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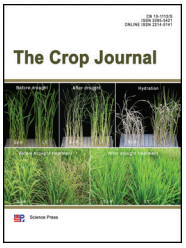

Allelic impacts on pre-harvest sprouting resistance and favorable haplotypes in *TaPHS1* of Chinese wheat accessions☆

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ABSTRACT

Pre-harvest sprouting (PHS) influences yield and end-use quality of bread wheat. Developing varieties with PHS resistance is the most effective way to reduce this problem. In this study, a panel of 725 Chinese wheat accessions were evaluated for PHS resistance in three environments. There was abundant variation in PHS resistance and 63 accessions showing high resistance had germination rates of less than 10% across three experiments. The distribution of three causal single nucleotide polymorphisms in *TaPHS1* at bases –222, +646, and +666 were assessed and frequencies were determined. Favorable alleles conferring PHS resistance were identified for each locus. Haplotype analysis showed that bases C, G, and A at each of the three loci comprised the best haplotype for PHS resistance, whereas TAT showed the highest sprouting rate. Accessions with the superior *TaPHS1* haplotypes proved to be resistant to PHS providing a basis to develop varieties with PHS resistance through marker assisted breeding.

1. Introduction

Wheat (*Triticum aestivum* L.) is one of the leading food crops, feeding about 40% of the world population and providing >20% of calories and proteins for humans [1]. China, the largest wheat-producing country, accounts for about 17% of global wheat production [2]. Maintaining high and stable wheat production is an important aspect of global food security. However, various types of biotic and abiotic factors, including pre-harvest sprouting (PHS), are ever-present and place significant constraints on wheat production.

PHS refers to the germination of physiologically mature kernels on spikes before or during the harvest period due to prolonged rainfall or periods of high humidity [3]. PHS occurs in almost all wheat growing regions worldwide such as North America, Asia, Europe and Oceania and can cause losses in both yield and end-use quality with annual economic losses of up to one billion U.S. dollars [4]. In China, PHS occurs in >80% of the production area, but especially the main production regions of the Middle and Lower Reaches of the Yangtze River, Northeast spring wheat region, and Southwest wheat region [5]. PHS is also common in the Yellow and Huai

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River Valleys wheat region and Northern wheat region of China. For instance, there were widespread occurrences of PHS in Shanxi province in 1983, 1987, and 1992, that caused losses of 100–150 million kilograms each time [5]. In 2013, 2015, and 2016, PHS was widespread throughout the Yellow and Huai River Valleys [6]. Developing and utilization of PHS resistant varieties are the only way to solve this problem.

PHS resistance is controlled by genetic and physiological factors such as seed dormancy (SD), low α-amylase activity in the grain, water-soluble germination inhibitors in the seed coat, especially those associated with red grain color, and the morphological structure of the spikes, and is also influenced by environmental factors such as humidity and temperature [7]. Wheat germplasm contains considerable variation in PHS characteristics that can be exploited to develop varieties with improved PHS resistance. Zhou et al. [8] evaluated the PHS responses of 717 Chinese wheat landraces, and identified 194 lines with germination rates lower than 20%. Jiang et al. [9] screened 260 wheat varieties and 183 accessions from the micro-core collection of China and identified 52 lines with high PHS resistance.

QTL for PHS resistance are distributed across all 21 chromosomes [3,10,11,17]. Among them, the QTL on chromosome arms 3AS and 4AL showed the highest effects and therefore were subjected to intensive investigation [12–19]. A QTL on chromosome 2B also was also a large contributor to PHS resistance [20].

The genes underlying PHS resistance QTL on chromosomes 3AS and 4AL were cloned and causal mutations were identified [21]. Nakamura et al. [22] established a 'Mother of Flowering Time' gene (*TaMFT*) as a candidate for the 3AS QTL. Liu et al. [23] cloned this gene by a comparative map-based cloning approach and named it *TaPHS1*. Another major QTL on chromosome 4AL was fine mapped and single nucleotide polymorphisms (SNPs) were identified [24–26]. A candidate gene underlying the QTL was cloned and named *TaMKK3* [27]. In addition, three seed dormancy related genes, *TaDOG1*, *TaSdr*, and *TaQsd1*, were associated with PHS resistance [28–30].

A SNP mutation at –222 in the promoter up-regulated *TaPHS1* expression and resulted in increased dormancy [22]. It was further validated to affect PHS resistance [19]. Liu et al. [23] characterized two additional SNP mutations at +646 and +666 that caused a nonfunctional *TaPHS1* transcript affecting PHS resistance. Liu et al. [31] evaluated the effect of all three SNPs on PHS resistance in wheat progenitors and wheat genotypes from North America after their conversion to competitive allele specific PCR (KASP) markers. The effectiveness of the two KASP assays were further validated on 223 cultivars that had been widely grown in China [32]. However, the allele distribution, frequency and effects on PHS resistance of the three SNPs in wheats from China were not well characterized. The objectives of this study were 1) to evaluate the PHS resistance of Chinese wheat varieties and breeding lines and to identify resistance sources for breeding; 2) to investigate the allele distribution of the above three SNPs and their effects on PHS resistance, and 3) to identify the favorable *TaPHS1* haplotypes in the expectation that it would be useful in marker-assisted breeding.

2. Materials and methods

2.1. Plant materials

The 725 accessions, including released varieties and elite breeding lines were mostly from the main wheat growing areas of China (Table S1). These accessions were planted at Shandong Agricultural University Experimental Station in Tai'an during the 2015–2016, 2016–2017, and 2017–2018 wheat growing seasons using a randomized complete block design (RCBD) with two replications. The accessions were seeded in one-row 3 m long plots; the row spacing was 25 cm and plant spacing was 5 cm. Standard local cultivation practices were followed.

2.2. Evaluation of PHS resistance

PHS resistance was evaluated as the level germination in a spike. In each year, 5–6 uniform spikes of each accession from each replication were harvested at physiological maturity defined by loss of spike greenness [33]. After air-drying for four days, the spikes were stored in a – 20 °C freezer to maintain seed dormancy until used. Sprouting assays were carried out following the methods described by Liu et al. [14]. Briefly, spikes of each accession were removed from the freezer and exposed to room temperature (~25°C) for two days; before immersion in deionized water for 10 h and transfer to a moist chamber (100% humidity) at 23 °C for seven days; when the numbers of germinated and non-germinated kernels per spike were determined and expressed as the sprouting rate.

2.3. Genomic DNA isolation

Leaf tissues sampled at the three-leaf stage and placed in a 2 mL centrifuge tube were freeze dried using a freezing vacuum dryer (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) for 2 days, and then ground to fine powder for 2 min at 30 cycles per second in a Mixer Mill (Coyote Bio-science, Beijing, China) with the aid of a 4.0 mm metal bead in each tube. Genomic DNA was isolated by a modified CTAB method [34].

2.4. Genotyping of causal SNP

Allele-specific primers were designed carrying FAM and HEX labelled oligo sequences. The KASP primers for the SNPs at –222, +646, and +666 in gene *TaPHS1* were from Liu et al. [31].

Protocols for preparation of KASP reactions followed the KASP manual (https://biosearch-cdn.azureedge.net/ assetsv6/Analysis-of-KASP-genotyping-data-using-cluster-plots.pdf). Assays were carried out in 384-well formats and set up as 6 μL PCR reaction volumes consisting of 3 μL of 2× KASP master mix, 0.0825 μL of KASP primer assay mix, and 3 μL of genomic DNA at a concentration of 20 ng μL^{-1}. The PCR protocols were: hot start at 94 °C for 15 min, followed by ten touchdown cycles (94 °C for 20 s; touchdown at 65 °C initially and decreasing by –0.8 °C per cycle for 25 s), followed by 30 additional cycles of denaturation and annealing/extension (94 °C for 10 s; 57 °C for

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60 s). An ABI Quant-Studio 12K Flex Real-time PCR System (Life Technology, Grand Island, NY, USA) was used to assay and visualize the PCR products.

2.5. Statistical analysis

Phenotypic analysis was conducted using a linear mixed model. For single-site analysis, accession (genotype) was regarded as fixed effect and replicate as random effect to obtain the best linear unbiased estimates (BLUEs) of accessions in each year. The ANOVA of phenotypic data and heritability were analyzed by the "aov" module implemented in QTL IciMapping V4.1 software [35]. Phenotypic differences between the alleles of each SNP and haplotypes were tested by ANOVA using SPSS software (version 20, http://www.spss.com). Linkage disequilibria (LD) were calculated by TASSEL 5.0 (https://tassel.bitbucket.io).

3. Results

3.1. Variation in PHS resistance in the panel

Sprouting rates continuously varied from 0 to 100% in the three experiments (Fig. 1, Table 1), indicating quantitative variation in the PHS resistance. The mean sprouting rates were 58.23%, 57.61%, and 41.14% in the 2015–2016, 2016–2017, and 2017–2018 experiments, respectively. Highly resistant accessions (sprouting rates <10%) and highly susceptible accessions (sprouting rates ≥90%) polarizations were observed in the panel (Fig. 1, Table 1). Sixty three accessions exhibited stable PHS resistance across the three years with sprouting rates lower than 10% (Table S2).

Variance analysis showed that of genotypes, environments, genotype × environment interactions, and replicates were highly significant (Table S3). The estimated broad sense heritability was 0.66, indicating that genotypes caused much of the variation in PHS resistance.

3.2. Distribution of variations and frequency

SNPs at the three positions in *TaPHS1* were characterized for each accession, and different frequencies of the bases variants were identified (Fig. 2, Table 2, Table S1). At –222, the resistance base C was present at a lower frequency than the susceptibility base T (Table 2). At +646 and + 666 the resistance the bases associated with resistance (G at +646 and A at +666) occurred at higher frequencies than the contrasting bases associated with susceptibility (A at +646 and T at +666) (Table 2).

Significant differences in sprouting rates were identified between bases at each position (Table 2). At –222, the average sprouting rates of accessions with C allele was about 30% lower than those with T allele in the three experiments. At +646 and + 666, accessions carrying the G and A bases exhibited 10.9%–12.4%, 11.8%–20.1% lower sprouting rates, respectively, than those carrying A and T in the three experiments indicating that C at –222, G at +646 and A at +666 were associated with lower sprouting rates in Chinese wheat and thus would be preferred for PHS resistance.

3.3. Association of haplotypes and PHS resistance

Five *TaPHS1* haplotypes (TGA, TAT, CGA, TGT, and TAA) were identified according to the genotypes at –222, +646, and + 666 positions among the 725 accessions (Table 3) with frequencies ranging from 1.24% to 73.84%. Among the haplotypes, TGA and TAT accounted for most of the accessions (94.12%).

Associations between haplotypes and sprouting rates were analyzed to identify the best haplotype. Since LD between +646 and + 666 SNPs was very high (R^2 = 0.81), the 5′ donor splicing site variation in intron 3 (+646) was the key variation causing mis-splicing in *TaPHS1* [23]. The TGT and TAA haplotypes were regarded as derived haplotype of TGA and TAT, respectively. Thus, only three groups of haplotypes CGA, TGA + TGT, TAT + TAA were used comparing haplotypes and PHS resistance (Fig. 3, Table S4).

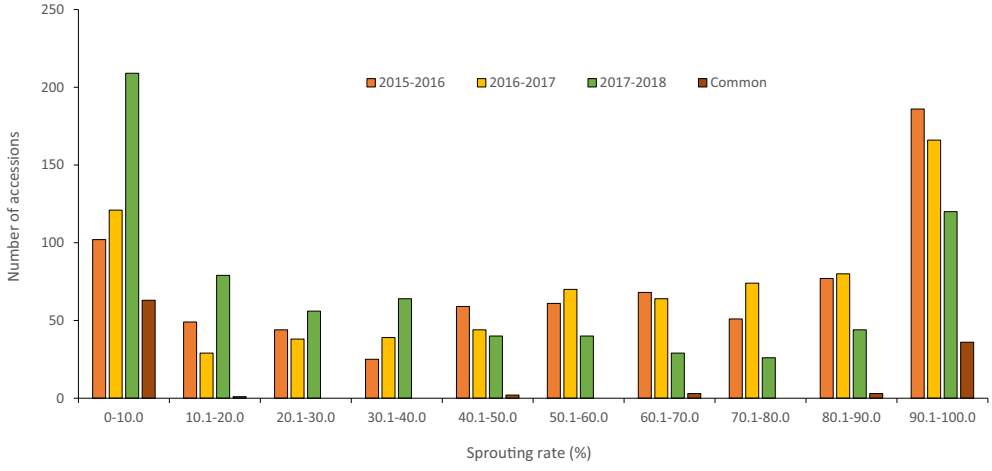

Fig. 1 – Frequency distribution of sprouting rate evaluated in the 2015–2016, 2016–2017, and 2017–2018 experiments. "Common" means the sprouting rates of accessions were in same category across three experiments.

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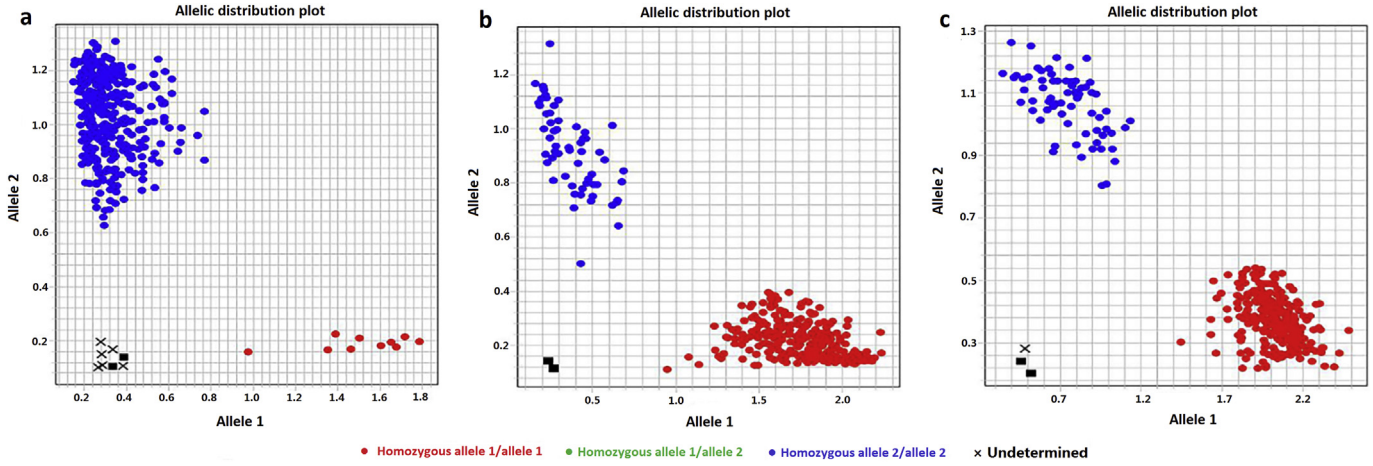

Fig. 2 – Illustration of KASP assays for SNP – 222 (a), +646 (b), and + 666 (c) analyzed in 725 wheat accessions. The blue and red dots represent the HEX and FAM labelled alleles, respectively. The black squares represent negative controls (ddH_2O) and the "×" means missing data.

The sprouting rates among the three groups of haplotypes were significantly different in all three years (Table S4). The CGA haplotype showed the best PHS resistance with average sprouting rates of 26.67%, 24.22%, and 8.33% in the three experiments. The TGA + TGT haplotypes showed moderate PHS resistance with average sprouting rates of 57.34%, 56.70%, and 36.91%, and TAA + TAT haplotypes showed the highest average sprouting rates of 69.29%, 66.96%, and 58.83%, respectively. Clearly, the CGA haplotype was associated with the best PHS resistance.

4. Discussion

4.1. *Variation of PHS resistance in wheat*

Modern common wheat cultivars have different degrees of PHS resistance. Seeds of most cultivars are dormant for only a few days post-harvest, whereas seeds of some cultivars can be dormant for months [20,36–38]. Liu et al. [31] concluded that most cultivated accessions were more susceptible to PHS than their wild progenitors. Zhou et al. [8] identified a large number of Chinese landraces displaying high levels of PHS resistance. Fakthongphan et al. [18] observed a wide range of PHS tolerance in wheat. In the present study, a panel of 725 wheat accessions were evaluated for PHS resistance, and as in other studies there was significant variation. Some accessions with high PHS resistance were identified, and these accessions can be used in breeding to improve PHS resistance.

The heritability of PHS resistance was 0.66, which was very close with other study [6], indicating genetic variation is the major component for PHS resistance, thus, selection of this trait in breeding would be reliable and effective. Environment and genotype × environment interaction also affected PHS resistance. The average sprouting rate in the 2017–2018 experiment was lower than that in the other two years. The average temperature during the grain filling stage in that year was also lower than normal, which is consistent with other study that lower temperature during grain filling stage usually caused longer seed dormancy and thus better PHS resistance [22].

Table 1 – Sprouting rates (%) of accessions in the 2015–2016, 2016–2017, and 2017–2018 experiments.

Experiments	Number of accessions	Mean ± SD	Sprouting rate range
2015–2016	722	58.23 ± 34.23	0–100
2016–2017	725	57.61 ± 33.98	0–100
2017–2018	707	41.14 ± 35.64	0–100

SD, standard deviation.

4.2. *Elite accessions for PHS resistance*

Genes for PHS resistance are widely present in wheat germplasm. Zhou et al. [8] identified 194 Chinese landraces displaying high PHS resistance. Some varieties or breeding lines with PHS resistance were also identified by Zhu et al. [6]. In this study, 63 resistant accessions with sprouting rates <10% over three years were identified, indicating that those accessions have stable PHS resistance and are therefore valuable sources for PHS resistance. Most of the accessions were released varieties, such as Kenong 213 and Annong 1124 that were released in Hebei province (Yellow and Huai River Valleys) and Anhui province (Middle and Lower Reaches of the Yangtze River). These varieties could be used as parents in crosses to develop PHS resistant varieties adapted to those environments.

Among the 63 accessions, six had haplotype CGA, 44 had haplotype TGA or TGT, thus, the *TaPHS1* was supposed to play an important role in PHS resistance in most of these accessions. However, some of the resistant accessions had the susceptible haplotypes TAT or TAA, suggesting the presence of other resistance genes or other unknown functional allelic variants of *TaPHS1*. Further investigation of these

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Table 2 – Allelic distribution of SNPs at – 222, +646, and +666 in the wheat accessions with mean sprouting rate (%).

SNP	Type	Number	Frequency (%)	Mean ± SD (%)		
				2015–2016	2016–2017	2017–2018
–222	C	21	2.98	28.64 ± 29.37 a	27.25 ± 31.17 a	12.58 ± 20.21 a
	T	683	97.02	59.33 ± 33.84 b	58.67 ± 33.49 b	41.96 ± 35.66 b
+646	A	151	21.33	68.28 ± 31.81 a	66.18 ± 31.58 a	57.79 ± 35.76 a
	G	557	78.67	55.85 ± 34.39 b	55.33 ± 34.18 b	36.60 ± 34.28 b
+666	T	152	22.16	67.95 ± 31.69 a	66.67 ± 30.84 a	56.29 ± 35.09 a
	A	534	77.84	55.60 ± 34.60 b	54.84 ± 34.58 b	36.18 ± 34.50 b

SD, standard deviation; a, b within columns, different letters indicate significant difference at $P < 0.05$.

accessions might reveal new allelic variations or mechanism of PHS resistance.

4.3. *Association of TaPHS1 haplotypes with PHS resistance*

The allele frequencies of –222, +646 and +666 in *TaPHS1* was considerably different (Table 2). At –222, the C allele frequency was low (2.98%) compared with the accessions from North America (24.3%) [31], indicating it is a minor variant in Chinese accessions. Considering that the C morph confers PHS resistance, selection of C could lead to improved PHS resistance. For +646 and +666 SNPs, G (+646) and A (+666) are far more frequent than A and T, respectively, as in North America accessions [31].

Several studies have tested the effectiveness of these variations for PHS resistance and concluded that they are highly predictive of PHS resistance in the North American, African and Asian germplasms [39,40]. In this study, the bases at each position were also tested for PHS resistance and the accessions with C at –222, G at +646 and A at +666 showed significantly lower sprouting rates than those with T, A, and T respectively.

4.4. *The best haplotype for PHS resistance in TaPHS1*

Five haplotypes were identified in the panel, and were significantly associated with PHS resistance (Fig. 3; Table S4). Among them, the CGA haplotype showed the highest PHS resistance. C was always present with G and A at +646 and +666, respectively, no other haplotype containing C at –222 was identified. When breeders apply this haplotype in marker-assisted selection, screening of the –222 KASP marker alone should be enough to obtain the entire resistant haplotype. The frequency of lines with base C at –222 and CGA haplotype was very low, thus, improve the C allele frequency and thus the CGA haplotype frequency, will improve PHS resistance effectively.

Table 3 – Haplotype frequencies identified among 725 accessions.

Haplotype	Number	Frequency (%)
CGA	18	2.79
TGA	477	73.84
TGT	12	1.86
TAA	8	1.24
TAT	131	20.28

Among the remaining haplotypes, TGA and TAT were the two most frequent haplotypes well characterized for PHS resistance with TGA having better PHS resistance compared with the TAT haplotype [31,40]. The results in this study were consistent with the earlier study with average sprouting rates for the TGA haplotype being significantly lower than those for the TAT haplotype (Fig. 3). The remaining haplotypes TGT and TAA were present at very low frequencies, because of the high LD between the +646 and + 666 positions.

Supplementary data for this article can be found online at https://doi.org/10.1016/j.cj.2019.12.003.

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Author contribution

Danfeng Wang and Shubing Liu wrote the manuscript; Danfeng Wang and Lei Dong performed experiments and

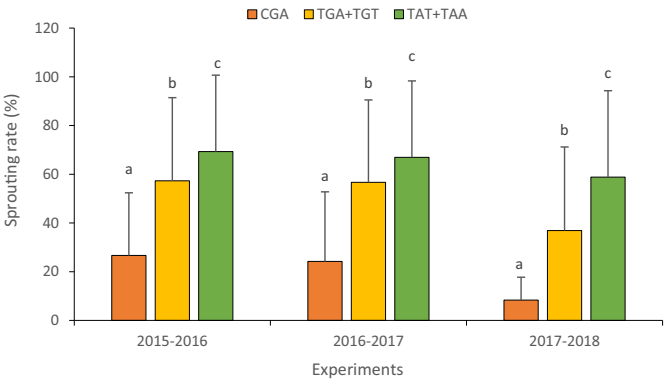

Fig. 3 – Comparison of sprouting rates of accessions with haplotypes CGA, TGA + TGT, and TAT + TAA evaluated over three years. Different letters indicate significance differences at $P < 0.05$.

PHS evaluation; Danfeng Wang, Yunlong Pang analyzed the data; all authors contributed in writing the manuscript.

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