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ORIGINAL PAPER

Fine mapping of a preharvest sprouting QTL interval on chromosome 2B in white wheat

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Abstract

Key message **Fine mapping by recombinant backcross populations revealed that a preharvest sprouting QTL on 2B contained two QTLs linked in coupling with different effects on the phenotype.**

Abstract Wheat preharvest sprouting (PHS) occurs when grain germinates on the plant before harvest, resulting in reduced grain quality. Previous mapping of quantitative trait locus (QTL) revealed a major PHS QTL, *QPhs.cnl-2B.1*, located on chromosome 2B significant in 16 environments that explained from 5 to 31 % of the phenotypic variation. The objective of this project was to fine map the *QPhs.cnl-2B.1* interval. Fine mapping was carried out in recombinant backcross populations (BC_1F_4 and BC_1F_5) that were developed by backcrossing selected doubled haploids to a recurrent parent and self-pollinating the BC_1F_4 and BC_1F_5 generations. In each generation, three markers in the *QPhs.cnl-2B.1* interval were used to screen for recombinants. Fine mapping revealed that the *QPhs.cnl-2B.1* interval contained two PHS QTLs linked in coupling. The distal PHS QTL, located between Wmc453c and Barc55, contributed 8 % of the phenotypic variation and also co-located with a major seed dormancy QTL determined by germination index. The proximal PHS QTL, between Wmc474 and CNL415-rCDPK, contributed 16 % of the variation. Several candidate genes including Mg-chelatase H subunit family protein, GTP-binding protein and calmodulin/Ca^{2+}-dependent protein kinase were linked to the PHS QTL. Although many recombinant lines were identified, the lack of polymorphism for markers in the QTL interval prevented the localization of the recombination breakpoints and identification of the gene underlying the phenotype.

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Introduction

Seed dormancy (SD) in wheat (*Triticum aestivum* L.) is an important agronomic trait. Lack of SD at harvest time results in preharvest sprouting (PHS). PHS occurs when seeds germinate on the spike before harvest. Prolonged rainfall and high humidity contribute to this preharvest germination. The main effects of PHS are a lower yield due to harvest losses and, more importantly, a reduction in end-product quality. Resistance to PHS is a quantitative trait that is affected by genotype, environment and genotype by environment interaction (Anderson et al. 1993; Zanetti et al. 2000; Imtiaz et al. 2008). Consequently, PHS resistance is difficult to achieve because of the influence of environmental conditions during the harvest season.

Fine mapping has been used to narrow the quantitative trait locus (QTL) intervals sufficiently for physical mapping and map-based cloning procedures and to determine if a QTL has pleiotropic effects. It also helps to identify precise markers for marker-assisted selection (MAS). In tomato, fine mapping in selected overlapping recombinants narrowed the QTL region contributing to agronomic traits to as little as 3 cM and separated the phenotypic effect of closely linked QTL from pleiotropic effects (Paterson et al. 1990). In rice, studies have reported that the significant correlation between heading date and SD might be due either to a pleiotropic effect of one gene, or two tightly linked genes (Lin et al. 1998). However, in a later study, fine mapping indicated that the correlation between heading date and SD QTLs on rice chromosome 3 was due to linkage (Takeuchi et al. 2003). In durum wheat, fine mapping provided a more precise location of grain protein content (GPC) within the QTL interval on chromosome 6B (Olmos et al. 2003). This was accomplished by genotyping of new markers in recombinant substitution lines (RSLs) containing additional crossover events in the region. Five candidate genes were identified within a collinear 64 kb region of rice chromosome 2 (Distelfeld et al. 2004). Later, in this interval, a single gene was found to have pleiotropic effects on grain protein, zinc and iron content (Uauy et al. 2006).

For fine mapping to be successful, markers must saturate the QTL region. Comparative mapping among grass species can be used as a tool to develop new markers for fine mapping in wheat. Collinearity between wheat, barley and rice over a 52 kb region was used to increase density of markers at the *SKr* gene region controlling crossability between wheat and rye and reduced the genetic interval to a 0.3 cM region that was small enough for a physical map to be constructed based on a 400 kb wheat BAC contig on chromosome 5BS (Alfares et al. 2009). The markers that were developed located the *SKr* gene region close to six genes including *GSP-1* (Grain softness protein). In another fine mapping study of wheat, the grain weight QTL (*gw1*) on chromosome 7D, explained 84.7 % of phenotypic variation (Röder et al. 2008). The interval was narrowed to 7.6 cM and cosegregated with a plant height reducing locus (*Rht),* which explained 70.7 % of the phenotypic variation. However, it was not possible to determine if it was a case of pleiotropy or closely linked genes. A SD QTL interval on rice chromosome 12 was narrowed to less than 75 kb, containing three genes encoding PIL5 (phytochrome-interacting factor 3-like 5), hypothetic protein, and bHLH (basic helix loop helix) DNA-binding domain containing protein (Gu et al. 2009).

In addition to dense markers, appropriate populations for fine mapping must be used for the trait to be mapped as a single Mendelian factor if cloning is to be achieved. The population should contain enough break points in recombinant lines and the recombinant population size should be large enough for sufficient statistical power to detect associations between the trait and the markers. The population can be recombinant inbred lines (RILs), nearly isogenic lines (NILs), advanced backcrossed lines or chromosome segment substitution lines (CSSLs). Nearly isogenic lines have been used to narrow down a QTL interval. Examples include a major fruit weight QTL in tomato (Alpert and Tanksley 1996), a SD QTL (*SD1*) in barley (Han et al. 1999), a heading date QTL (*Hd6*) in rice (Yamamoto et al. 2000), a grain weight QTL (*gw3.1*) in rice (Li et al. 2004), a SD QTL (*Sdr4*) in rice (Sugimoto et al. 2010), and a PHS QTL (*TaPHS1*) in wheat (Liu et al. 2013). NILs have the advantage of a uniform genetic background except in the targeted region. However, population development is very time-consuming and many individuals are needed. For example, to have a 95 % recovery rate of the target region, a 0.1 cM interval would require approximately 3,000 individuals (Tanksley 1993). Alternatively, advanced backcrossed lines could decrease the time for development of genetic population. The populations such as BC_3 and BC_4 have been used successfully in fine mapping studies of rice (Yamamoto et al. 1998; Takeuchi et al. 2003), maize (Vladutu et al. 1999), soybean (Nichols et al. 2006), wheat (Röder et al. 2008), and tomato (Paterson et al. 1990). However, one disadvantage of advanced backcross populations is that they have a less uniform genetic background than NILs.

QTL studies of PHS and SD phenotypes revealed genetic regions contributing to these traits. The PHS and SD QTLs coincided in many studies, especially the major SD and PHS QTLs for both traits (Mares et al. 2005; Tan et al. 2006; Ogbonnaya et al. 2008; Munkvold et al. 2009; Kulwal et al. 2010). In previous work, PHS QTL mapping utilized a doubled haploid (DH) white winter wheat population from a cross between Cayuga and Caledonia.

Approximately, 15 different PHS QTLs, located across the wheat genome, were detected at $p < 0.05$ in at least one environment (Munkvold et al. 2009). The major QTLs were located on chromosomes 2B, 2D, 3D and 6D and were significant ($p < 0.01$) in four or more environments. All PHS QTL resistance alleles were contributed by Cayuga. The PHS QTL on 2B, *QPhs.cnl-2B.1*, was significant ($p < 0.01$) in all 16 environments and explained approximately 24 % of the phenotypic variation. The QTL interval was broad, ranging from 2 to 31 cM across environments. The QTL peak for the PHS QTL mean from all environments was at 14 cM, between the SSR markers Barc55 and Wmc474 on chromosome 2B (Munkvold et al. 2009). Based on PHS QTL data for 2B.1 on 209 DH lines, the QTL interval covered nearly the entire 2B.1 deletion. The broad QTL interval could result from either a major QTL or multiple closely linked minor QTLs. Fine mapping of PHS QTL requires comparing PHS means of individual recombinants with those of non-recombinants. A high proportion of recombinant individuals can provide the statistical test with sufficient power to resolve multiple linked QTLs. In this study, fine mapping in recombinant populations for the *QPhs.cnl-2B.1* interval was used to determine if there is one or more linked QTLs. Furthermore, using the comparative genomics approach, candidate genes for the PHS QTLs have been identified.

Materials and methods

Development of genetic populations for fine mapping

The fine mapping population was created using selected homozygous recombinant BC_1F_4 and BC_1F_5 lines (Fig. 1; Table 1). They were developed from backcrossing the selected doubled haploids (a cross between Cayuga as the female and Caledonia as the male) to Caledonia and then self-pollinating to the F_4 and F_5 generation. Cayuga is a PHS-resistant variety derived from a cross between Geneva (PHS susceptible) and Clark's Cream (PHS resistant), which was then backcrossed to Geneva. Caledonia is a PHS-susceptible variety selected as an off type from Geneva. Development of the Cayuga × Caledonia doubled haploid population was previously described (Munkvold et al. 2009). The selected DH individuals that contained a high percentage of Caledonia background (63.5–72.6 %), but with the Barc55 allele at *QPhs.cnl-2B.1* coming from Cayuga, were chosen to backcross with Caledonia. The DH #86 line, that was used to produce backcross family 04173, contained 63.5 % Caledonia background. DH #95 was crossed to Caledonia to produce backcross family 04175 contained 64.5 % Caledonia background. The DH #87 had 72.6 % Caledonia background and was used to produce backcross family 04174. One selected DH individual (DH #129) that contained

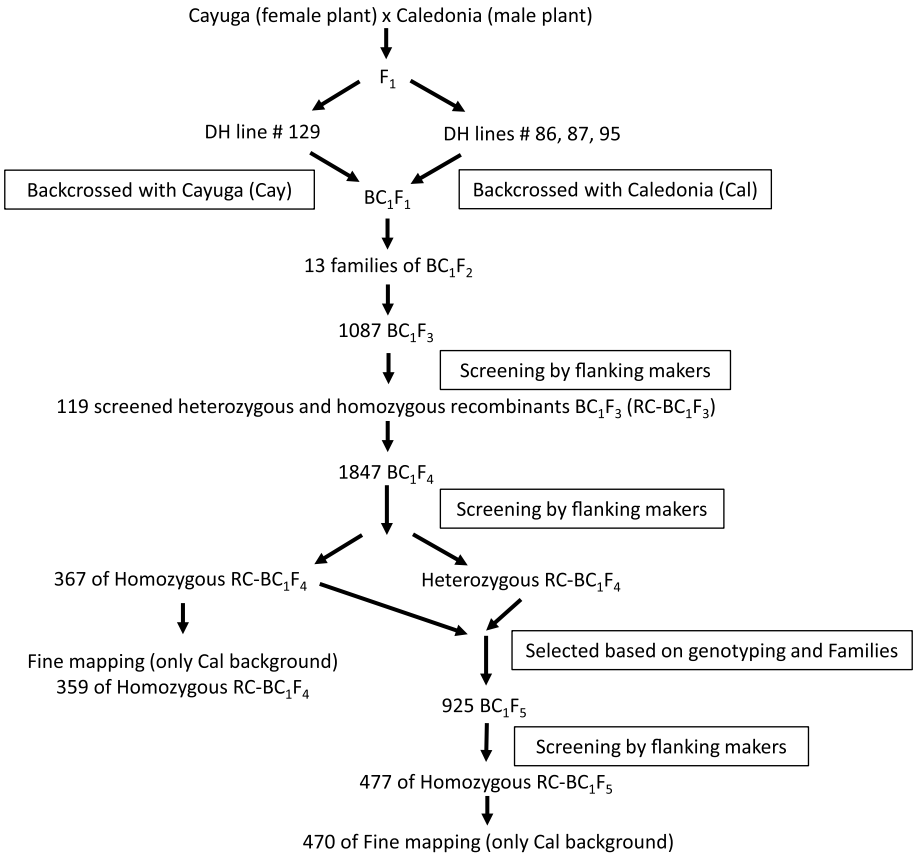


Fig. 1 The populations used for fine mapping were screened for homozygous BC_1F_4 and BC_1F_5 recombinants. The recombinant screening was started at the BC_1F_3 generation by selecting both heterozygous and homozygous recombinants resulting from genotyping with three flanking markers; Gwm429, Barc55 and Wmc474. These recombinants were used to produce 1,087 BC_1F_4 lines. After screening using the flanking markers, 359 BC_1F_4s containing Caledonia background were homozygous recombinants. After narrowing the QTL region in homozygous BC_1F_4 recombinants, more recombinants in BC_1F_5 were needed at the new interval region. After screening with the flanking markers, 470 BC_1F_5s were found to be homozygous recombinants and were used for fine mapping analysis

Table 1 Summary of population sizes of BC_1F_3, BC_1F_4 and BC_1F_5 used in fine mapping population development. The population was developed with both Cayuga and Caledonia background. Only Caledonia background was used for fine mapping

	Initial population Size			Non-recombinants			Heterozygous recombinants		Homozygous recombinants	
	Cayuga background (Cay-BG)	Caledonia background (Cal-BG)	Total	Cay-BG	Cal-BG	Missing	Cay-BG	Cal-BG	Cay-BG	Cal-BG
BC_1F_3	130	957	1087	121	841	6	5[a]	72[a]	4[a]	38[a]
BC_1F_4	131	1716	1847	91	633	18	32	706	8	359[b]
BC_1F_5	10	915	925	0	391	12	3	42	7	470[c]

[a] The heterozygous and homozygous recombinants BC_1F_3 that were used to produce the 1,847 BC_1F_4

[b] The homozygous recombinant BC_1F_4 that was used for fine mapping

[c] The homozygous recombinant BC_1F_5 that was used for fine mapping

60.8 % Cayuga background, but with the Caledonia allele at the Barc55 locus, was crossed with Cayuga to produce backcross family 04176. The BC_1F_1 of the backcrossed DH lines with Caledonia and Cayuga generated 13 BC_1F_2 lines. All 13 lines were grown in the field in separate rows designated 04173-1, 04173-2, 04175-0, 04176-1, 04176-2, 04174-1, 04174-2, 04174-3, 04174-4, 04174-5, 04174-6, 04174-7 and 04174-8. All lines contained Caledonia background, except 04176-1 and 04176-2, which contained Cayuga background. Two spikes of all individual plants in each family were collected to produce 1,087 BC_1F_3 lines that were planted in the fall 2006. All BC_1F_3 lines were screened for heterozygous and homozygous recombinants containing a break point in the *QPhs.cnl-2B.1* interval using three flanking markers, Wmc474, Barc55 and Gwm429. All recombinant plants were used to produce 1,847 BC_1F_4 lines that were planted at two locations; Snyder and Ketola, Ithaca, NY, during the fall of 2007.

For fine mapping of the BC_1F_4 population, homozygous recombinants containing the break point at *QPhs.cnl-2B.1* were selected by screening all 1,847 BC_1F_4 lines, using the three flanking markers. Additional homozygous recombinants were needed in BC_1F_5, so both selected heterozygous and homozygous recombinants were planted at two locations, Snyder and Ketola, in Ithaca, NY, during the fall of 2008. For fine mapping of the BC_1F_5 population, homozygous lines containing a recombination in the *QPhs.cnl-2B.1* interval were selected by screening all 925 BC_1F_5 lines with the same three flanking markers. All BC_1F_4 and BC_1F_5 recombinant lines used for fine mapping contained Caledonia background. The number of recombinants that contained Cayuga background was not sufficient and was not used for further mapping.

DNA isolation

All populations BC_1F_3, BC_1F_4 and BC_1F_5 grown in the field were used for DNA extraction. After planting in the field for 3 weeks, five leaves of different plants in the same row were collected for each line. Approximately, 0.8–1.0 cm of five leaves were placed in each well of a 96-well plate containing 0.25 g of silica gel. The leaf samples were dried for 5–10 days at room temperature and ground using a homogenizer (TALBOYS). The protocol for DNA extraction was modified from general SDS/chloroform: isoamyl alcohol DNA extraction reported by McCouch et al. (1988). After the extraction buffer (0.1 M Tris–HCl pH 7.5, 0.05 M EDTA pH 8, 1.25 % (w/v) SDS) to the sample mixture, it was extracted with chloroform: isoamyl alcohol to remove protein and pigments.

Marker development

Sixteen markers were used for fine mapping, including six SSR markers (Barc55, Wmc474, Gwm429, Cdo64-PCR, Wmc453c, Gwm319) and 10 newly designed primers reported by Somyong et al. (2011) based on ESTs in the *QPhs.cnl-2B.1* interval (CNL406-BE405569, CNL407-BF202468, CNL408-BE500206, CNL409-BE606438, CNL410-BE636824, CNL411-BE498254, CNL412-BF201533, CNL413-BQ294702, CNL414-BE494262 and CNL415-rCDPK) (designed from calcium/calmodulin-dependent protein kinase). Primer sequences of the SSR markers were reported in the GrainGenes database (http://wheat.pw.usda.gov/GG2/index.shtml) except Cdo64 (Forward primer sequence: ggaggggcaagttcacataa and reverse primer sequence: gctgatgttgatggtgatgg).

In addition to the markers mentioned above, primer sets were designed using PCR-based Landmark Unique Gene (PLUG) system (Ishikawa et al. 2007). PLUG markers are EST-PCR markers developed based on the orthologous gene conservation between rice and wheat. Using the similarities in gene structures between the two species, primers are designed so that PCR products include intron sequences. One hundred and twenty primer sets were designed based on homologous rice genes in the targeted QTL region (Table S1).

PCR product detection and homozygous recombinant screening

Approximately, 5–10 pmol of genomic DNA were used for PCR. The annealing temperatures of some markers were different. Barc55 annealed at 55 °C, Wmc474 at 56 °C, Gwm429 at 50 °C, Cdo64 at 56 °C, Wmc453c at 61 °C, and Gwm319 at 55 °C. The PCR products were detected on a 4 % polyacrylamide (PAGE) gel, except the three flanking markers which were detected with fluorescent labeling. Nine PCR products amplified by seven newly designed markers were also detected on a 10 % single-strand conformation polymorphism (SSCP) gel for 16–19 h, depending on the PCR product size. The annealing temperature of newly designed markers and the types of detection gels were described by Somyong et al. (2011). There were three types of PCR machines used. MJ Research PTC-225 Peltier Thermal Cycler, BIO-RAD C1000^{TM} Thermal Cycler for 384 well plates and BIO-RAD MyCycler^{TM} Thermal cycler for 96 well plates.

Fluorescent labeling was used only for screening homozygous recombinants. Three co-dominant markers, Barc55, Wmc474 and Gwm429, were used to screen heterozygous and homozygous recombinants. There were 1,087 lines screened from the BC_1F_3 population, by selecting heterozygous and homozygous recombinants, using the Barc55 marker with an additional 18 base-pair M13 sequence (5′-TGTAAAACGACGGCCAGT-3′) at the 5′ end of the Barc55 forward primer (Barc55F-M13). The PCR reaction and condition for M13 labeled markers were described by Schuelke (2000). The PCR cycles were 30 cycles of normal PCR and 7 additional cycles at 53 °C annealing temperature for dye binding. For BC_1F_3 genotype screening, the PCR products amplified by Wmc474 and Gwm429 were run on a 4 % PAGE gel. For the 1,847 BC_1F_4 and 925 BC_1F_5 lines screening, all three flanking markers were labeled directly, each with a different fluorescence color. The DNA fragments that were amplified from both markers labeled with M13 and directly labeled fluorescence markers were analyzed using an ABI 3730 fluorescence-detection system at Cornell University Life Science Core Laboratories Center. The results were visualized on Peak Scanner ^{TM} Software v.10 (http://marketing.appliedbiosystems.com/mk/get/PS1_login). After screening with the three flanking markers, the homozygous recombinants were used in genotyping for fine mapping.

For PLUG markers, PCR was used to amplify genomic DNA of Cayuga and Caledonia. Each 25-μL PCR mixture included either 50–100 ng of DNA, 0.2 pmol of each primer, 0.2 mM dNTP (each), 1 × standard buffer, and 0.5 U of *Taq* polymerase (New England BioLabs Inc., Ipswich, MA, USA). The PCR cycle consisted of an initial 5 min denaturation at 95 °C, followed by 32 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for one min, followed by a final extension at 72 °C for seven min. DNA amplification was performed using a BIO-RAD MyCycler^{TM} Thermal cycler for 96-well plates. An 8-μL aliquot of the amplification product was analyzed by electrophoresis on a 2 % agarose gel in TBE buffer. For PCR–RFLP analysis, an 8-μL aliquot of the product was digested overnight with 1.0 U of *Hae*III or *Taq*I in incubators set at 37 or 65 °C, respectively. Digested fragments were fractionated by electrophoresis on a 2 % agarose gel in TBE buffer. Band sizes were estimated using a '1 kb DNA Ladder' (Invitrogen, Carlsbad, CA, USA). Polymorphic markers were genotyped using a 209 DH population for mapping (Munkvold et al. 2009). To narrow the region of the QTL, a marker TNAC9025 was genotyped using 456 BC_1F_5 lines.

PHS and SD phenotyping

For PHS phenotyping, five spikes were sampled on the same day for BC_1F_2, BC_1F_3 and BC_1F_4, at each location. Because of climatic conditions, the BC_1F_5 spikes had a wider range in maturity, thus requiring two harvests on different days in each location. Three additional spikes were collected for a BC_1F_4 germination test. The spikes were determined to be at physiological maturity by loss of green color from the glumes. The spikes were dried indoors for 5 days at ambient humidity and temperature. After drying for 5 days, the phenotyping was conducted as described by Anderson et al. (1993). Peduncles were cut to around 8 cm and spikes were inserted upright into pre-drilled holes in foam blocks. A fine mist of water was applied over the spikes every 30 s throughout the experiment. After 4 days under simulated rainfall, individual spikes were rated on a scale of 0 (no evidence of sprouting) to 9 (extensive sprouting throughout the spike). The rating takes into account both the number of sprouted kernels observed and the rate at which they germinate. PHS scores for individual plots were calculated by averaging the scores for the five spikes. For germination testing, three additional spikes were dried for 5 days then stored at −20 °C to preserve dormancy until the germination test was conducted. The germination test was used to measure SD in 359 BC_1F_4 recombinants at one location, Ketola 2008. The spikes were stored at −20 °C for 8 months. The primary and secondary seeds from the middle two-thirds of the spike were used for the germination test. Seeds having black point, disease, or shriveling were discarded. Twenty seeds from each spike were sterilized by soaking in 10 % Clorox® bleach and rinsing with sterile water five times. The sterilized seeds were placed crease-down on a 9-cm diameter germination paper soaked with three ml of sterilized water in Petri plates. The plates were placed in plastic bags to retain moisture and stored

at 22 °C in the dark. Germinated seeds were evaluated every 24 h for 10 days by looking at radicle protrusion. Each day the number of germinated seeds was counted as they were discarded. The seeds contaminated with fungus during germination were discarded and excluded from calculations. The germination test consisted of three replicates, each with seed from a different spike. The germination index (GI) was calculated by the following method: $GI = (10 \times g_1 + 9 \times g_2 + \ldots\ldots + 1 \times g_{10})/(\#$ of viable seeds), where g is the number of seeds that germinated on the given day (Munkvold et al. 2009).

Fine mapping analysis

Statistical data analysis

PHS scores were compared for non-recombinant lines, containing Barc55 and flanking markers for Cayuga and Caledonia alleles at the *QPhs.cnl-2B.1* interval. Also, homozygous recombinant lines containing different recombination break points at the *QPhs.cnl-2B.1* interval were evaluated for PHS. The *t* test of the JMP statistical software package (http://www.jmp.com/) was used to determine the level of significance and to determine phenotype distribution of recombinants that were used for fine mapping.

Linkage map construction

All markers used in this project were located in the same *QPhs.cnl-2B.1* interval based on linkage analysis in the Cayuga × Caledonia population (Munkvold et al. 2009). MapManager QTXb20 (Manly et al. 2001) and MapDisto v.1.7.5 (Lorieux 2012) were used to determine the marker order and a new linkage map was created using the Kosambi mapping function, and linkage threshold significance of $p < 0.001$.

QTL analysis

The QTL analyses used the software package Windows QTL Cartographer (Version 2.5 http://statgen.ncsu.edu/qtlcart/WQTLCart.htm) to evaluate the new PHS QTL data from BC_1F_4 recombinants. For QTL Cartographer, composite interval mapping (CIM) with model 6 was used. Precise selection walking speed (cM) was 0.5. The significance threshold was calculated at $p < 0.01$ by 1,000 permutations.

Results

Development of screening populations for fine mapping

After first backcrossing the selected DH individuals to Caledonia and Cayuga (BC_1), the background of the BC_1 lines in this project contained more of the recurrent parent genome than an average BC_1, ranging from 81.75 to 86.3 % Caledonia background. This was comparable to a BC_2 individual, which would average 87.5 % of the recurrent parent background. The mean PHS scores of lines in the Caledonia background ranged from 3.52 in family 04174-6 to 5.53 in family 04174-8 with a mean of 4.53. The mean PHS score in the two lines that contained Cayuga background, ranged from 1.94, in family 04176-2, to 2.54, in family 04176-1 with an overall mean of 2.24.

The three flanking markers at *Qphs.cnl-2B.1* were used to genotype the 1,087 BC_1F_3 lines. For the interval between Barc55 and Wmc474, there were 46 recombinant BC_1F_3 lines, 42 of which were in Caledonia background and 4 in Cayuga background. For the interval between Barc55 and Gwm429, there were 70 recombinant BC_1F_3 lines in the Caledonia background, but only five in the Cayuga background. Of those recombinants, two lines were recombinants between both Barc55 and Wmc474 and Barc55 and Gwm429 resulting from double crossovers. In total, 119 BC_1F_3 lines were either heterozygous or homozygous recombinants. To increase recombinants without increasing the size of the population that was used for fine mapping, only heterozygous and homozygous recombinant BC_1F_3 lines were selected with 15 plants per BC_1F_3 to produce 1,847 BC_1F_4 lines. After screening with flanking markers, 1,065 of the 1,847 lines were heterozygous or homozygous recombinants in the Caledonia background but only eight Cayuga lines were homozygous recombinant. Among the Caledonia lines, 706 were heterozygous and 359 were homozygous BC_1F_4 recombinants. Because of the few Cayuga background containing recombinants, only the 359 BC_1F_4 Caledonia background containing lines were used for QTL analysis and fine mapping. For BC_1F_5 development, not all heterozygous and homozygous BC_1F_4 recombinant lines were used. The selection was based on both genotyping and the origin of BC_1F_4 lines. For BC_1F_5 population development, five individual plants of each selected BC_1F_4 line were used. One hundred BC_1F_5 lines out of 706 heterozygous BC_1F_4 recombinants containing Caledonia background were selected. Also, the selection of homozygous lines was based on the fine mapping results of 359 BC_1F_4 recombinants. In summary, 184 lines of both heterozygous and homozygous BC_1F_4 recombinants were used to produce 915 BC_1F_5 lines. After screening for homozygous recombinants, 470 homozygous BC_1F_5 lines containing Caledonia background were used further for fine mapping at the *QPhs.cnl-2B.1* interval (Table 1).

QTL effect on preharvest sprouting and seed dormancy in non-recombinant groups

The PHS or SD QTL effect from the *QPhs.cnl-2B.1* interval was determined by comparing the phenotypic mean

Table 2 PHS mean score and germination index (GI) mean score for seed dormancy in non-recombinant groups at different populations and years

Trait-generation-trial	Parent score			Non-recombinant (NR)		
	Cayuga (cay)	Caledonia (Cal)	Significant *p* value	NR-Cay	NR-Cal	Significant *p* value
PHS-BC_1F_3 -Snyder 2007	3.17	6.17	<0.0001	3.95 ± 0.67	5.39 ± 0.37	<0.0001
PHS-BC_1F_4 -Ketola 2008	2.48 ± 0.92	6.16 ± 0.35	<0.0001	4.0 ± 0.71	4.55 ± 0.86	<0.05
PHS-BC_1F_4 -Snyder 2008	1.64 ± 0.58	5.42 ± 1.06	<0.0001	4.17 ± 1.53	4.73 ± 1.52	NS (0.18)
PHS-BC_1F_5 -Ketola 2009	0.93 ± 0.78	3.27 ± 0.85	<0.01	1.90 ± 0.95	3.21 ± 1.88	<0.01
PHS-BC_1F_5 -Snyder 2009	1.03 ± 0.83	5.67 ± 1.26	<0.0001	2.07 ± 1.13	3.89 ± 1.48	<0.0001
SD-BC_1F_4 -Ketola 2008	5.86 ± 0.61	8.14 ± 0.70	<0.0001	6.99 ± 0.62	7.49 ± 0.89	<0.05

Score 0 = no seed germination at day 10, Score 10 means all seed germination at day 1

PHS Preharvest sprouting, scoring from 0 to 9, *0* no sprouting *9* the most sprouting, *SD* Seed dormancy, evaluated by germination index from day 1 to day 10, *NS* Non-significance

between non-recombinant lines containing Cayuga alleles (NR-cay) or Caledonia alleles (NR-cal) in the PHS interval (Table 2). In 2007 (BC_1F_3), the NR-cay mean score was 3.95 compared to the NR-cal mean score of 5.39 ($p < 0.0001$). The NR-cay family mean was higher than the Cayuga parent (3.17) and the NR-cal family mean score was lower than the Caledonia parent (6.17). In 2008 (BC_1F_4), the mean PHS scores for 20 non-recombinant BC_1F_4 lines in each group were compared at two locations, Ketola and Snyder. At the Ketola location, the NR-cay mean score was 4.0 versus 4.55 for the NR-cal ($p < 0.05$). At Snyder, the NR-cay mean score was 4.17 versus 4.73 for the NR-cal (non-significant). In 2009 (BC_1F_5), the mean PHS score of 19 non-recombinant BC_1F_5 lines in each group was significant at both Ketola ($p < 0.01$) and Snyder ($p < 0.0001$). At Ketola, the NR-cay mean score was 1.90 whereas the NR-cal mean score was 3.21. At Snyder, the NR-cay mean score was 2.07 whereas the NR-cal mean score was 3.89.

The germination index (GI) mean score of both parents was significantly different ($p < 0.0001$). Seed germination of Caledonia began on Day 1 with an average of 15 % germination whereas Cayuga began on Day 3 with an average of 12 % germination. The highest percent germination of Caledonia was on Day 2 (30 % germination), whereas the highest percent germination for Cayuga was on Day 5 (30 % germination). After Day 5, more than 95 % of seeds from Caledonia had germinated whereas Cayuga exceeded 95 % after Day 8. Mean GI score for Cayuga was 5.86 versus 8.14 for Caledonia ($p < 0.0001$). Mean GI scores for NR-cay and NR-cal were 6.99 and 7.49, respectively ($p < 0.05$). The level of significance for both PHS and SD effects was similar at the Ketola location.

QTL effect on preharvest sprouting and seed dormancy in BC_1F_4 recombinants

The PHS score and germination index for the 359 BC_1F_4 recombinants in the Caledonia background were significant (Fig. 2). The range of PHS phenotypic values at Snyder 2008 was approximately 20 % greater than at Ketola 2008. The Goodness-of-Fit Tests for the PHS scores within each environment were significant indicating that they were not normally distributed, while the distributions of PHS means and germination indices were normal (Fig. 2).

Linkage map analysis resulted in 15 out of 16 markers being separated (Fig. 3). Because only 359 recombinants were used for QTL analysis, the genetic distance was normalized with 633 BC_1F_4 non-recombinants (Table 1). The 2B.1 linkage for the QTL interval in the BC_1F_4 population was around 20 cM, 10 cM greater than the linkage distance in the DH population (Munkvold et al. 2009). The average marker distance was one marker per 0.91–1.32 cM. Based on comparisons between markers CNL407-BF202468 and CNL409-BE606438 and markers CNL411-BE498254 and CNL414-BE494262 and their location on the rice physical map, there was about 120–128 kb of rice physical map distance per one wheat cM.

Linkage analysis revealed two PHS QTLs located in the *QPhs.cnl-2B.1* interval, both contributed by Cayuga (Fig. 3). The distal PHS QTL was between markers Wmc453c and Barc55 in a 4.2 cM interval. This QTL was significant for Ketola 2008, for the mean PHS at Ketola and Snyder 2008 and for SD at Ketola 2008. LOD scores ranged from 3.10 to 6.19 ($p < 0.01$). The second (proximal) PHS QTL was between Wmc474 and CNL415-rCDPK and was also significant for

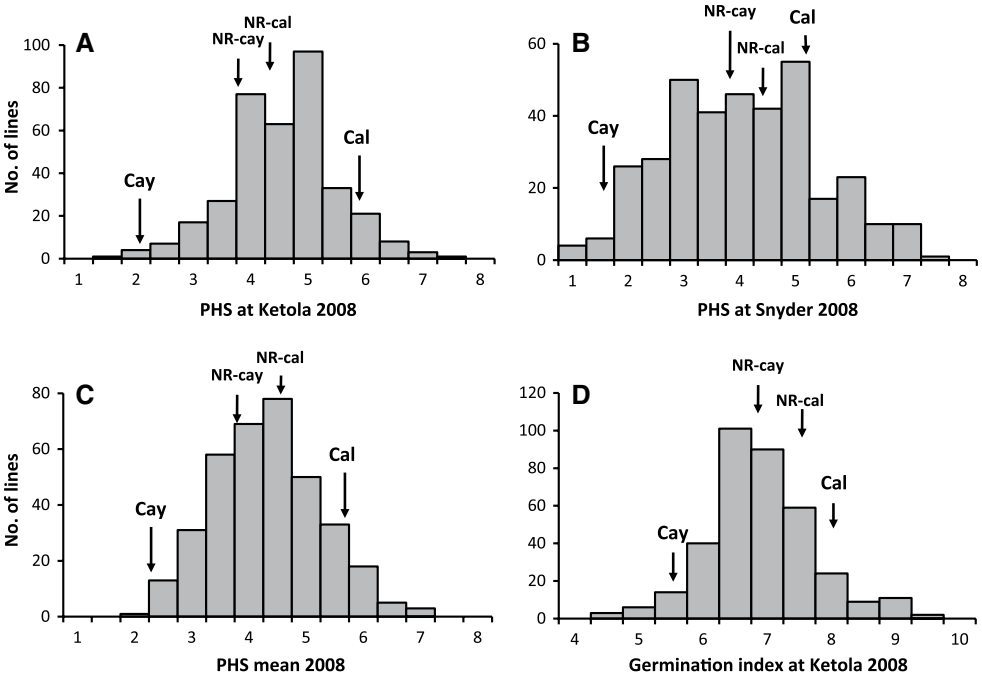


Fig. 2 PHS phenotype distribution and germination index of 359 recombinant BC_1F_4 at Ketola 2008 (**a**, **d**) and Snyder 2008 (**b**) and PHS mean of the two environments (**c**). Cay = Cayuga, Cal = Caledonia, NR-cay = non-recombinant containing Cayuga at *QPhs.cnl-2B.1* interval. NR-cal = non-recombinant containing Caledonia at *QPhs.cnl-2B.1* interval

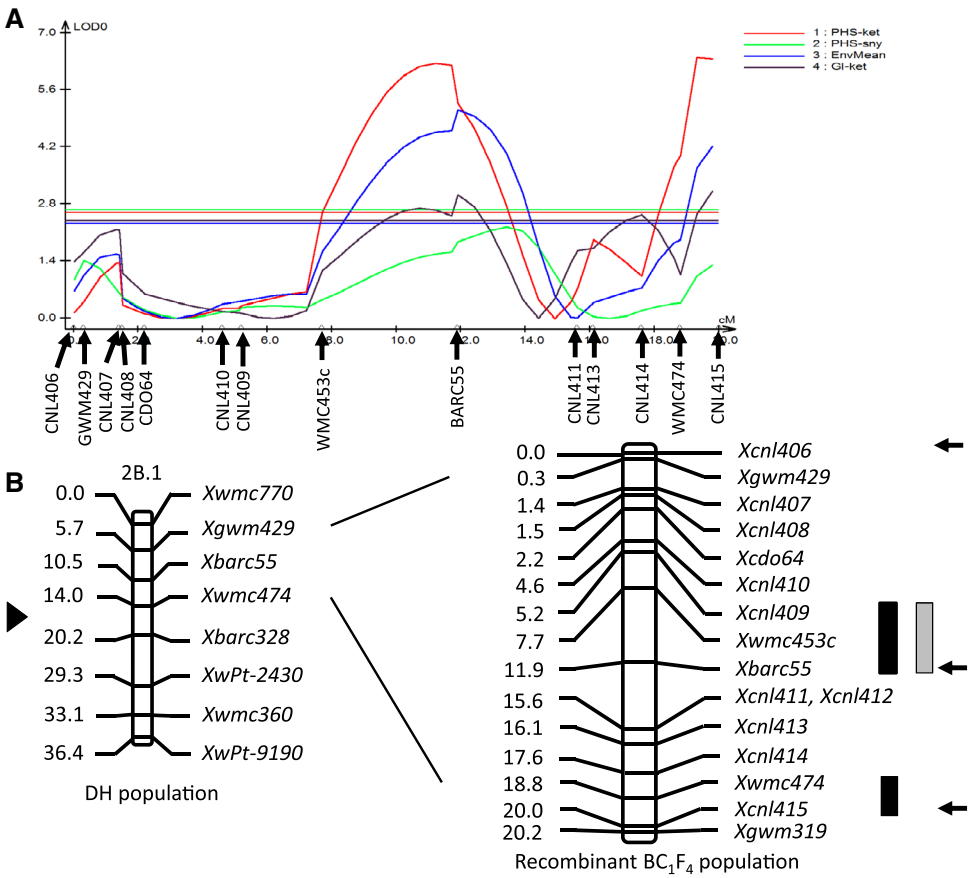
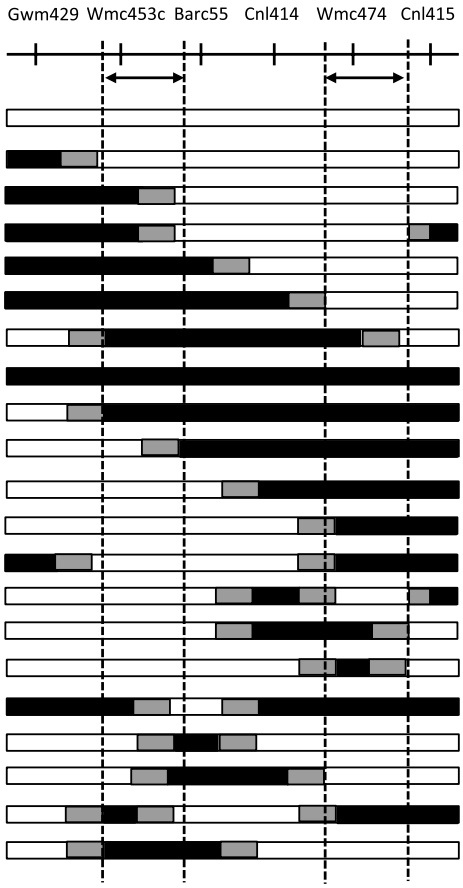


Fig. 3 QTL analysis in recombinant BC_1F_4 results (**a**) and the genetic distance after normalization with non-recombinant BC_1F_4 (**b**). The major PHS QTL, *QPhs.cnl-2B.1*, was narrowed down to the Wmc453c to Barc55, and Wmc474 to CNL415-rCDPK marker intervals, both contributed from the Cayuga parent. Two significant PHS QTLs were identified in two environments whereas SD QTL tested in one environment was co-located between marker Wmc453c and Barc55. ◗ = Putative position of centromere, ▮ = PHS, ▮ = SD, → = Location of flanking markers



		Ketola09		Snyder09		Mean09	
RC-group	RC # (491)	ME±SD	P-value	ME±SD	P-value	ME±SD	P-value
gr.01-111111	16	3.21±1.88	NS	3.89±1.48	NS	3.55±1.38	NS
gr.02-011111	33	3.41±1.06	NS	4.08±0.91	NS	3.75±0.74	NS
gr.03-001111	53	2.85±1.18	0.0486	3.43±1.27	0.0113	3.15±0.93	0.0059
gr.04-001110	12	2.71±1.27	NS	3.42±1.16	NS	3.06±1.15	0.0383
gr.05-000111	120	2.89±1.22	0.0394	3.42±1.18	0.0035	3.16±1.00	0.0023
gr.06-000011	35	3.03±1.23	NS	3.41±0.90	0.018	3.22±0.82	0.0264
gr.07-100001	4	1.75±0.91	0.0138	2.55±0.64	0.0123	2.15±0.49	0.0021
gr.08-000000	19	1.90±0.95	<.0001	2.07±1.13	<.0001	1.98±0.65	<.0001
gr.09-100000	15	1.69±1.41	<.0001	2.73±1.34	0.0002	2.21±1.17	<.0001
gr.10-110000	22	2.88±1.63	NS	2.84±1.33	<.0001	2.85±1.25	0.001
gr.11-111000	72	2.23±1.20	<.0001	3.28±1.09	0.0009	2.75±0.91	<.0001
gr.12-111100	22	2.29±1.36	0.0014	3.37±0.90	0.0255	2.83±0.93	0.0007
gr.13-011100	3	2.67±0.23	NS	3.12±1.14	NS	2.89±0.66	NS
gr.14-111010	3	2.40±0.92	NS	4.27±1.53	NS	3.33±1.07	NS
gr.15-111001	12	1.60±1.75	<.0001	1.82±1.60	<.0001	1.70±1.50	<.0001
gr.16-111101	6	1.96±1.17	0.0177	2.27±1.01	0.0004	2.15±1.03	0.0002
gr.17-001000	4	1.50±0.50	0.0047	1.35±1.02	<.0001	1.43±0.69	<.0001
gr.18-110111	16	3.26±1.49	NS	3.78±0.88	NS	3.52±0.89	NS
gr.19-110011	10	2.91±1.49	NS	3.78±0.92	NS	3.31±0.96	NS
gr.20-101100	4	2.75±0.34	NS	2.60±1.34	0.0154	2.68±0.80	0.0382
gr.21-100111	10	3.22±0.92	NS	4.16±1.36	NS	3.69±0.76	NS

Fig. 4 Fine mapping at *QPhs.cnl-2B.1* in selected homozygous recombinant BC_1F_5. Six markers were used in fine mapping genotyping. The order of markers was based on the marker order of the double haploid and selected recombinant BC_1F_4 genotypes from Gwm429 to CNL415-rCDPK. There were 19 recombinant groups (RC) (456 lines) and two non-recombinant groups (35 lines) for Caledonia (NR-cal, gr.01) and Cayuga (NR-cay, gr.08). PHS scores were evaluated in two environments, Ketola09 and Snyder09, and the mean between these two environments (Mean09). Two QTLs were identified in the *QPhs.cnl-2B.1* interval. The distal QTL was located between marker Wmc453c and Barc55, significant in two environments and also mean09. The proximal QTL region was between Wmc474 and CNL415-rCDPK, significant in two environments and also mean09. RC # = the number of recombinants; ME ± SD = PHS score mean ± standard deviation; *P* value = the significance value of each recombinant group, compared with a recombinant group that has no PHS-affecting region (gr.02); NS = Non-significant *p* value; gr. = recombinant group; Two regions between the *vertical dotted lines* on the figure represent PHS QTL regions, *Empty bar* Caledonia (PHS-susceptible parent) region, *Black bar* Cayuga (PHS-resistant parent) region, *Gray bar* Cayuga (PHS-resistant parent) region without knowing the boundary region

Ketola 2008 and for the mean PHS at Ketola and Snyder 2008. Marker CNL415-rCDPK was designed from the transcript for a calmodulin/Ca^{2+}-dependent protein kinase gene. The QTL LOD scores were 3.26 and 8.07 between markers Wmc474 and CNL415-rCDPK ($p < 0.01$). This result was confirmed in fine mapping of selected BC_1F_5 recombinants. The high QTL LOD score (8.07) between markers Wmc474 and CNL415-rCDPK suggested that the PHS effect in this interval was greater than between Wmc453c and Barc55.

Fine mapping of preharvest sprouting QTL in BC_1F_5 recombinants

Fine mapping analyses in BC_1F_5 were performed using six markers (Fig. 4). Screening with the three flanking markers identified 470 BC_1F_5 homozygous lines but only 456 BC_1F_5 lines were used in fine mapping because of missing data for Wmc453c or CNL415-rCDPK. The 456 recombinant BC_1F_5 lines and non-recombinant lines were grouped to 21 groups (Nineteen recombinant lines and two non-recombinant lines were assigned to groups 1–21) according to their recombination break points at six flanking markers at *QPhs.cnl-2B.1* (Fig. 4). The mean PHS scores of the 21 groups were used to estimate the PHS effects in the PHS QTL interval. PHS scores were evaluated for two environments, Ketola in 2009 (Ketola09), Snyder in 2009 (Snyder09) and the mean (Mean09). The QTL analysis again resolved the two PHS QTLs with the resistance alleles from Cayuga in the *QPhs.cnl-2B.1* interval (Fig. 4) and both were significant in both environments. The distal QTL was

between markers Wmc453c and Barc55 and the proximal QTL was between markers Wmc474 and CNL415-rCDPK.

In addition, nineteen groups of recombinant lines containing different break points were assigned to four categories according to whether they had no PHS QTL, one PHS QTL between Wmc453c and Barc55, one PHS QTL between Wmc474 and CNL415-rCDPK or both PHS QTLs. The recombinant groups in the first category included recombinant groups 02, 14, 18 and 19. These groups had no PHS QTL effect because they were not significantly different from NR-cal (ns). The PHS mean scores ranged from 3.31 to 3.75 based on the Mean09 whereas the mean score for NR-cal lines was 3.55. The recombinant groups in the second category included recombinant groups 03, 04, 05 and 06. These recombinant groups had one PHS QTL between Wmc453c and Barc55. The PHS mean scores ranged from 3.06 to 3.22 and were significantly different from recombinant groups that had no PHS QTL (p value ranged from 0.0023–0.0383) based on Mean09. The recombinant groups in the third category included recombinant groups 10, 11, 12, 15, 16 and 20. These recombinant groups had one PHS QTL between Wmc474 and CNL415-rCDPK with mean PHS scores ranging from 1.70 to 2.85 and were significantly different from recombinant groups that had no PHS QTL ($p < 0.001$) based on Mean09. The recombinant groups in the fourth category included groups 07, 09 and 17. These recombinant groups as well as all NR-cay groups had both of the PHS QTLs. The PHS mean scores of these groups ranged from 1.43 to 2.21 based on Mean09. These groups were compared with group 06, which had one PHS QTL region between Wmc453c and Barc55 and with group 10, which had one PHS QTL region between Wmc474 and CNL415-rCDPK. These groups with two PHS QTL regions were significantly different ($p < 0.01$) from the groups that had only one PHS QTL region. The PHS mean scores suggest that the contribution from the second PHS QTL region, between Wmc474 and CNL415-rCDPK (mean score = 2.67), had a larger effect than the first PHS QTL between markers Wmc453c and Barc55 (mean score = 3.16) and these scores were significantly different ($p < 0.0001$). The PHS QTL region between Wmc474 and CNL415-rCDPK contributed 16 % of the PHS QTL effect, whereas the PHS QTL region, between markers Wmc453c and Barc55 contributed 8 % of PHS QTL effect for the *QPhs.cnl-2B.1* interval. Some recombinant groups that had only one PHS QTL region (group 15 and group 16) between Wmc474 and CNL415-rCDPK had a larger PHS effect ($p < 0.0001$). However, two recombinant groups (group 13 and group 21) should contain one PHS QTL, but their mean scores were not significantly different from the recombinant groups that had no PHS QTL region, possibly because of the small sample size in group 13. Group 21 mean score was 3.69, based on Mean09 suggesting that it might contain the break point between markers Wmc453c and Barc55, resulting in no PHS effect. Interaction plots for the two QTLs were shown in Fig. 5. Effect of proximal QTL (Wmc474) was consistent between the two environments, while that of distal QTL (Barc55) was observed only in Snyder09. For the Snyder09 and Mean09, the distal QTL showed a weak interaction with the proximal QTL. It is possible that there is a combined effect of resistance alleles for the two QTLs that will require further investigation.

Marker TNAC9025 is the closest marker for the distal PHS QTL

Out of 120 PLUG primer sets, five markers were polymorphic among the DH lines and all mapped on group 2 chromosomes (Fig. S1). Two markers were mapped on each of chromosomes 2A and 2D, while TNAC9025 was tightly linked to Barc55 on 2B. From 456 BC_1F_5 lines, eight recombinants between TNAC9025 and Barc55 were detected. Mean PHS scores of six lines with the Cayuga allele for TNAC9025 were 2.40 and 1.53 at Snyder and Ketola, respectively (Table S2). Whereas scores of the other two lines with the Caledonia allele were 4.30 and 3.10 at Snyder and Ketola, respectively. This result indicates TNAC9025 is closer to the distal QTL than Barc55.

Discussion

Fine mapping population

In this study, selected homozygous recombinant BC_1F_4 and BC_1F_5 lines were used for fine mapping the *QPhs.cnl-2B.1* interval on wheat chromosome 2B. These populations were estimated to contain 81.75–86.3 % Caledonia background outside the PHS QTL interval. These populations contained more of the recurrent parent (Caledonia) background than a normal BC_1 because doubled haploid individuals containing more of the recurrent parent background were backcrossed to the recurrent parent. The proportion of recurrent parent background was close to that expected for a BC_2. This strategy saves a considerable amount of population development time in a winter cereal crop. The recombinants were identified in BC_1F_3, BC_1F_4 and BC_1F_5 generations resulting in many recombinants in a relatively small population, thus facilitating phenotyping and fine mapping. Advantages to this strategy are shorter development time than for NILs and advanced backcrossed populations and fewer lines required for phenotyping. Population development and fine mapping occurred simultaneously rather than sequentially. Although the populations may have some residual heterogeneity for recurrent parent background, it

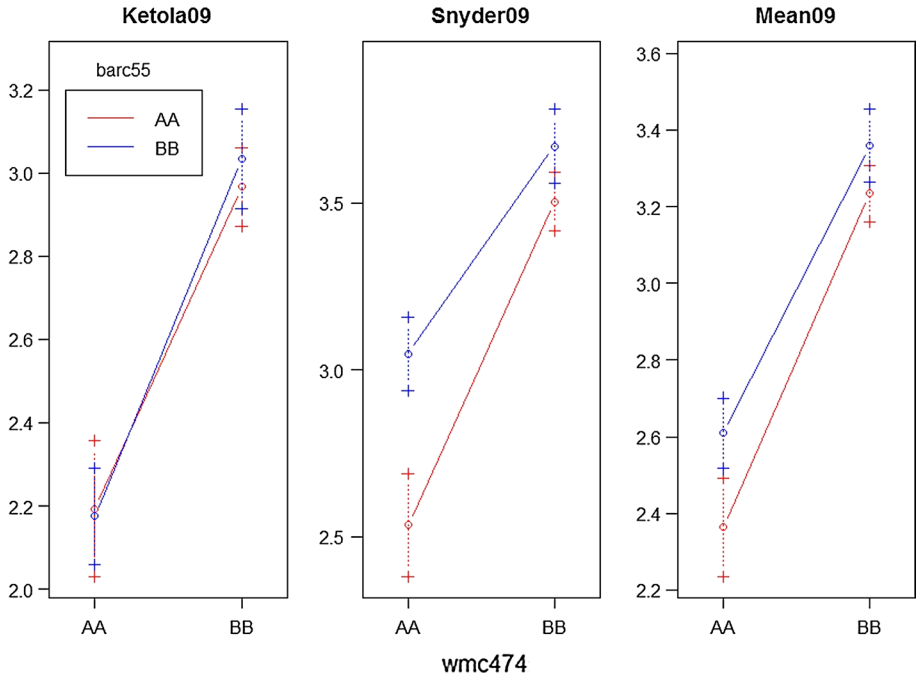


Fig. 5 Interaction plots for Barc55 (distal QTL) and Wmc474 (proximal QTL) using PHS scores of BC_1F_5 homozygous recombinants. AA and BB represent Cayuga (PHS-resistant parent) and Caledonia (PHS-susceptible parent) genotype, respectively. *Open circle* indicates mean value of each genotype; *Error bars* are plotted at ±1 Standard error

was offset using more lines and by sampling within lines to increase phenotyping accuracy. In addition, fine mapping was augmented by comparing phenotypic means among the recombinant and randomly selected, non-recombinant lines for the PHS QTL interval. It is likely that both groups had a similar background so the phenotypic difference results mostly from the PHS QTL interval.

Most fine mapping studies have used NILs and advanced backcrossed populations (Paterson et al. 1990; Alpert and Tanksley 1996; Yamamoto et al. 1998; Han et al. 1999; Vladutu et al. 1999; Yamamoto et al. 2000; Takeuchi et al. 2003; Nichols et al. 2006; Röder et al. 2008). However, some studies were also successful using BC_1 and large segregating populations for fine mapping. The Diageotropica (*Dgt*) gene in tomato was fine mapped using 1,308 BC_1 individuals. Ten recombinants narrowed the region to 0.8 cM (Oh et al. 2002). Another study used 1,050 BC_1F_2 individuals to narrow the hybrid embryo sac sterility (*S32(t)*) locus in rice to 1.9 cM. Based on the recombinant events, it was located in a 64 kb region containing six recombinants and seven predicted open reading frames (Li et al. 2007). In another study, fine mapping used a large segregating population of 1,849 (1,256 F_2 and 593 BC_1F_2) to fine map a frost resistance locus (*Fr-H2*) in barley. It was narrowed to 0.81 cM, containing six recombinations over 0.03–0.32 cM (Francia et al. 2007). These studies, as well as our study, showed that fine mapping can be done in any population that contains enough recombinants in the targeted region.

The low polymorphism between two hexaploid wheat parents in this study that were used to develop the mapping populations limited the progress to narrow the QTL region. Another limiting factor included the lack of a physical map that could be used to develop new markers. The available sequences were mapped ESTs, transcripts and STS (sequence tag site) markers that generally show low polymorphism.

Fine mapping versus doubled haploid genetic mapping

In previous work, the PHS QTL *QPhs.cnl-2B.1* was identified as a major QTL on chromosome 2B in 209 individuals from a Cayuga × Caledonia doubled haploid population (Munkvold et al. 2009). While, in this study, fine mapping in the BC_1F_4 (359 lines) and BC_1F_5 (456 lines) recombinants revealed that the PHS QTL interval resulted from two closely linked QTLs in coupling with resistance coming from Cayuga. Factors that may cause a single broad QTL in one population to be two QTLs in another population include missing data for a marker at the middle of the peak, dominant markers used in a heterozygous or heterogeneous population, wrong marker order and double crossovers in some recombinants. However, in this study, those factors were eliminated in the fine mapping of the homozygous recombinant lines. For the first factor, there was almost no missing data (only 0.01 %) for the middle markers between the two PHS QTLs. Second, only homozygous recombinants were used for fine mapping so genotypes based on dominant markers were not different from those based on co-dominant markers. For the third factor, the order of both previous and newly designed primers was first determined using the Cayuga × Caledonia doubled

haploid population, and then later the BC_1F_4 recombinants to order the markers in the QTL interval, which were found to agree. For the last factor, double crossovers can happen, but at very low frequency. In BC_1F_5 recombinants, some recombinant groups contained two separate Cayuga fragments with a Caledonia fragment in the middle resulting from double crossover but the frequency was only 5.7 % for all BC_1F_5 recombinants (26 out of 456 recombinants).

Candidate gene identification based on homology with rice and Brachypodium

Fine mapping revealed that the *QPhs.cnl-2B.1* interval was 3 and 2.7 Mb based on the rice and *Brachypodium* homologous regions, respectively (Somyong et al. 2011), and contained two closely linked QTLs. The PHS interval on the short arm of wheat chromosome 2B was orthologous with the long arm of rice chromosome 7. Comparative genetic analysis reported by Somyong et al. (2011) revealed that six candidates, CIPK3, CIPK2, CIPK11, ABAR/GUN5, Calmodulin-binding protein and GTP binding, were located at the *QPhs.cnl-2B.1* interval. The distal PHS QTL between Wmc453c and Barc55 was located within the *QPhs.cnl-2B.1* interval so the ABAR/GUN5, which is annotated as Mg-chelatase H subunit (CHLH) family protein in the Rice Annotation Project Database (RAP-DB) (Sakai et al. 2013; http://rapdb.dna.affrc.go.jp), was proposed to be closest to the distal QTL. The CHLH protein is known to play a role in seed dormancy and was a possible receptor for ABA in Arabidopsis (Shen et al. 2006). Additional markers were developed using the polymorphic marker, TNAC9025, closest to Barc55. TNAC9025 was designed from homologous rice gene Os07g0659100 (LOC_Os07g46500). This homologous gene located within 150 kb from homologous gene Os07g0656500 (LOC_Os07g46310) encodes the CHLH protein. This was supporting evidence that CHLH remains a strong candidate for the distal QTL.

The other PHS QTL between Wmc474 and CNL415-rCDPK was located proximally within the *QPhs.cnl-2B.1* QTL region making the GTP-binding protein the closest candidate. However, the proximal QTL also covered the region outside of the *QPhs.cnl-2B.1* interval. Thus, there may be other candidate genes not revealed by comparative genetic analysis reported by Somyong et al. (2011). The RAP-DB shows that there were 68 annotated rice genes in this PHS interval stretching from Os07g0619500 (LOC_Os07g42740) to Os07g0627800 (LOC_Os07g43470, homologous with BE494262). Those genes included 44 expressed genes, 10 hypothetical protein genes, three non-protein coding genes, one transposon, and 10 predicted genes. There were three known genes that may be involved in seed dormancy. A calmodulin-dependent protein kinase (Os07g0619800, LOC_Os07g42770) was under rice SD QTL (Gu et al. 2004). The other two genes were serine/threonine protein kinase SAPK2 (Os07g0622000, LOC_Os07g42940), and myb-DNA-binding gene (Os07g0627300, LOC_Os07g43420). However, the lack of polymorphism for markers in the QTL interval prevented the localization of the recombination breakpoints and identification of the gene underlying the phenotype.

Recent advances in wheat genome-sequencing projects will overcome this difficulty; 5X genome sequences of Chinese Spring wheat have been released (Brenchley et al. 2012), and the international wheat genome-sequencing consortium (IWGSC, http://www.wheatgenome.org/) that launched in 2005 is working on physical mapping and sequencing using sorted chromosomes and chromosome arms. A high-quality reference genome sequence of bread wheat will enable direct searches for polymorphisms in the specific regions of interest facilitating the saturation of markers around the target QTL regions. The population developed in this study contains high amounts of recombinations across the target QTLs. Therefore, accomplishing by genotyping of newly developed markers, these materials will be useful resources for identification of genes associated with preharvest sprouting.

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

Ethical Standards The authors declare that the experiments described in this manuscript comply with the current laws of the United States.

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