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ORIGINAL PAPER

Genotyping-by-sequencing (GBS) identified SNP tightly linked to QTL for pre-harvest sprouting resistance

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Abstract

Key message **Using a GBS-SNP map, a QTL for pre-harvest sprouting resistance on 4AL of Totoumai A was delimited to 2.9-cM interval, and SNP closely linked to several other QTL were identified.**

Abstract Pre-harvest sprouting (PHS) of wheat is a major constraint to wheat production in many wheat-growing areas worldwide, because it reduces both wheat grain yield and the end-use quality. To identify markers tightly linked to the quantitative trait loci (QTL) for PHS resistance and seed dormancy (SD), we evaluated 155 recombinant inbred lines (RIL) derived from a cross between a PHS-resistant parent 'Tutoumai A' and a PHS-susceptible parent 'Siyang 936' for single-nucleotide polymorphisms (SNP) using genotyping-by-sequencing (GBS), and for PHS resistance and SD using both field and greenhouse grown plants. Two SNP, *GBS109947* and *GBS212432*, were mapped to a major QTL region for PHS resistance and SD on chromosome 4AL, and delimited the QTL to a 2.9-cM interval. Two and nine additional SNP were mapped to minor QTL regions for SD on chromosome 5B and 5A, respectively. Critical SNP in these QTL regions were converted into KBioscience Competitive Allele-Specific PCR (KASP) assays that can be easily used for marker-assisted selection to improve PHS resistance.

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Introduction

Pre-harvest sprouting (PHS) in wheat (*Triticum aestivum L.*), or germination of grain in a matured wheat spike before harvest, can cause significant reductions in wheat grain yield and grain end-use quality, thus substantial reductions in grain price (Groos et al. 2002; Mares et al. 2005). PHS usually occurs when weather is continuously wet before harvest. Growing PHS-resistant cultivars is the most effective way to minimize the PHS damage, especially in wheat-growing areas where wet weather occurs frequently during harvest season.

Seed dormancy (SD) has been considered the major factor that determines PHS resistance in wheat and other cereal crops (Bewley and Black 1982; Anderson et al. 1993; Mares and Mrva 2001; Ogbonnaya et al. 2008), although several other factors also have been considered to contribute to overall PHS resistance, including physical barriers to water penetration (Gale 1989), spike morphology (King and Richards 1984), red seed color (Gfeller and Svejda 1960; Groos et al. 2002) and environmental factors such as temperature and moisture (Argel and Humphreys 1983; Ceccato et al. 2011). Both PHS resistance and SD are complex traits controlled by several quantitative genetic loci (QTL). For PHS resistance, one major QTL was mapped on chromosome 3AS (Osa et al. 2003; Mori et al. 2005; Liu et al. 2008), and the casual gene of this QTL for both SD and PHS resistance has been cloned (Nakamura et al. 2011; Liu et al. 2013). Another major QTL has been identified on chromosome 4AL in different genetic backgrounds (Kato et al. 2001; Mares et al. 2005; Torada et al. 2005;

Chen et al. 2008; Ogbonnaya et al. 2008). In addition, QTL with minor effects have been reported on 2B (Kulwal et al. 2004; Munkvold et al. 2009), 3D (Imtiaz et al. 2008), 4B and 4D (Kato et al. 2001), 6B and 7D (Roy et al. 1999), and several other chromosomes (Anderson et al. 1993). For SD, major QTL were reported on 3A (Osa et al. 2003; Mori et al. 2005) and 4A (Kato et al. 2001; Noda et al. 2002; Mares et al. 2005). However, how much SD contributes to PHS resistance remains unknown. Therefore, mapping QTL for PHS resistance and SD simultaneously may reveal the genetic relationship between the two traits.

High-density genetic maps are essential for fine mapping QTL and delimiting the causal genes to very narrow genetic intervals (Liu et al. 2014). More recently, next-generation sequencing (NGS) technology has been used for QTL mapping in many crops (Wicker et al. 2008; Kobayashi et al. 2014; Chen et al. 2014). Wheat is a polyploid with a large genome (~17 GB) and abundant repetitive DNA sequences, which complicates the analysis of genetic variations and development of high-resolution genetic maps. A genotyping-by-sequencing (GBS) protocol recently was adapted in wheat using restriction digestion to reduce the complexity of the genome (Poland et al. 2012). GBS takes advantage of NGS and keeps sequencing costs down by multiplexing samples using barcodes. Although complete reference genome sequences can increase the efficiency of SNP identification in different species (Poland et al. 2012; Spindel et al. 2013), such sequences are unavailable for wheat. Fortunately, analytical tools are now available for species with incomplete or no reference genome sequences (Mascher et al. 2013). The objectives of this study were to (1) fine map QTL for both PHS resistance and SD in a Chinese landrace using GBS-SNP, (2) develop DNA markers closely linked to the QTL for marker-assisted selection in wheat breeding programs, and (3) elucidate the genetic relationship between SD and PHS resistance.

Materials and methods

Plant materials and experimental design

A mapping population of 155 F_6 RIL derived from the cross 'Tutoumai A' × 'Siyang 936' was developed by single-seed decent. Tutoumai A is a white PHS-resistant Chinese landrace, and Siyang 936 is a white PHS-susceptible cultivar from China. Both parents and the RIL were evaluated for PHS resistance using plants collected from two field experiments (2005 and 2006) at Jiangsu Academy of Agriculture Sciences (JAAS), Nanjing, China, and from three greenhouse experiments (2005–2007) at Kansas State University (KSU), Manhattan, KS, USA. Seed dormancy was evaluated using plants grown in the five experiments from 2004 to 2006 in both locations. Each experiment was arranged in a randomized complete block design with two replicates.

Evaluation of SD and PHS

In the greenhouse experiments, plants were grown at 22 ± 5 °C day/17 ± 2 °C night temperature with supplemental daylight of 12 h. Pre-harvest sprouting was evaluated in the laboratory using intact spikes. When wheat spikes reached physiological maturity, five spikes per RIL harvested from each replicate were air-dried for 5 days in a greenhouse. Spikes were then stored at −20 °C to maintain dormancy. After all RIL were collected, spikes were air-dried again for 2 days and immersed in de-ionized water for 5 h. The wet spikes were incubated in a moist chamber set up in the laboratory at 22 ± 1 °C with 100 % humidity maintained by running a humidifier for 30 min twice a day. On the 7th day of incubation, the numbers of germinated and non-germinated seeds in each spike were counted, and PHS resistance was measured as the percentage of visible sprouted kernels (PVSK) in a spike. For SD testing, 50 hand-threshed kernels from the remaining spikes in each RIL that were stored at −20 °C for PHS test were evaluated for seed germination rate in the laboratory after they were air-dried for 2 days. Germinated kernels were counted daily and removed after counting, and a weighted germination index (GI) was calculated to reflect SD as previously described (Chen et al. 2008).

In the field experiments, each RIL and their parents were sowed in a two-row plot with 4-m-long at 0.25 m apart. At physiological maturity, when the spike and peduncle turned yellow, 20 spikes per plot (10 spikes per row) were harvested. Harvested spikes were stored and evaluated for both PHS and SD as previously described for the greenhouse experiment, with the exception that 10 spikes per RIL were used for PHS evaluation in field experiments instead of five in greenhouse experiments.

GBS library construction and SNP identification

Genomic DNA of parents and their RIL were extracted using a modified cetyltrimethyl ammonium bromide (CTAB) method (Saghai-Maroof et al. 1984). DNA concentration was quantified using the Quant-iT™ PicoGreen® dsDNA Assay (Life Technologies Inc., NY) and normalized to 20 ng/μl. The GBS library was constructed as previously described (Poland et al. 2012). In brief, DNA samples were digested with *HF-PstI* and *MspI* (New England BioLabs Inc., Ipswich, MA, USA), then ligated to barcoded adaptors and a Y common adaptor using T4 ligase (New England BioLabs Inc.). Ligation products were pooled and cleaned up using the QIAquick PCR Purification Kit

(Qiagen Inc., Valencia, CA, USA). Primers complementary to both adaptors were used for PCR. The PCR product was then cleaned up again using the QIAquick PCR Purification Kit and size-selected for a range of 250–300 bp in an E-gel system (Life Technologies Inc.), then concentration was estimated by the Qubit 2.0 fluorometer using Qubit dsDNA HS Assay Kit (Life Technologies Inc.). The size-selected library was sequenced on an Ion Proton system (Life Technologies Inc.).

SNP detection used the software package developed by Saintenac et al. (2013). Reads were removed if more than 20 % of bases had quality scores of <15. Sequences from each parent were clustered, and the clusters that differed from each other by no more than three mismatches were used as reference sequences. Reads were aligned to the reference using *bowtie* (Langmead et al. 2009) with a parameter set at -v 3 –k 1. Because RIL were used in library construction, SNP with heterozygotes >10 % of total RIL were discarded to reduce the false positive results. SNP with missing data <50 % were used for mapping.

Genetic map construction and QTL analysis

A linkage map was constructed using SNP data from GBS (GBS-SNP) and previously reported SSR data (Liu et al. 2011) using the Regression function in JoinMap version 4.0 (Van Ooijen 2006). Recombination fractions were converted into centiMorgans (cM) using the Kosambi function (Kosambi 1944). Composite interval mapping (CIM) was performed for the data from each experiment and from line mean sprouting and SD ratings using WinQTLCart 2.5 (Wang et al. 2005). A LOD threshold of 2.24 was determined from 1000 permutation tests (Doerge and Churchill 1996) to claim significant QTL. QTL nomenclature followed Liu et al. (2011).

Results

GBS-SNP identification

A combination of *PstI* and *MspI* restriction enzymes was used to reduce wheat genome complexity. GBS generated a total of 87 million reads in one run of Ion Proton. After initial filtering, 82 million reads met the quality score. A total of 3180 GBS-SNP were called at <20 % missing data, and 8623 GBS-SNP were called at <50 % missing data in the population.

Map construction

All GBS-SNP with <50 % missing data and 93 SSR were used to construct the linkage map, and 2029 GBS-SNP and 43 SSR were mapped into 63 linkage groups. The map covers all the 21 chromosomes with the lengths of the individual linkage groups ranging from 18.37 to 119.18 cM. The total length of the map is 2646.82 cM with an average marker density of 1.28 cM per marker and 5–175 markers per linkage group.

Seed dormancy and PHS resistance in parents and RIL

The PVSK ranged from 6.8 to 48.4 % for Tutumai A and from 43.9 to 90.8 % for Siyang 936, and the GI ranged from 18.2 to 62.3 % for Tutoumai A and from 61.2 to 92.7 % for Siyang 936 in the five experiments conducted at JAAS and KSU. Tutoumai A had about 35 and 40 % lower PVSK and GI ratings than these for Siyang 936 in an average, although large variations in each trait were observed for each parents among experiments. Both traits showed continuous distributions in the RIL population, and transgressive segregation was observed for both traits, indicating that both parents might contribute favorable alleles.

QTL mapping

CIM detected four QTL on different linkage groups. A major QTL was detected on chromosome 4A (*Qphs.pseru-4A.1*) for both PHS resistance and SD (Fig. 1a). One QTL each for PHS resistance was detected on chromosome 5B (*Qphs.pseru-5B.1*) and 5A (*Qphs.pseru-5A.1*; Fig. 1b, c), and one QTL for both PHS resistance and SD was detected on chromosome 4B (*Qphs.pseru-4B.1*; Fig. 1d). Two GBS-SNP were mapped to the *Qphs.pseru-4A.1* region, two were mapped to *Qphs.pseru-5B.1* region, nine were mapped to *Qphs.pseru-5A.1* region, and none was mapped to *Qphs.pseru-4B.1* region.

To verify the genotypic data generated by GBS and to eliminate missing data for markers in QTL regions, 26 KASP assays were designed from the corresponding GBS sequences harboring SNP that were mapped within or around these QTL regions. Eleven KASP-SNP markers amplified well and showed polymorphism between parents and among the RIL, and seven of them were remapped to three of the QTL regions (Table 1). The other four SNP shifted position and moved outside the QTL regions after all missing data at these loci were filled by KASP-SNP and errors were corrected. Comparison between GBS-SNP and KASP-SNP data found that seven SNP showed identical genotypes in RIL between GBS and KASP assays, and four KASP-SNP did not match with GBS-SNP because two GBS-SNP had a SNP calling error in one RIL, one had errors in five RIL, and one had errors in 16 RIL. Therefore, the average error rate for the seven SNP caused by either sequencing or SNP determination was 1.35 %.

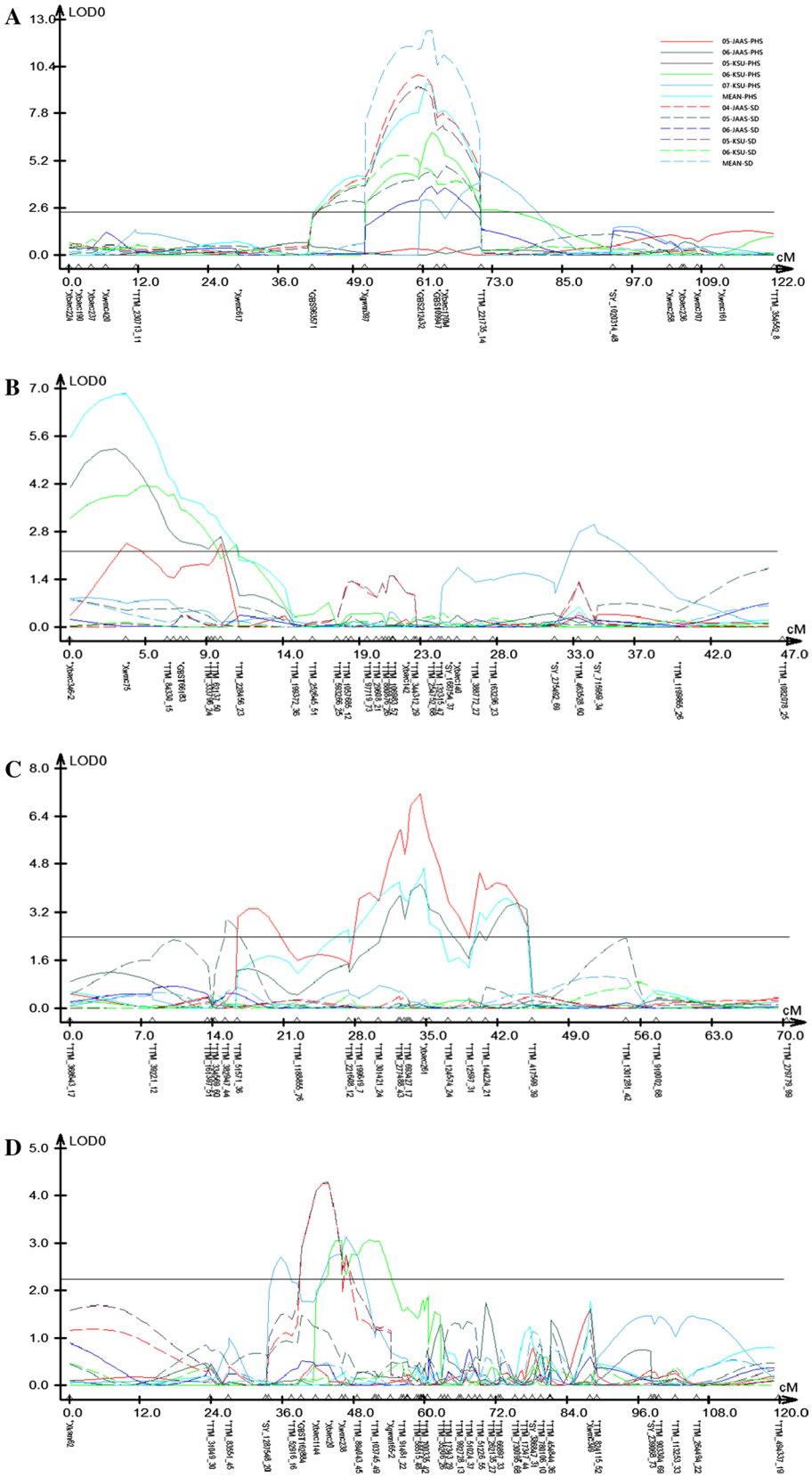

Fig. 1 Composite interval mapping (CIM) of QTL for long seed dormancy (SD) and pre-harvest sprouting (PHS) resistance on chromosome 4A (**a**), 5B (**b**), 5A (**c**) and 4B (**d**) using SSR and SNP markers and phenotypic data from 10 experiments. The line parallel to the *X*-axis is the threshold line for the significant LOD value of 2.24 ($p < 0.05$). Genetic distances are shown in centiMorgans (cM)

Table 1 List of KASPar primers developed from GBS sequences

Primer name[a]	Position	Primer sequence (5′–3′)
GBS_212432_T	*Qphs.pseru-4A*	TTCACAGCGCCTCGGC-CGCCC
GBS_212432_S	*Qphs.pseru-4A*	TTCACAGCGCCTCGGC-CGCCA
GBS_212432_R	*Qphs.pseru-4A*	GTACCACTCTGGTG-CACTCC
GBS_109947_T	*Qphs.pseru-4A*	TTAGCCGTGTGACGC-CGTGT
GBS_109947_S	*Qphs.pseru-4A*	TTAGCCGTGTGACGC-CGTGC
GBS_109947_R	*Qphs.pseru-4A*	GCGTGAATTGCTGAC-CTCTC
GBS_963571_T	*Qphs.pseru-4A*	CGATCATAGCAGTG-GAACGC
GBS_963571_S	*Qphs.pseru-4A*	CGATCATAGCAGTG-GAACGT
GBS_963571_R	*Qphs.pseru-4A*	CTCGCACAGTGAAGGT-CATT
GBS_T240557_T	*Qphs.pseru-5B*	CAGCTTCAGTGCCTTC-CTCG
GBS_T240557_S	*Qphs.pseru-5B*	CAGCTTCAGTGCCTTC-CTCA
GBS_T240557_R	*Qphs.pseru-5B*	GAGTGACGTCATC-CACAAGG
GBS_T66183_T	*Qphs.pseru-5B*	GGTGGAGGGATTTGGAT-GATC
GBS_T66183_S	*Qphs.pseru-5B*	GGTGGAGGGATTTGGAT-GATA
GBS_T66183_R	*Qphs.pseru-5B*	CGTCCTCTTGCTTGATGGTA
GBS_T169803_T	*Qphs.pseru-5B*	GCAGTAATTTTAGTAG-CATTC
GBS_T169803_S	*Qphs.pseru-5B*	GCAGTAATTTTAGTAG-CATTT
GBS_T169803_R	*Qphs.pseru-5B*	TATTGCTTCATTAGAGGACA
GBS_T162884_T	*Qphs.pseru-4B*	CAAATGTCGCATGTG-GCTGC
GBS_T162884_S	*Qphs.pseru-4B*	CAAATGTCGCATGTG-GCTGA
GBS_T162884_R	*Qphs.pseru-4B*	CGCGTATGAGCATGATACCT

[a] T forward primer with Tutoumai Aallele, S forward primer with Siyang 936allele, R reverse primer

The QTL with the largest effect, *Qphs.pseru-4A.1*, was delimited to a 2.9-cM interval between SNP *GBS212432* and *GBS109947* (Fig. 1a) and explained 8.3–17.2 % phenotypic variances for PHS resistance and 9.4–26.5 % for SD (Table 2). On one side of the QTL, both markers *Xbarc170* and *GBS109947* showed the largest effect on PHS resistance and SD among all markers tested in all the experiments (Table 3), and the difference in the mean sprouting rates between two groups carrying contrasting alleles of *GBS109947* was similar to that of *Xbarc170* (Table 4); on the other side of the QTL, however, *GBS212432* had much greater effects than *Xgwm397* on both traits measured (Table 3), with SNP *GBS212432* showing a larger difference in spouting rates between two contrasting alleles than that for *Xgwm397* (Table 4), so *GBS212432* was the more closely linked marker to the QTL than *Xgwm397*, and *GBS212432* and *GBS109947* flanked the QTL.

Qphs.pseru-5B.1 was detected in two JAAS experiments and one KSU experiment that accounted for 5.5–12.5 % of phenotypic variances on PHS, but this QTL was not detected in any SD experiment (Fig. 1b; Table 2). Two SNP were mapped to this QTL region, which was linked closely to the SSR marker *Xbarc275* in these experiments (Fig. 1b).

Qphs.pseru-5A.1 was another QTL identified for PHS resistance. It was detected in the two JAAS experiments and significant for the overall mean of germination rate, and explained 7.7–15.5 % phenotypic variances (Fig. 1c; Table 2). Nine GBS-SNP together with two SSR were mapped to this QTL region, and the SSR were the most closely linked markers to the QTL (Fig. 1c).

Qphs.pseru-4B.1 was identified for both PHS resistance and SD in four experiments, and explained 6.3–8.7 % phenotypic variances, but GBS-SNP were not mapped to the QTL region (Fig. 1d; Table 2).

Discussion

Evaluation of PHS and SD

PHS is a complicated trait, and many factors may contribute to PHS resistance, including SD, seed color, and other morphological characteristics. Environmental factors, such as temperature and moisture during the maturation period, also can interfere with the expression of PHS resistance. Therefore, repeated experiments are critical to providing increased accuracy in PHS resistance estimation. In this study, we conducted five experiments to estimate PHS resistance and SD. To minimize the environmental interference on phenotyping, spikes were harvested at physiological maturity, dried for a fixed period, and soaked in distilled water for 5 h. The sprouting index (SI), a visual estimation of the germination rate of intact spikes on a 1–6 or 1–10 scale, has been used as a standard method to measure the germination rate (Anderson et al. 1993; Kulwal et al. 2004). Chen et al. (2008) and Imtiaz et al. (2008) used percentage of visually sprouted seeds (VSS) to measure germination rate, and proved that VSS gave a more accurate PHS rating than SI. The current study used this same measurement to measure overall PHS resistance.

Table 2 Putative QTL for pre-harvest sprouting resistance (PHS) and seed dormancy (SD) identified by composite interval mapping using spikes and seeds harvested from recombinant inbred lines grown in field trials of 2004, 2005 and 2006 (JAAS, Nanjing, China) and greenhouse trials of 2005, 2006 and 2007 (KSU, Manhattan, KS, USA)

QTL and its location	Marker interval	2004 JAAS		2005 JAAS		2006JAAS		2005 KSU		2006 KSU		2007 KSU		Mean over all experiments	
		LOD[a]	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2
PHS															
Qphs.pseru-4A	*GBS_212432/GBS_109947*	–[b]	–	0.448	0.009	0.506	0.010	3.810*	0.102	6.768*	0.166	4.009*	0.083	9.490*	0.172
Qphs.pseru-5B	*Xbarc346-2/TTM_62137_50*	–	–	2.473*	0.055	5.237*	0.125	0.247	0.006	4.150*	0.098	0.881	0.017	6.849*	0.127
Qphs.pseru-4B	*Xbarc20/Xwmc238*	–	–	0.465	0.009	0.282	0.006	0.523	0.012	3.084*	0.070	3.138*	0.063	0.290	0.004
Qphs.pseru-5A	*TTM_199619_7/TTM_12597_31*			7.171*	0.155	4.149*	0.089	0.160	0.004	0.336	0.007	0.779	0.014	4.680*	0.077
SD															
Qphs.pseru-4A	*GBS_212432/GBS_109947*	9.933*	0.216	4.927*	0.115	3.731*	0.094	9.234*	0.203	4.820*	0.133	–	–	11.029*	0.265
Qphs.pseru-5B	*Xbarc346-2/TTM_62137_50*	0.359	0.007	0.797	0.017	0.248	0.006	0.342	0.006	0.153	0.004	–	–	0.810	0.016
Qphs.pseru-4B	*Xbarc20/Xwmc238*	4.255*	0.084	1.499	0.033	0.523	0.012	4.281*	0.087	0.205	0.005	–	–	0.487	0.009
Qphs.pseru-5A	*TTM_199619/TTM_12597_31*	0.256	0.007	0.718	0.017	0.159	0.001	0.190	0.004	0.201	0.005	–	–	0.175	0.004

* Significant quantitative trait locus (QTL) with LOD value greater than the threshold (2.24)

[a] LOD refers to logarithm of odds

[b] Trait was not evaluated in this location

Table 3 Closely linked or flanking markers, LOD values, and coefficients of determination (R^2) of QTL for pre-harvest sprouting PHS) resistance and seed dormancy (SD) on chromosome 4AL estimated using the recombinant inbred lines (RIL) from Tutoumai A/Siyang 936 grown in JAAS Jiangsu Academy of Agricultural Sciences (JAAS), and Kansas State University (KSU), respectively

Close or flanking markers of 4A QTL	Position	2004 JAAS		2005 JAAS		2006JAAS		2005 KSU		2006 KSU		2007 KSU		Mean over experiments	
		LOD[a]	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2
PHS															
Xgwm397	51.04	–[b]	–	0.094	0.002	0.144	0.003	1.619	0.042	2.872	0.070	0.078	0.001	4.352	0.080
GBS212432	60.52	–	–	0.337	0.007	0.000	0.000	3.245	0.083	5.011	0.119	3.010	0.062	8.417	0.147
GBS109947/GBS212432	62.53	–	–	0.321	0.006	0.028	0.001	3.810	0.102	6.768	0.166	3.107	0.067	9.490	0.172
GBS109947	63.43	–	–	0.232	0.004	0.172	0.003	3.365	0.085	6.577	0.152	2.607	0.054	7.854	0.135
Xbarc170	64.78	–	–	0.448	0.009	0.109	0.002	3.726	0.094	5.535	0.130	2.019	0.042	7.984	0.137
SD															
Xgwm397	51.04	4.243	0.102	2.933	0.070	1.621	0.042	3.846	0.092	3.923	0.104	–	–	7.324	0.188
GBS212432	60.52	9.933	0.216	4.362	0.103	3.248	0.083	9.234	0.203	4.722	0.124	–	–	11.393	0.273
GBS109947/GBS212432	61.53	9.865	0.223	4.622	0.113	3.814	0.103	9.276	0.210	4.820	0.133	–	–	12.426	0.311
GBS109947	63.43	7.582	0.170	4.248	0.100	3.369	0.085	6.894	0.157	3.948	0.105	–	–	10.475	0.254
Xbarc170	64.78	7.604	0.171	4.927	0.115	3.730	0.094	6.928	0.158	3.934	0.104	–	–	11.029	0.265

[a] LOD logarithm of odds

[b] Trait was not evaluated in this location

Table 4 Difference (Dif) in ratings of pre-harvest sprouting (PHS) and seed dormancy (SD) as reflected by a percentage of germinated seeds between resistance (R) and susceptible (S) alleles of two SNP and two SSR for the PHS resistance QTL on chromosome 4A

Locus	Genotype	PHS						SD					
		2005 JAAS	2006 JAAS	2005 KSU	2006 KSU	2007 KSU	Mean over experiments	2004 JAAS	2005 JAAS	2006 JAAS	2005 KSU	2006 KSU	Mean over experiments
GBS109947	S	71.52	53.45	39.88	53.28	57.24	56.82	71.17	34.21	39.87	71.20	69.89	57.12
GBS109947	R	65.26	51.86	22.51	33.99	38.31	45.56	54.05	18.54	22.50	54.59	59.86	41.64
GBS109947	Dif	6.26	1.59	17.37*	19.29*	18.92*	11.27*	17.12*	15.67*	17.38*	16.61*	10.04*	15.48*
Xbarc170	S	72.44	54.41	39.88	52.87	57.80	57.29	71.64	34.15	39.88	71.70	69.93	57.33
Xbarc170	R	66.08	52.09	22.49	34.98	38.84	46.11	54.03	18.63	22.48	54.59	59.87	41.63
Xbarc170	Dif	6.36	2.32	17.39*	17.89*	18.96*	11.18*	17.61*	15.52*	17.40*	17.12*	10.06*	15.70*
GBS212432	S	72.50	54.98	38.51	52.55	58.03	57.13	71.55	33.73	38.50	71.61	70.10	56.96
GBS212432	R	64.83	50.52	23.10	34.19	36.29	45.04	53.00	18.32	23.09	53.57	58.89	41.10
GBS212432	Dif	7.67	4.46	15.40*	18.36*	21.74*	12.09*	18.55*	15.42*	15.41*	18.04*	11.21*	15.86*
Xgwm397	S	72.30	53.56	38.93	52.71	57.07	56.78	70.86	33.81	38.92	70.85	70.18	56.92
Xgwm397	R	66.23	51.89	25.19	36.73	40.97	47.02	57.00	20.00	25.18	56.89	60.13	43.72
Xgwm397	Dif	6.06	1.68	13.74*	15.98*	16.10*	9.75*	13.86*	13.81*	13.75*	13.95*	10.06*	13.20*

* Significant differences between resistant and susceptible genotypes with p value <0.01 in student t test

QTL for PHS resistance and SD in wheat

In this study, four QTL were detected for PHS resistance and two of them were detected for SD. Many QTL for PHS resistance have been reported on different chromosomes in previous studies. Anderson et al. (1993) detected several genetic regions on chromosomes 1AS, 3BL, 4AL, 5DL and 6BL associated with PHS resistance, whereas Zanetti et al. (2000) reported QTL on chromosomes 3B, 5A, 6A and 7B. QTL for PHS resistance were detected on chromosome 5A and group 3 chromosomes where the kernel color genes were previously reported (Groos et al. 2002), and on chromosomes 6B and 7B (Roy et al. 1999). For SD, major QTL were mainly reported on chromosomes 3A (Osa et al. 2003; Mori et al. 2005) and 4A (Kato et al. 2001; Noda et al. 2002; Mares et al. 2005). In this study, both PHS resistance and SD were evaluated in the same experiments, which enables estimation of QTL effects on both PHS resistance and SD.

The QTL on chromosome 4A, designated as *Qphs.pseru-4A.1* previously (Liu et al. 2011), was detected in three KSU greenhouse experiments for PHS resistance and all experiments for SD, and explained up to 17.2 and 26.5 % of phenotypic variance for PHS resistance and SD, respectively. This result indicates that *Qphs.pseru-4A.1* is a stable QTL with a large effect on both PHS resistance and SD, and that SD is the most important factor in PHS resistance.

Another QTL on chromosome 5B, previously designated as *Qphs.pseru-5B.1* (Liu et al. 2011), was detected only for PHS resistance, suggesting this QTL may contribute to PHS resistance due to factors other than SD. The QTL for PHS resistance on chromosome 5B have been reported in previous studies (Groos et al. 2002; Tan et al. 2006), but we were unable to determine whether they were the same QTL owing to the lack of common markers among these QTL. Similarly, the QTL *Qphs.pseru-5A.1* was also only for PHS resistance. Groos et al. (2002) and Nakamura et al. (2007) reported QTL on chromosome 5AS for PHS resistance, but common markers were not found between those and our studies. One QTL was detected on chromosome 4B, *Qphs.pseru-4B.1,* that showed minor effects on PHS resistance and SD. QTL for PHS resistance and SD were also previously reported on chromosome 4B (Kato et al. 2001; Mori et al. 2005; Mohan et al. 2009; Rasul et al. 2009), but common markers among these QTL are lacking to determine if they are the same QTL. In this study, a previously reported QTL, *Qphs.pseru-5B.2*, (Liu et al. 2011) was not detected. This may be due to addition of new markers that changed the map. In the previous map, *Qphs.pseru-5B.2* was mapped between markers *Xwmc363* and *Xbarc1176* in the end of linkage group for 5B, but *Xwmc363* in the end of the linkage group was not mapped in the new map after a dozen of SNP linked to *Xbarc1176* were added. Thus, the new map does not cover the region where the previous QTL *Qphs.pseru-5B.2* was located.

We are not able to detect the QTL for PHS resistance on chromosome 3A, *TaPHS1*, in this study. The functional SNP of *TaPHS1* is not polymorphic between Tutoumai A and Siyang 936. Two SSR closely linked to the 3A QTL, *Xbarc57* and *Xbarc321*, also are not polymorphic in the population. *Xwmc11* was the closest polymorphic marker to this QTL in this study (data not shown), but it was at least 30 cM away from the QTL (Song et al. 2005; Liu et al. 2008). Therefore, it is more likely that both parents carry the same allele at the 3A QTL.

Efficiency of GBS and KASP

The application of GBS facilitates generation of high-density genetic maps at a low cost (Poland et al. 2012). High-resolution maps have been created with GBS-SNP in sorghum, wheat, rice and barley, and maps saturated with GBS-SNP have proven very useful for fine mapping of QTL for different traits and identification of candidate genes for gene cloning (Poland et al. 2012; Saintenac et al. 2013; Liu et al. 2014; Spindel et al. 2013). One disadvantage of GBS-SNP for mapping is a large amount of missing data for some markers because of limited sequencing depth; therefore, the imputation method is recommended to predict genotypes with missing data (Poland et al. 2012; Spindel et al. 2013; Sonah et al. 2013). Another way to increase data quality is to use high-quality SNP with <20 % missing data without imputation (Liu et al. 2014), but this approach would probably result in loss of some important SNP. In this study, we used a different strategy. First, we used GBS-SNP with <50 % missing data to construct an initial map to scan QTL, then we convert GBS-SNP from the QTL regions to KASP-SNP to confirm GBS-SNP in the QTL regions. Using this method, more than 8000 SNP were scored from one Ion Proton run, and together with SSR anchoring markers, a high-density genetic map was generated with 2029 SNP and 43 SSR. Missing data and sequencing errors may cause an expansion of genetic distance between markers in the initial genetic map, but it includes many more SNP than the map developed using SNP with <20 % missing data. We validated GBS-SNP with KASP-SNP assays, which minimized the negative effect of missing data and corrected sequencing errors in the QTL regions, thus improving the accuracy of fine mapping results in the QTL regions. Among the 26 KASP assays designed, 11 worked well in the RIL. Among these working KASP-SNP, seven agreed with GBS-SNP calls among RIL, but four had SNP call errors and one had the wrong SNP calls in 16 RIL. These errors could occur in either sequencing or the SNP calling pipeline. Thus,

reducing sequence error and improving SNP call quality will minimize genotyping error. Conversion of GBS-SNP to KASP-SNP could improve QTL mapping quality. Other KASP assays did not amplify well, mainly because short sequence reads resulted in primer design difficulties that cannot generate optimal primers for SNP amplification.

With a new GBS-SNP map developed from the same population reported in the previous study (Liu et al. 2011), we identified not only the same QTL on chromosomes 4A, 5B and 4B, but also a new QTL on 5A. The new QTL on 5A was detected in this study because it was mapped in a large linkage group of GBS-SNP and two SSR; in the previous study, the two SSR did not form a linkage group and were not used in QTL analysis. Therefore, GBS is an effective marker system for SNP discovery and is useful for QTL identification and QTL fine mapping.

In this study, mapping resolution was significantly increased in the 4A and the 5B QTL regions by adding GBS-SNP. In our previous study, the QTL in 4A was mapped in a 9.1-cM genetic interval (Chen et al. 2008); using GBS-SNP in this study, it was mapped to a 2.9-cM interval between two SNP, *GBS212432* and *GBS109947*. The 4A QTL shows major effects and is a good candidate for map-based cloning of the PHS resistance gene, and the SNP identified in this study laid a solid foundation for such work.

Application of SNP in MAS

Because PHS is easily affected by environmental factors and phenotyping of PHS is time-consuming and labor-intensive, marker-assisted selection provides a desirable approach to quick deployment of PHS-resistant QTL in breeding programs. *GBS212432* and *GBS109947* are the closest markers associated with QTL on chromosome 4A in the population used in this study. As *Xbarc170* showed effects similar to *GBS109947*, it remains a valuable marker for MAS. Therefore, *GBS212432* can be used together with either *Xbarc170* or *GBS109947* to increase selection accuracy. In addition, SNP and SSR in *Qphs.pseru-5B*, *Qphs.pseru-5A* and *Qphs.pseru-4B* regions can be valuable in pyramiding multiple PHS resistance QTL to achieve an increased level of PHS resistance.

Author contribution statement GB conceived the experiments; ML, SC, SL conducted experiments; SC developed mapping population; ML and SW conducted SNP and data analysis; GZ and GB provided reagents; ML and GB wrote the manuscript.

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

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