Mapping of QTL for Pre-Harvest Sprouting Resistance and Seed Dormancy in a Doubled Haploid Population

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ABSTRACT Pre-harvest sprouting (PHS) of wheat reduces grain yield and has deleterious effects on wheat flour quality. PHS resistance is strongly associated with grain color and seed dormancy. Resistance to PHS is a priority of wheat breeding program. We have studied a population of 122 Doubled Haploid (DH) lines from the cross between two cultivars, 'Keumkang' and 'Olgeuru', in order to detect QTLs correlated with PHS resistance. PHS was evaluated using mist spray in the humid chamber (PHS-MS), and the germination index (GI) was determined for two years. The variety 'Keumkang' has white grains and higher percentage of PHS rate (30.2%), while 'Olgeuru' has red grains and lower PHS rate (2.8%). There was significant difference in grain color according to the GI. PHS-MS was positively correlated with GI ($r = 0.732$, $P < 0.001$). Two molecular markers, *Xbarc12* and *Xgwm3*, were found correlated with PHS resistance and GI. These markers were mapped on chromosomes 3A and 3D with LOD scores of 4.95 and 4.02, respectively. *Xbarc12* explained the highest amount of phenotypic variation (R^2) which is 11.99% in GI and 27.25% in PHS. *Xgwm3* explained 9.10% of phenotypic variation in GI. The respective value of PHS-MS and GI in Korean wheat cultivars ranged from 1.37% to 86.75% and from 0.03 to 0.73. This study provides useful information for marker-assisted wheat breeding for PHS resistance improvement in Korea.

Keywords Doubled haploid line, Pre-harvest sprouting resistance, Simple sequence repeats, Wheat

INTRODUCTION

Pre-harvest sprouting (PHS) of wheat, which causes a major loss of grain yield and a reduction of end-use quality, has been frequently observed in southern part of Korea due to the prevailing rainy seasons during the harvesting period (Kim *et al.* 2012). Improvement of PHS resistance has been one of the most important efforts carried out in Korean wheat breeding programs to solve this problem. However, PHS is generally influenced by both genotype and environment, and PHS resistance could be also influenced by seed dormancy, seed coat permeability and color, α -amylase activities, endogenous hormones levels and genes (Gao *et al.* 2013). Molecular markers associated with PHS resistance have been reported which consequently

facilitated in understanding the genetic control of PHS resistance and in developing PHS resistance wheat cultivars, which is otherwise hard to achieve through phenotypic selection only (Finkelstein *et al.* 2008; Gupta *et al.* 2010).

Several sequence-tagged sites (STS) markers were evaluated to determine the relationship between PHS resistance and allelic variations of Korean wheat cultivars in our previous studies. Allelic variations at *TaPHS1* and *TaVp-1A* loci, the molecular markers associated with PHS and seed dormancy, were not significantly different from PHS resistance in Korean wheat cultivars, although *TaSdr-B1a* allele showed lower PHS value than *TaSdr-B1b* allele (Kim *et al.* 2012, in press). Kim *et al.* (in press) reported the relationship between seed dormancy and STS markers associated with grain weight, *TaSus2-2B*, *TaGW2-6A*

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and *TaCwi-A1* alleles. Seed dormancy was correlated positively with grain traits, including length, width, thickness and thousand kernel weight of grain (Shin *et al.* 2013). Allelic variations at *TaGW2-6A* and *TaCwi-A1* loci were not significantly different from seed dormancy, although *Hap-H* haplotype showed a higher seed dormancy than *Hap-L* haplotype in *TaSus2-2B* alleles.

Quantitative trait loci (QTLs) associated with PHS resistance have been found in all chromosomes, except in chromosomes 1D and 4D (Groos *et al.* 2002; Flintham *et al.* 2002; Kulwal *et al.* 2004, 2005, 2012; Lohwasser *et al.* 2005; Mori *et al.* 2005; Kottearachchi *et al.* 2006; Chen *et al.* 2008; Liu *et al.* 2008; Ogonnaya *et al.* 2008; Fofana *et al.* 2009; Mohan *et al.* 2009; Munkvold *et al.* 2009; Rasul *et al.* 2009; Liu and Bai 2010; Miao *et al.* 2013), while QTLs associated with seed dormancy have been identified on chromosomes 1A, 1B, 2A, 2B, 2D, 3A, 3B, 4A, 4B, 4D, 5B, 6B, 7A, 7B and/or 7D (Kato *et al.* 2001; Mares and Mrva 2001; Osa *et al.* 2003; Lohwasser *et al.* 2005; Mares *et al.* 2005, 2009; Tan *et al.* 2006; Chen *et al.* 2008; Ogonnaya *et al.* 2008; Torada *et al.* 2005, 2008; Zhang *et al.* 2008; Munkvold *et al.* 2009; Singh *et al.* 2010; Rehman *et al.* 2012). A major QTL has been identified on chromosomes 3A, 3B, 3D and 4A which explained up to 58.0% of phenotypic variation in PHS resistance and 44.8% in seed dormancy (Groos *et al.* 2002; Osa *et al.* 2003; Mares *et al.* 2005; Kulwal *et al.* 2004, 2005; Mori *et al.* 2005; Torada *et al.* 2005; Liu *et al.* 2008; Ogonnaya *et al.* 2008; Chen *et al.* 2009; Miao *et al.* 2013).

Despite the extremely low self-sufficiency of wheat in Korea, the per capita wheat consumption is the second highest following rice. But just recently, wheat production in Korea is improving by settlement of contract farming and demand of domestic wheat products by consumers. The expansion of wheat cultivation has been more important than ever before in Korea. Only a few cultivars, however, have been grown in southern part of Korea and these cultivars have been susceptible to PHS. Therefore, an efficient and reliable molecular markers should be developed to boost programs in improving PHS resistance in Korean wheat cultivars. This study was conducted to understand the genetic basis of improvement in Korean wheat breeding programs through the investigation of QTLs

related with PHS and seed dormancy with DH population derived from the Keumkang and Olgeuru cross. Keumkang, the most commonly grown in Korea, is a hard-white grain cultivar susceptible to PHS, while Olgeuru is a soft-red grain cultivar with moderate resistance to PHS (Kim *et al.* 2012).

MATERIALS AND METHODS

Plant materials

A population of 122 DH lines was derived from F₁ plants of the Keumkang and Olgeuru cross using the wheat × maize system of Inagaki and Mujeeb-Kazi (1995) at CIMMYT (El Batán, Mexico). The DH lines were grown at the Upland Crop Experimental Farm of National Institute of Crop Science, RDA (Iksan, Korea) for seed multiplication in 2009. Field trials of DH population were established in 2012/2013 on 50% clay loam soil where seeds were sown in randomized complete blocks with three replications. Twenty-five Korean wheat cultivars were also cultivated at the same conditions for DH population. The seeds were sown on each plot consisted of three 4.0 m rows spaced 25 cm apart. Sowing was carried out on October 25. The plots were combine-harvested on June 20 in three years. Fertilizer was applied at 5: 7: 5kg/10a (N: P: K) before sowing, and weeds, insects and diseases were stringently controlled. No supplemental irrigation was applied. Spikes of each line in the field were harvested at maturity, when all the green color had disappeared from the leaves and stems. Spikes were dried under room conditions for seven days until the moisture content of the grain was ~14% and they were then gently threshed by hand and kept at -20°C to preserve dormancy (Mares 1983).

Phenotypic traits evaluation

Pre-harvest sprouting was conducted by mist spray in the humid chamber (PHS-MS) according to the procedure described in Liu *et al.* (2008) with some modifications. Ten spikes per line were immediately placed in a mist chamber for sprouting assays. Sprouting assays were conducted with a humidifier (Humidifier HR-25, Faran Industrial Co., Korea) which was sprayed at 3,400 rpm with 2,500 ml of

tap water every hour for two weeks at $20 \pm 2^\circ\text{C}$ with 100% humidity. After humidification, spikes were air-dried at room temperature for one week. Air-dried spikes were hand-threshed and sprouted and sound kernels were counted. Pre-harvest sprouting was described as the percentage of sprouted kernels in a spike.

Germination index (GI) was measured according to the procedure provided in Walker-Simmons (1988). Sound grains were incubated on a single layer of filter paper, moistened with distilled water, in the dark condition for seven days at 20°C . Germinated grains were counted daily and removed. Germination index was calculated using the following formula: Germination index = $(7 \times n_1 + 6 \times n_2 + \dots + 1 \times n_7) / (7 \text{ days} \times \text{total grains})$, where $n_1, n_2, n_3, \dots$, and n_7 are the number of grains that had germinated on Day 1, Day 2, ..., and Day 7. The maximum index is 1.0 if all grains germinate by Day 1, with lower indices indicating increasing levels of grain dormancy or reduced germinability (Mares and Mrva 2001).

Grain color was determined by the procedure described in Ram *et al.* (2002). A total of 30 grains were soaked with 50 ml of 5M NaOH solution for 10 min at 60°C . Red-grained cultivars showed a dark-red color and white-grains exhibited straw yellow color. Statistical analysis of the phenotypic data was performed by SAS software (SAS Institute, NC, USA) using Fisher's least significant difference test (LSD), analysis of variance (ANOVA) and pair-wise t-test.

PCR Primer

A total of 537 microsatellite markers were used to detect polymorphisms survey between the parents. These markers were developed based on the DNA sequences

available at GenBank database (www.ncbi.nlm.nih.gov). A set of 140 markers successfully produced amplicons, among them, *Xbarc12* primer amplified 203 and 185 bp bands (Table 1). Another primer, *Xgwm3*, amplified 90 and 83 bp bands (Table 1).

PCR condition

Leaf tissue was collected from a single plant for each DH line after germination for two weeks which was flash-frozen in liquid nitrogen and stored at -80°C until needed. Genomic DNA was extracted from young leaf tissue (100 mg) using a genomic DNA prep kit (Solgent Co., Korea) according to the manufacturer's instructions. DNA was quantified by Biodrop (Biodrop Ltd, UK). The PCR reactions with simple sequence repeats (SSRs) were performed in a total volume of 15 μL containing 100 ng genomic DNA, 1X PCR buffer, 1.5 mM MgCl_2 , 0.2 mM dNTPs, 1 unit Taq DNA polymerase, and 0.2 $\mu\text{g}/\mu\text{L}$ each primer. After an initial denaturation at 94°C for 5 min., 10 cycles (1°C -lower per cycle) were performed for 1 min at 94°C , 1 min at 63°C , 1 min at 72°C , followed by 35 cycles of 1 min at 94°C , 1 min at 55°C , 1 min at 72°C and final extension at 72°C for 10 min. The PCR products were analyzed with QIAxcel Advanced system using a 12-capillary QIAxcel DNA high resolution Cartridge (Qiagen Co., USA).

Statistical analysis

Data were analyzed with SAS software (SAS Institute, NC, USA) using Fisher's least significant difference test (LSD), analysis of variance (ANOVA) and t-test. Analysis of variance was conducted using the general linear model procedure and sources of variation in the model were considered to be fixed effects. Pearson's correlation

Table 1. The primer sets used for amplification of the genomic sequence and PCR analysis by *Xbarc12* and *Xgwm3* markers.

Marker	Forward and reverse primers (5'→3')	Annealing temperature ($^\circ\text{C}$)	Fragment size (bp)
<i>Xbarc12</i>	F: CGACAGAGTGATCACCCAAATATAA R: CATCGGTCTAATTGTCAATGTA	52	203, 185
<i>Xgwm3</i>	F: GCAGCGGCACTGGTACATT R: AATATCGCATCACTATCCCA	55	90, 83

analysis was also conducted with significance level of $P < 0.05$ unless otherwise specified.

QTL analysis

Individual map for initial QTL detection was constructed using MapDisto (<http://mapdisto.free.fr>). Composite interval mapping (CIM) analysis (Zeng 1993, 1994) was performed using Windows version QTL Cartographer v2.5 (Wang *et al.* 2012) based on a model 6, Forward and Backward Regression method. The walking speed for all QTL was 2.0 cM and the probability in stepwise regression was $p < 0.001$. Centimorgan units were calculated using Kosambi mapping function. Empirical logarithm of the odds (LOD) value thresholds were estimated using 1,000 permutations at an experiment-wise $P < 0.01$. (Churchill and Doerge 1994). The LOD score for declaring a QTL was significant at 3.0. The additive value and the coefficient of determination

(R^2) which is based on the partial correlation of putative QTL with the trait adjusted for cofactors in the multi-locus model, were estimated based on the most significant dataset.

RESULTS

The means and ranges of PHS-MS and GI percentage are shown in Table 2 and Fig. 1. Olgeuru was more resistant to pre-harvest sprouting than Keumkang, showing lower percentage of PHS-MS (2.80%) and GI (0.17) than Keumkang (30.20% and 0.41, respectively). The PHS-MS and GI in DH lines ranged from 0.00% to 73.03% and from 0.01 to 0.73, respectively. The frequency distribution of 122 DH lines was continuous, and transgressive segregants were observed in PHS-MS and GI (Fig. 1). Of the 122 DH lines, 24 had white seed coat and 98 lines showed red color.

Table 2. Means, ranges and ANOVA of percentage of pre-harvest sprouting induced by the mist spray (PHS-MS) and germination index (GI) in 122 DH population derived from the cross of white × red seeded cultivar, cvs. Keumkang and Olgeuru.

Traits	Parents ^{z)}		DH population				
	Keumkang	Olgeuru	Mean ± SD	Range	Mean square	F-value ^{y)}	Root MSE ^{x)}
PHS-MS (%)	30.20a	2.80b	8.62 ± 13.65	0.00 – 73.03	601.69	544.51***	1.05
GI	0.41a	0.17b	0.18 ± 0.18	0.01 – 0.73	0.10	103.06***	0.03

^{z)}Values followed by the same letters are not significantly different at $P < 0.05$.

^{y)}*** indicates significant at $P = 0.001$.

^{x)}Square root of the error mean square from ANOVA.

Fig. 1. Frequency distributions of pre-harvest sprouting parameters in 122 DH population. (A) Percentage of pre-harvest sprouting induced by the mist spray (PHS-MS); (B) germination index (GI). Arrows indicate PHS-MS and GI of parental cultivars.

Red-grained DH lines showed lower GI (0.15) than white-grained (0.30, Fig. 2A), but there was no significant difference in PHS-MS according to the grain color. PHS-MS correlated positively with GI in DH population (r

$= 0.732$, $P < 0.001$, Fig. 2B). From a total of 537 SSR markers, 140 were polymorphic between Keumkang and Olgeuru. Linkage analysis of the 140 segregating markers identified a partial genetic map spanning 2,340.7 cM over

Fig. 2. The differences in germination index according to the grain color (A) and relationship between percentage of pre-harvest sprouting induced by the mist spray (PHS-MS) and germination index (GI) in 122 DH population (B). Each bar represents mean $\pm$ standard error and values on the bars with different letters are significantly different at $p < 0.05$. 122 DH population with red grain color (●) and white grain color (○).

Fig. 3. Linkage maps for percentage of pre-harvest sprouting induced by the mist spray (PHS-MS) and germination index (GI) on chromosomes 3A and 3D in 122 DH population. Markers are indicated on the right and genetic distances (cM) are indicated on the left. Markers are underlined by line for GI and dotted line for PHS-MS.

21 chromosomes.

Three putative QTLs associated with GI and PHS were detected as summarized in Table 3 and Fig. 3. Two significant QTLs associated with GI, *Xbarc12* and *Xgwm3*,

were detected and mapped on chromosomes 3A and 3D with LOD scores of 4.95 and 4.02, respectively. Only *Xbarc12* was associated with PHS with LOD score of 10.43. *Xbarc12* explained the highest phenotypic variation

Table 3. QTL for pre-harvest sprouting resistance detected using composite interval mapping in 122 DH population derived from the cross of white × red seeded cultivar, cvs. Keumkang and Olgeuru.

Traits	Marker	Chromosome	LOD ^{z)}	R ² (%) ^{y)}	Additive effect ^{x)}
GI	<i>Xbarc12</i>	3A	4.95	11.99***	-0.12
	<i>Xgwm3</i>	3D	4.02	9.10**	0.11
PHS-MS (%)	<i>Xbarc12</i>	3A	10.43	27.25***	-14.38

^{z)}The logarithm of the odds.

^{y)}The ratio of phenotypic variation explained (R^2) explained by individual QTL. The mean of each QTL was compared by one-way ANOVA, where ** and *** are levels of significance at $P \leq 0.01$ and $P \leq 0.001$, respectively.

^{x)}Additive effect of allele substitution. The units are those of the corresponding traits. A positive sign indicated that the Keumkang allele increased the quantitative trait, and vice-versa.

Table 4. Percentage of pre-harvest sprouting induced by the mist spray (PHS-MS) and germination index (GI) of homozygous recombinants among *Xbarc12*, and *Xgwm3* in 25 Korean wheat cultivars.

Cultivar	Genotype ^{z)}		PHS-MS (%)	GI
	<i>Xbarc12</i>	<i>Xgwm3</i>		
Alchan	<i>b</i>	<i>a</i>	2.17	0.07
Anbaek	<i>b</i>	<i>b</i>	25.93	0.11
Baekjoong	<i>b</i>	<i>b</i>	41.74	0.55
Cheonggkye	<i>a</i>	<i>a</i>	5.96	0.03
Dahong	<i>a</i>	<i>a</i>	1.37	0.05
Eunpa	<i>b</i>	<i>a</i>	18.44	0.26
Geuru	<i>b</i>	<i>b</i>	25.87	0.26
Gobun	<i>b</i>	<i>a</i>	19.30	0.13
Jeokjoong	<i>a</i>	<i>b</i>	48.79	0.59
Jinpoom	<i>a</i>	<i>a</i>	4.99	0.12
Joeun	<i>b</i>	<i>b</i>	14.48	0.38
Jokyung	<i>a</i>	<i>a</i>	86.75	0.43
Jonong	<i>a</i>	<i>b</i>	11.41	0.08
Jopoom	<i>b</i>	<i>a</i>	17.60	0.12
Keumkang	<i>a</i>	<i>a</i>	30.20	0.41
Milsung	<i>a</i>	<i>a</i>	3.14	0.05
Namhae	<i>a</i>	<i>a</i>	4.19	0.12
Ol	<i>b</i>	<i>a</i>	3.31	0.73
Olgeuru	<i>b</i>	<i>b</i>	2.80	0.17
Saeol	<i>a</i>	<i>a</i>	14.00	0.06
Seodun	<i>a</i>	<i>a</i>	43.20	0.35
Sukang	<i>b</i>	<i>b</i>	1.72	0.03
Tapdong	<i>b</i>	<i>a</i>	6.09	0.08
Uri	<i>a</i>	<i>a</i>	2.12	0.05
Younbaek	<i>a</i>	<i>b</i>	80.44	0.60
LSD ^{y)}			0.88	0.06

^{z)}*a* and *b* represent homozygous to Keumkang and Olgeuru genotypes, respectively.

^{y)}Least significant difference ($P < 0.05$).

(R^2) in GI and PHS, accounting for 11.99% and 27.25%, respectively. *Xgwm3* explained 9.10% of phenotypic variation in GI. Negative additive effects of *Xbarc12* were -0.12 for GI and -14.38 for PHS-MS, respectively, with Olgeuru alleles causing 12% reduction in GI and 14% lower PHS-MS. Keumkang alleles, on the otherhand, tended to increase GI by 11% at *Xgwm3*.

Validation for PHS and GI of 25 Korean wheat cultivars using two QTLs, *Xbarc12* and *Xgwm3* is summarized in Table 4 and Fig. 4. The PHS-MS and GI in Korean wheat cultivars ranged from 1.37% to 86.75% and from 0.03 to 0.73, respectively. Korean wheat cultivars carrying Keumkang and Olgeuru alleles at *Xbarc12* and *Xgwm3* loci showed higher percentage of PHS-MS than others (Fig.4-B). On the contrary, Olgeuru and Keumkang alleles at respective *Xbarc12* and *Xgwm3* loci showed lower percentage of PHS-MS than others.

DISCUSSION

PHS is the germination of seeds while still on plants when rainfall and high humidity prevail before harvest. PHS has resulted in yield losses and decreased end-used quality of wheat around the world (Lin *et al.* 2009). PHS is mainly influenced by seed dormancy and complex traits affected by environmental factors (Chang *et al.* 2010; Singh and Singh 2012). Seed dormancy evaluated by germination index (GI) is generally used to predict PHS resistance because GI is more stable with regard to growing condition including high temperature and drought during the grain filling stage (Gubler *et al.* 2005; Shorter *et al.* 2005; Chang *et al.* 2010). In Korea, the percentage of PHS rate increased up to 30% due to climate change especially during monsoon season. Therefore, resistance to PHS is an important breeding target trait to improve the wheat

Fig. 4. PCR products produced from QIAxcel system of two QTLs, *Xbarc12* and *Xgwm3* (A) and difference of percentage of pre-harvest sprouting induced by the mist spray (PHS-MS) in Korean wheat cultivars (B). 1, Alchan; 2, Anbaek; 3, Baekjoong; 4, Cheongkye; 5, Dahong; 6, Eunpa; 7, Geuru; 8, Gobun; 9, Jeokjoong; 10, Jinpoom; 11, Joeun; 12, Jokyeung; 13, Jonong; 14, Jopoom; 15, Keumkang; 16, Milseong; 17, Namhae; 18, Ol; 19, Olgeuru; 20, Saeol; 21, Seodun; 22, Sukang; 23, Tapdong; 24, Uri; 25, Younbaek. *a* and *b* represent homozygous to Keumkang and Olgeuru genotypes, respectively, and QTLs order is *Xbarc12* and *Xgwm3*. Error bar denotes standard error. Different letters, *a* and *b*, indicate significant difference at $P < 0.05$.

production in Korea.

The relationship between grain color and PHS resistant was reported in previous studies (Flintham *et al.* 1999; Mares *et al.* 2005; Mori *et al.* 2005). Red grain color controlled by R gene which located on the long arms of chromosomes 3A, 3B and 3D is associated with coat-imposed dormancy. Red-grained wheat is usually more resistant to PHS than white-grained wheat (Flintham 2000; Croos *et al.* 2002; Liu *et al.* 2013). Of the 122 DH lines, 24 had white seed coat and 98 showed red color. The red-grained DH lines showed lower GI value (0.15) than white-grained (0.30), however there was no significant difference in PHS-MS according to the grain color. However, PHS-MS correlated positively with GI in DH population ($r = 0.732$, $P < 0.001$, Fig. 2B). Shin *et al.* (2012) also reported that white-grained Korean wheat cultivars have higher flour yields than red grains. But white-grained Korean wheat cultivars were susceptible to PHS than red-grained wheat cultivars. Thus, improvement of PHS resistance in white-grained wheat is important in Korean wheat breeding programs.

PHS has been proved to be controlled by multigenes or quantitative trait loci (QTLs) on chromosomes 2, 3, 4, 6, and 7 from different wheat mapping populations (Flintham 2000; Groos *et al.* 2002; Chen *et al.* 2008; Ogonnaya *et al.* 2008; Depauw *et al.* 2009; Chang *et al.* 2011; Liu *et al.* 2013). Major QTLs on chromosomes 2B, 3A, and 4A have demonstrated major effects on PHS resistance (Mares *et al.* 2005; Liu *et al.* 2008; Munkvold *et al.* 2009; Liu *et al.* 2011). Chang *et al.* (2010) reported six variant alleles of *Vp-1B* depending on PHS resistance in Chinese wheat varieties. The two markers, *ZXQ118* and *Xhbe3* on chromosome 4A, were successfully validated on each country's wheat lines (Torada *et al.* 2008; Zhang *et al.* 2008). But, *Vp-1B* and SSR markers did not correlate with PHS-MS and GI in Korean wheat varieties (Kim *et al.* 2012). Groos *et al.* (2002) detected that QTLs for grain color located on chromosomes 3A, 3B and 3D included QTLs for seed dormancy (Osa *et al.* 2003). Also, Liu *et al.* (2013) reported that a QTL on chromosome 3A for *TaPHS1*, which is a homolog of flowering time-like gene, is a PHS resistance-linked gene. Linkage analysis of the 140 segregating markers positively correlated with two

markers, *Xbarc12* and *Xgwm3*, on chromosomes 3A and 3D. *Xbarc12* and *Xgwm3* explained the highest amount of phenotypic variation (R^2) in GI and PHS. These markers will be further fine-mapped to explore their potential uses in Korean wheat breeding programs.

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