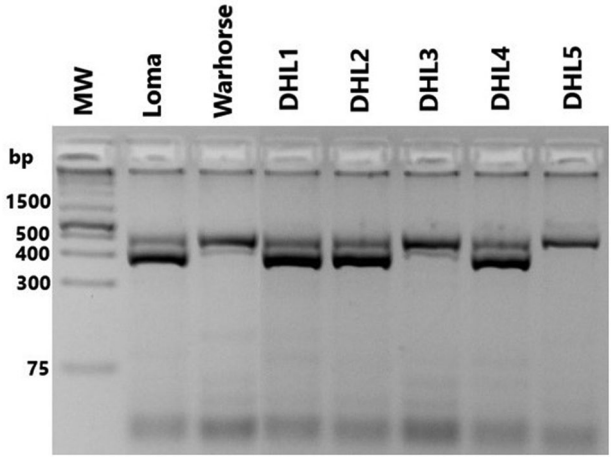


**FIGURE 2** The *MFT-3B2b* positive–negative dominant marker amplifies a 347 bp product only from genotypes containing the *MFT-3B2b* allele. *Puroindoline b* primers were included as a positive control and produce a 469-bp product in each wheat genotype. DHL, double haploid line

FASTA format (see Table 3 for Refseq v1.0 gene identification numbers). The following Qseq advanced options were adjusted from default settings: (a) read match was set to 100%, (b) minimize mers (may degrade results) was unselected, and (c) all other settings were default. Statistical comparison between the average expression levels for each genotype were generated within ArrayStar using a student's *t* test. 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 *Triticum aestivum Cyclophilin A* (CYP18-2, AY456122) expression.

## 2.7 | 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 obtained from each trial. Overall entry means were obtained by averaging the entry means from the two 2018 trials and the 2017 trial. Allelic class means for *TaMFT* (*3A*, *3B2*), *TaMKK3*, and *TaVp1* were computed with a *t* statistic with unequal variances using the *t* test function in R v4.1 (R Core Team, 2018) using entry means from each trial and the overall entry means. The proportion of variation in PHS score accounted for by each *TaMFT* homolog was obtained as the proportion of sums of squares accounted by the model from fitting each *MFT* homolog as the independent variable using the lm function in R v4.1. Interaction of *TaMFT* homolog with trial were examined with a model which included trial, *TaMFT* homolog, entries within *TaMFT* homolog, and trial × *TaMFT* homolog using the entry means from each trial where entries within *TaMFT* homolog as random and other factors as fixed effects using the lmer function in the lme4 package in R v4.1 (Bates et al., 2015).

# 3 | RESULTS

A total of 162 hard red winter wheat DHLs derived from Loma//Warhorse $BC_1F_1$ and Loma and Warhorse were planted in a single replication in one year and in two triple-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. Heading date, physiological maturity, seed protein, and 1,000-seed weight are included in data set to evaluate whether selection for alleles of the genes in this study also impact these important agronomic traits. All lines were also assessed for PHS susceptibility. Entries were then genotyped for *TaMFT-3A*, *TaMFT-3B1*, *TaMDT-3D*, and *TaMKK3* by direct sequencing of portions of the genes. *TaMFT-3B2* and *TaVp1-3B* genes were genotyped using the markers *TaMFT-3B2b* (developed in this study) and *Vp1B3* (Yang et al., 2007). Loma and Warhorse contained different alleles for *TaMFT-3A*, *TaMFT-3B2*, *TaMKK3*, and *TaVpl-3B* varied in protein content and varied greatly in their susceptibility to PHS. Warhorse had an average PHS score of 1.1 and Loma had an average PHS score of 4.5 on the 1-to-7 PHS scale, making Warhorse very PHS resistant and Loma moderately PHS susceptible (Vetch et al., 2019a). 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; Yang et al., 2007. *TaMFT-3B1* and *TaMFT-3D* did not vary between Loma and Warhorse (Table 1).

## 3.1 | TaMFT

Genotyping the DHL population for *TaMFT-3A* 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 $< .001$) for heading date and PHS score. Lines containing the *R* allele had an average PHS score of 2.9, whereas lines containing the *S* allele had an average sprouting score of 3.7 indicating that *R* allele DHLs have reduced PHS susceptibility at physiological maturity.

*TaMFT-3B2* polymorphism was also detected between the parental lines. Loma was found to have the *TaMFT-3B2b* allele, as found in the parent line Yellowstone and verified with the developed *TaMFT-3B2b* marker. Warhorse lacked this allele as verified by the *TaMFT-3B2b* marker. There were 31 DHLs carrying the Warhorse *TaMFT-3B2* allele and they

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**TABLE 1** Parental preharvest sprouting (PHS) susceptibility gene genotypes and agronomic trait means (± standard error)

| Parent cultivar | *TaMFT* allele | | *TaMKK3* allele | *Vp1B3* allele | Heading date | Physiological maturity | Pre-harvest sprout score (1–7) | Seed protein | 1,000-seed weight |
|---|---|---|---|---|---|---|---|---|---|
| | *3A* | *3B2* | *4A* | *3B* | Julian day | | | % | g |
| Loma | *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* | *S* | *R* | 169.9 ± 0.2 | 214.9 ± 0.5 | 1.1 ± 0.1 | 12.8 ± 0.2 | 35.2 ± 0.5 |

*Note*. *S*, susceptible; *R*, resistant.

[a]Sprout scoring scale (1–7): A score of 1 is completely dormant after 7 d of wetting, 2 is <10% sprouted, 3 is 10–24% sprouted, 4 is 25–49% sprouted, 5 is 50–74% sprouted, 6 is 75–99% sprouted, and a score of 7 represents completely nondormant heads that have sprouted 100% and shoots are >5.05 cm.

**Significant at the .01 probability level.

***Significant at the .001 probability level.

had a mean sprouting score of 2.8, whereas the 126 lines carrying the Loma *TaMFT-3B2b* allele had a mean sprout score of 3.6. The Loma allele contained multiple insertions in the first intron relative to Warhorse and Chinese Spring *TaMFT-3B2* sequences and *TaMFT-3B2b* carrying $BC_1$ lines had a significantly higher mean sprout compared with lines carrying the Warhorse allele. Because of this, the Loma *TaMFT-3B2b* allele was designated the susceptible allele and the Warhorse *TaMFT-3B2* allele the resistant allele. Genotype was not acquired for five lines resulting in an *n* of 157. Only three DHL out of 162 contained both the susceptible allele for *TaMFT-3A* and *TaMFT-3B2*, preventing a proper statistical analysis of the additive effect of these homologues together.

Both *TaMFT-3A* and *TaMFT-3B2* loci interacted with environment (Supplemental Table S3). The difference between the *R* and *S* alleles was less for the 2017 trial than both 2018 trials. The impact of allelic difference for the *TaMFT-3A* and *TaMFT-3B2* loci was essentially equal on PHS score. No polymorphism was detected between Loma, Warhorse, and the Chinese Spring reference genome for the exons of *TaMFT-3B1* and *TaMFT-3D*.

## 3.2 | TaMKK3

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

## 3.3 | Vp1B3

It was observed through *Vp1* genotyping with the *Vp1B3* marker that 24 lines carried the resistant allele (845- or 569-bp repeat) and 136 lines carried the susceptible allele (652-bp repeat) as described by Yang et al. (2007). The *Vp1* alleles were associated with heading date ($P < .01$). Lines that carried the resistant allele had a shorter heading time with that difference being detected ($P < .001$) in one of the two 2018 trials.

## 3.4 | Expression

RNA sequencing (RNA-seq) was conducted on the parental line embryos at 25 d after flowering. Three dormancy genes—*MFT*, *MKK3*, and *Vp1*—and all associated homeologs were targeted for RNA-seq analysis (Table 3). *TaMFT_3A* (TraesCS3A02G006600) was down regulated 6.5-fold in the susceptible genotype Loma vs. the resistant genotype Warhorse, *TaMFT_3B1* (TraesCS3B02G010100) was upregulated 3.8-fold ($P < .05$), *TaMFT_3B2* (TraesCS3B02G007400) was downregulated 50.5-fold ($P < .01$), and *TaMFT_3D* was downregulated 8.8-fold ($P < .001$). *Vp1_3D* (TraesCS3D02G412800.1) was upregulated 5.67-fold in Loma vs. Warhorse, however this may be an artifact of experimental error. Of the three biological reps preformed in the RNA-seq experiment, two showed significantly closer transcripts between Loma and Warhorse; however, one of the reps did not find transcripts for *Vp1_3D* in Warhorse, exaggerating the difference in expression between parental lines. All other genes observed displayed consistent results across biological reps. No significant differences were observed in transcript levels of any other dormancy genes in this study.

# 4 | DISCUSSION

The aim of this study was to investigate the effects of three genes on PHS 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; Vetch et al., 2019a; Yang et al., 2007). These three genes all act to promote seed dormancy and have a complex regulation that includes multiple genes.

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**TABLE 2** Allelic class means for preharvest sprout (PHS) score and agronomic and seed traits across all trials (± standard error)

| Allele | No. lines | $\chi^2$ | Heading date | Physiological maturity | Pre-harvest sprout score (1–7) | Seed protein | 1,000-seed weight |
|---|---|---|---|---|---|---|---|
| | | | Julian day | | | % | g |
| *TaMFT-3A R* | 53 | 3.86 | 169.9 ± 0.06*** | 213.9 ± 0.1 | 2.9 ± 0.1*** | 13.1 ± 0.1 | 32.6 ± 0.3 |
| *TaMFT-3A S* | 109 | 1.29 | 169.6 ± 0.04 | 214.0 ± 0.1 | 3.7 ± 0.1 | 13.0 ± 0.1 | 32.3 ± 0.2 |
| *TaMFT-3B2 R* | 31 | 1.73 | 169.7 ± 0.10 | 214.2 ± 0.14 | 2.8 ± 0.2*** | 13.0±0.16 | 31.7±0.4* |
| *TaMFT-3B2 S* | 126 | 0.65 | 169.8 ± 0.04 | 213.9 ± 0.08 | 3.6 ± 0.1 | 13.0±0.05 | 32.6±0.2 |
| *TaMKK3* R | 99 | 3.68 | 169.7 ± 0.0* | 214.1 ± 0.1 | 3.5 ± 0.1 | 13.07 ± 0.1 | 32.3 ± 0.2 |
| *TaMKK3* S | 61 | 11.03 | 169.8 ± 0.1 | 213.9 ± 0.1 | 3.4 ± 0.1 | 13.02 ± 0.1 | 32.5 ± 0.3 |
| *Vp1B3* R | 24 | 6.4 | 169.5 ± 0.1** | 214.1 ± 0.2 | 3.6 ± 0.1 | 12.9 ± 0.1 | 31.8 ± 0.5 |
| *Vp1B3* S | 136 | 2.13 | 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 |

*Note*. Sprout scoring scale (1–7): A score of 1 is completely dormant after 7 d of wetting, 2 is <10% sprouted, 3 is 10–24% sprouted, 4 is 25–49% sprouted, 5 is 50–74% sprouted, 6 is 75–99% sprouted, and a score of 7 represents completely nondormant heads that have sprouted 100% and shoots are >5.05 cm. Only three of 162 DHL contained both the susceptible allele for *TaMFT-3A* and *TaMFT-3B2* preventing a proper statistical analysis of the additive effect of these homologues together.

*Significant at the .05 probability level.

**Significant at the .01 probability level.

***Significant at the .001 probability level.

The effects of *TaMFT_3A*, *TaMKK3_4A*, and *TaVp1_3B* on the dormancy of Northern Great Plains winter wheats has not been well documented compared with populations or cultivars 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 13.7% of the observed variation, whereas, 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 biparental recombinant inbred population was used and *TaMFT_3A* homeologs (*3B1*, *3B2*, and *3D*) were also assessed to determine their association with susceptibility to PHS.

The population investigated consisted of 162 DHLs generated from a PHS susceptible cultivar (Loma) and a resistant cultivar (Warhorse), both of which have been recently released and are adapted to Montana, USA. The population was nonconventional in that Loma was crossed to Warhorse and the resulting $F_1$ was backcrossed to Loma before generation of the double haploids. The 162 double haploids differed from expected segregation ratios for *TaMFT-3A*, *TaMFT-3B2*, *TaMKK3*, and *TaVP1* indicating mutations may confer a fitness cost, specifically during the generation of double haploids, or there may be a preferential crossover from certain chromosomes; see Table 2 for Chi-square results.

The *TaMFT-3A* missplicing 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 ($P < .001$), explaining ~14% of the observed phenotypic variation. This effect was expected because of 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 *TaMFT_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 *TaMFT_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 d postanthesis. RNA was extracted, and a targeted RNA-seq analysis were conducted. *TaMFT*_3A and all homeologs were detected to be significantly up- or down-regulated in the susceptible parent (Loma) vs. the resistant parent (Warhorse). Both *TaMFT-3A* and *TaMFT-3B2* showed significant downregulation in Loma, the susceptible parent, vs. Warhorse, the resistant parent. Segregation of the resistant allele found in Warhorse for *TaMFT-3A* and *TaMFT-3B2* was associated with increased dormancy in both cases, indicating that *TaMFT* acts as a positive regulator of the dormancy trait. The effect of the 8.8-fold downregulation of Ta*MFT-3D* was not determined in this study, as no polymorphism was detected in the coding region of the gene between Warhorse

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**TABLE 3** RNA sequencing analysis of embryos from 'Loma' and 'Warhorse' cultivars at 25 d after flowering

| Name | Ensembl Transcript ID (Refseq 1.0)[a] | Linear total reads per kilobase of transcript, per million mapped reads | | Fold change |
|---|---|---|---|---|
| | | 'Warhorse' | 'Loma' | |
| *MFT-3A* | TraesCS3A02G006600.1 | 93,167 ± 10,221 | 14,304 ± 838 | 6.513 down |
| *MFT-3B1* | TraesCS3B02G010100.1 | 43,514 ± 2,231 | 165,530 ± 2,160 | 3.804 up |
| *MFT-3B2* | TraesCS3B02G007400.1 | 142,362 ± 6,264 | 2,822 ± 264 | 50.450 down |
| *MFT-3D* | TraesCS3D02G004100.1 | 170,782 ± 9,894 | 19,402 ± 347 | 8.803 down |
| *MKK3-4A* | BAU88551.1 (NCBI ID) | 1,823 ± 178 | 1,698 ± 569 | 1.073 down |
| *MKK3-5B* | TraesCS5B02G565100.6 | 2,117 ± 280 | 1,514 ± 432 | 1.398 down |
| *MKK3-5D* | TraesCS5D02G549600.1 | 1,891 ± 283 | 1,963 ± 566 | 1.038 up |
| *Vp1-3A* | TraesCS3A02G417300.1 | 1,021 ± 59 | 1,218 ± 102 | 1.193 up |
| *Vp1-3B* | TraesCS3B02G452200.1 | 2,252 ± 142 | 2,438 ± 206 | 1.083 up |
| *Vp1-3D* | TraesCS3D02G412800.1 | 425 ± 259 | 2,410 ± 145 | 5.670 up |
| Actin | TraesCS1A02G274400 | 43,170 ± 2,639 | 42,921 ± 6,303 | 1.005 down |
| *CYP18-2* | AY456122 (NCBI ID) | 453,413 ± 0 | 453,413 ± 0 | none |

[a]Genome assembly IWGSC GCA_900519105.1 accessed on 15 Oct. 2019.

and Loma. This suggests the potential of an allelic difference in the promoter region for this gene between Warhorse and Loma. Given the effect demonstrated on PHS by *TaMFT-3A* and *TaMFT-3B2*, it is postulated that a similar effect may be observed for *TaMFT-3D* so subsequent studies will explore the promoter region of *TaMFT-3D* for polymorphism that can be exploited to make this determination. Similarly, there was no polymorphism in the coding region of *TaMFT-3B1* detected between Warhorse and Loma that could account for the 3.8-fold expression increase, therefore, exploring the promoter region of *TaMFT-3B1* for polymorphism is also warranted for future study. At present, wheat *TaMFT*_3A homeologs and their independent impacts on dormancy have not been well characterized.

Other dormancy genes and gene homeologs investigated in the RNA-seq analysis included *MKK3* and *Vp1*. A 5.67-fold upregulation was detected between Warhorse and Loma for *Vp1_3D*; however, the impact of this difference in expression on PHS has yet to be determined. It may be the case that this differential expression is an artifact of experimental error as the fold change was not consistent across biological reps for only this homolog of this gene. All other expression data observed in this study appeared balanced across biological reps, which brings questions to the validity of the reported expression difference for *Vp1_3D*. Expression of the other *Vp1* homologs and all *MKK3* homologs was not significantly different between the two parents at 25 d after anthesis. This data cannot be used to rule out the potential impacts of the other genes but does support the observations that *TaMFT* is associated with differences in dormancy, whereas *MKK3* and *Vp1* were not and may indicate that *TaMFT_3A* homeologs are playing an important, undocumented role in seed dormancy.

To determine if there was any association between the *TaMFT* homeologs and PHS score a marker was developed (*MFT-3B2b*) to track allelic variation in the DHL population. As noted earlier, there were multiple polymorphisms between the parents in the *TaMFT-3B2* sequence we obtained, with the Loma allele having a total of 14 bp inserted into the first intron. Additionally, the *TaMFT-3B2* transcripts were found to be downregulated 50-fold in Loma vs. Warhorse. This could indicate that the Loma copy of *TaMFT-3B2* is nonfunctional or only partially functional. The *TaMFT-3B2* homeolog has not previously been shown to affect PHS or seed dormancy and, as can be seen in this study, appears to be associated with a nearly one-point change in the sprout scoring scale. Allelic variation at *TaMFT3B2* explained 13.6% of the variation in PHS score. The difference in PHS determined by *TaMFT-3A* and *TaMFT-3B2* was not found to be associated with changes in the important agronomic traits of seed protein or philological maturity. The 1,000-seed weight was shown to be similarly unaffected with the exception of one field trial from 2018 that found slightly lower 1,000-seed weight associated with the *TaMFT-3B2* resistant allele. This new-found association between *TaMFT* homeologs and PHS tolerance can potentially be used in breeding programs to tailor the amount of dormancy wanted in a specific cultivar near harvest time without incurring negative effects to important agronomic traits.

## 5 | CONCLUSION

Variation was detected in all three genes investigated in this study (*TaMFT* [*3A*, *3B2*], *TaMKK3*, and *TaVp1*); however,

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only variation in *TaMFT-3A* and *TaMFT-3B2* was significantly associated with PHS scores ($P < .001$) (Table 2). Little work has been done to analyze the impacts of *TaMFT* homeologs or even to identify variation. In this study, variation in one *TaMFT* homeolog, *TaMFT-3B2*, was detected and observed to be consistently associated with sprouting score values and may provide more control of dormancy or nondormancy 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 (Vetch et al., 2019a), where alleles from all three genes were compared in a diverse set of winter wheat cultivars adapted to Montana.

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## AUTHOR CONTRIBUTIONS

Justin Michael Vetch: Formal analysis; Investigation; Methodology; Writing-original draft; Writing-review & editing. Brandon Tillett: Data curation; Formal analysis; Investigation; Methodology; Validation; Writing-original draft; Writing-review & editing. Philip Bruckner: Conceptualization; Supervision. Jack Martin: Conceptualization; Investigation; Methodology; Supervision; Writing-original draft; Writing-review & editing. Karol Marlowe: Methodology; Writing-review & editing. Marcus Alan Hooker: Methodology; Writing-review & editing. Deven Robert See: Methodology. Michael J Giroux: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing-original draft; Writing-review & editing.

## CONFLICT OF INTEREST

The authors declare they have no competing financial or nonfinancial interests.

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## SUPPORTING INFORMATION

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