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Mapping the *Bo$_d$2* gene associated with boron efficiency in wheat

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ABSTRACT: Genotypic variation for response to boron (B) deficiency in wheat (*Triticum aestivum* L.) was determined as grain set index (GSI, %) and found to be controlled by two major genes, *Bo$_d$1* and *Bo$_d$2*. Breeding for B efficiency may be enhanced by molecular markers associated with quantitative trait loci (QTL) for B efficiency. Two mapping populations, (Bonza × SW 41) BC_2 and Fang 60 × Bonza, were made from Bonza (B inefficient), SW 41 (B moderately inefficient), and Fang 60 (B efficient) parents. Leaf samples from F_2 plants from each cross were used for DNA analysis. GSI was measured in F_2-derived F_3 families. Seven hundred and eighty-seven simple sequence repeat (SSR) markers were used. Bulked segregant analysis and QTL analysis were performed to identify the chromosomal location for B efficiency. One QTL for B efficiency was identified and mapped in (Bonza × SW 41) BC_2F_3, and was found to be flanked by two SSR markers, gwm192, and gwm165. This QTL is located on the long arm of chromosome 4D between gwm165 and gwm192. This QTL region corresponded for 21% of the variation in GSI, suggesting that the chromosome segment of SW 41 parent containing the *Bo$_d$2* locus was inherited by the progenies and that is located on 4D. Additional markers and further examination are required to locate *Bo$_d$1*, the other B efficient gene. Linked markers will enable applications of marker-assisted selection for B-efficient genotypes in wheat.

KEYWORDS: quantitative trait loci (QTL), SSR markers, *Triticum aestivum*

INTRODUCTION

Boron (B), an essential micronutrient for plants, is often present at insufficient levels for crops to growth in coarse textured soil in humid regions[1]. Boron deficiency usually affects young growing parts of plants. In cereals including wheat, symptoms mostly occur in reproductive tissues[2]. In wheat, B deficiency affects reproductive growth by causing abnormal development of the pollen, resulting in male sterility and failure to set grain. Boron deficiency in wheat has been reported in several wheat-growing countries including Bangladesh, Brazil, Bulgaria, China, Finland, India, Madagascar, Nepal, Pakistan, South Africa, Sweden, Tanzania, Thailand, USA, Russian Federation, Yugoslavia, Zambia[3].

A wide range of genotypic variation in B efficiency in wheat has been reported[4–6]. As B deficiency is closely associated with grain set index (GSI), which measures grain set in basal florets of the central spikelets of the wheat ear, it has been used effectively as a measure of B efficiency in wheat[6]. The range of B efficiency of genotypes is expressed as the GSI ranging from 0–100%.

The use of B efficient genotypes was suggested as means of avoiding sterility induced by B deficiency of wheat in low B soils[7]. To do this effectively, the genetics of B efficiency must be better understood so that the trait can be incorporated in breeding programs, targeting areas prone to B deficiency. Recently, molecular markers are increasingly used to accelerate selection. Mapping traits for a desirable quality, disease resistance, and nutrient uptake by molecular means has allowed considerable improvement in the efficiency of wheat breeding[8] which can pinpoint gene location and quantitative trait loci (QTL) controlling traits of interests.

For wheat, B efficiency has been reported to be controlled by two major genes, *Bo$_d$1* and *Bo$_d$2*[7]. These were identified by assessing grain set index in F_1- hybrids and F_2-derived F_3 populations in three wheat genotypes with different levels of B efficiency; however, mapping and QTL for B efficiency have yet to be described. In the case of B toxicity, several

major additive genes have been reported to control the accumulation of B in both root and shoots in tolerant genotypes[9]. In addition, Jefferies et al[10] mapped and validated genes for B toxicity tolerance on the chromosome 7B and 7D in wheat, using RFLP markers which were associated with leaf symptom and root growth. On the other hand, previous studies reported that genetic control of B efficiency entails a single gene in some plants such as celery[11], tomato[12], and red beet[13]. For barley, B efficiency is controlled by incomplete to completed dominance, depending on cross combination and the severity of B deficiency[14]. In *Brassica napus*, it is expressed as a dominant trait, controlled by a major gene[15], but by additive gene action in sunflower[16]. A QTL for B efficiency has been identified in *Arabidopsis thaliana*[17] and *Brassica napus*[18,19].

The objectives of this study were (1) to identify the chromosomal location(s) of genes conferring B efficiency in wheat and (2) to use QTL analysis to assess the genetic effect of the B efficiency gene(s). Understanding chromosomal location and genetic effects will facilitate breeding for B efficiency by marker-assisted selection.

MATERIALS AND METHODS

Genetic materials

Three bread wheat (*Triticum aestivum* L.) genotypes, B-inefficient Bonza (genotype $bo_d1bo_d1bo_d2bo_d2$), B-moderately inefficient SW 41 (genotype $bo_d1bo_d1Bo_d2Bo_d2$) and B-efficient Fang 60 (genotype $Bo_d1Bo_d1Bo_d2Bo_d2$)[7] were used as parental lines to construct three mapping populations. Two F_2 populations were made from crosses between (Bonza × SW 41) BC_2 (BSW) (Bonza was used as the recurrent parent and SW 41 as Bo_d2-donor parent) and Fang 60 × Bonza (FB).

Screening for response to B

Boron efficiency evaluation was made with GSI (percentage grain set in the first two florets of 10 central spikelets[20]) in parents and their F_2-derived F_3 families. Plants were grown in sand culture in earthenware pots (0.3 m diameter, 0.3 m deep) containing washed quartz river sand with undetectable B. The sand substrate contained no added B (B0) as described by Jamjod et al[7]. Seeds were pre-germinated on moist filter paper in Petri dishes and stored at room temperature for two days and transplanted at 10 plants/pot for F_2 and 11 plants/family/pot for F_3 with one plant of Bonza as a control at the centre of the pot. There were also six pots of each parent with 10 plants/pot. Plants in each pot were watered twice daily with 1.0 l of complete nutrient solution containing 1000 μM $CaCl_2$, 250 μM $MgSO_4$, 500 μM KH_2PO_4, 10 μM Fe-EDTA, 250 μM K_2SO_4, 1 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.1 μM $CoSO_4$, 0.1 μM Na_2MoO_4 (modified from Broughton and Dilworth[21]), and 5000 μM KNO_3.

Phenotyping for B efficiency

Parents and F_2s of all crosses were grown in sand culture as above, but with 10 μM B (B10) added to the nutrient solution. At tillering, leaf samples from each plant were collected and kept in silica gel for simple sequence repeat (SSR) analysis. At maturity, each F_2 plant was harvested separately, and the progeny represented each F_3 family. The number of families from each cross were 70 for BSW and 126 for FB. Response to B was evaluated in the F_3 generation and F_3 families from the two crosses were grown in sand culture without added B (B0). Each pot contained one family with 11 plants/family. Six pots of each parent were included as controls. At maturity, two spikes from each plant were collected for GSI determination. Frequency distributions were drawn to display segregation for B efficiency in terms of GSI (%) in F_3 populations. Mean, range, and variance within family related to those of parents were calculated to indicate the variation induced by B deficiency in each cross. The significant difference between means of families within each cross was assessed using the least significant difference (LSD) at 95% confidence.

SSR analysis

The DNA of each leaf sample of F_2 individuals was extracted using the CTAB method[22]. PCR and fragment detection were performed as described[23]. DNA amplification was carried out using 50 ng/μl of DNA from each sample. Denaturation was induced by heating to 94 °C, and published annealing temperatures were used for each marker. Finally, the temperature was shifted to 72 °C. The cycle was then repeated 30–35 times (30–35 cycles). Annealing temperatures of each marker used touchdown cycling (the annealing temperature was decreased by one degree every cycle). Fragments of PCR products were identified and separated on 8% polyacrylamide gel electrophoresis (PAGE), run at 80 V, 400 mA, for 14–17 h overnight and visualized by ethidium bromide staining. Three parents, Bonza, SW 41, and Fang 60, were screened for polymorphism at 787 SSR markers. Polymorphic markers between parents were selected for each cross and then screened in the F_2 progenies.

Bulked segregant analysis

Bulked segregant analysis[24] was performed with polymorphic markers to find markers linked to the trait. For each cross, two bulks of DNA representing the 6–12 plants with the highest and the lowest GSI were formed. Polymorphic markers between the bulks were selected and screened among individuals of each bulk. Markers identified as potential linked markers in individuals from the bulks were then used for the final screening of F_2 individuals.

QTL analysis

Goodness of fit between the expected and observed segregation ratios was tested by χ^2 analysis. Markers that skewed from the expected distribution were omitted from the analysis. Single factor ANOVA was performed to compare marker classes and phenotypic means to describe locus effects. Significant difference ($P < 0.05$) was determined using SX release 8.0 (Analytical software, Tallahassee, USA). Linkage maps were constructed and QTL analysis was performed by MAP MANAGER QTX b20[25] to establish a map and identify associations between markers and the B efficiency trait. Recombination frequencies were converted using the Kosambi mapping function[26]. Significant QTL were accepted with the logarithm of odds (LOD) threshold at 3.0 ($P = 0.001$) to indicate the marker to be tightly linked with the trait[27].

RESULTS

Evaluation for B efficiency

The three parents and two F_3 populations showed a large range of responses to low B in sand culture (Table 1). The range in GSI of Bonza, SW 41, and Fang 60 were 26–49, 75–83, and 98–100%, respectively. Mean GSI of F_2-derived F_3 families were within the range of their parents. Within line variance of B inefficient Bonza was 13–60, variance of B efficient Fang 60 was 1–8 whereas variance of moderately inefficient SW 41 was 50–217. Within family variances of F_3 varied from 0–1897.

Variation in GSI was observed within all mapping populations. There was a continuous distribution of GSI values with a large range of response to B deficiency in the crosses FB and BSW (Fig. 1).

SSR analysis

Among the 787 markers tested, three parents, Bonza, SW 41, and Fang 60, differed in about 344–380 markers (44–48%) depending on the pair being compared. SSRs revealing polymorphisms between two bulks and the individual plants are shown in Table 2. In the BSW cross, eight potentially linked markers were found and that distributed on chromosomes 2B, 3D, 4D, and 7B. In the FB cross, there were four potential linked markers, gdm35 (chromosome 2D),

Table 1 Range of mean GSI (%) and variance within family of parents and F_2-derived F_3 populations. Values are based on 11 plants per family.

Genotypes	N	GSI (%)			Variance		
		min	mean	max	min	mean	max
Parents							
Bonza (I)	6	26	40	49	13	39	60
SW 41 (MI)	6	75	78	83	50	91	217
Fang 60 (E)	6	98	98	100	1	4	8
F_3 populations							
(Bonza × SW 41) BC_2F_3	70	26	80	98	0	281	1137
(Fang 60 × Bonza) F_3	126	30	81	100	0	420	1897

N = Number of parental lines or F_3 families; I = Inefficient, MI = Moderately Inefficient, E = Efficient.

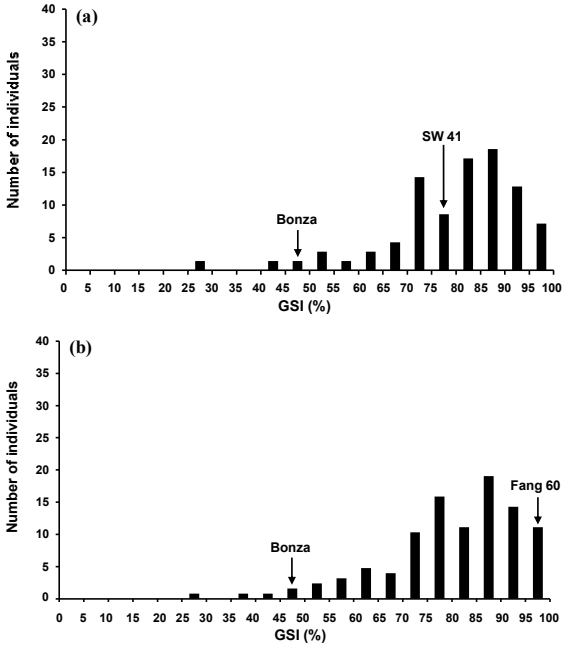

Fig. 1 Frequency distributions of mean GSI (%) in F_2-derived F_3 families and their parents grown in sand culture without added B. Mean and variance (in parentheses) presented for F_3 and their parents. (a) (Bonza × SW 41) BC_2F_3, 80(281); and (b) (Fang 60 × Bonza) F_3, 81(420). Parents: Bonza, 40(39); SW 41, 78(91); and Fang 60, 98(4). Data were transformed by arcsine transformation.

Table 2 Allele patterns between parents and bulks (BS and BT) and between individuals within bulks detected by eight polymorphic markers in (Bonza × SW 41) BC_2F_3 and four markers in (Fang 60 × Bonza) F_3 crosses. The number of individuals from the bulks of each cross that showed an allele different from their parents is shown.

P1 × P2	Marker	Chromosome	Parents		Bulk		Number of individuals					
							BS			BT		
			P1	P2	BS	BT	+	+/−	−	+	+/−	−
Bonza × SW 41	barc200	2BS	−	+	−	+			6	3		7
	gwm120	2BL	−	+	−	+	1		5	2		8
	gwm52	3DL	−	+	−	+			6	3		7
	gwm341	3DL	−	+	−	+			6	3		7
	gwm192	4DL	−	+	+	−	6					10
	gwm165	4DL	−	+	+	−	4	2			3	7
	gwm131	7BL	−	+	−	+			6	3		7
	gwm111	7BL 7DS	−	+	−	+			6	3		7
Fang 60 × Bonza	gdm35	2DS	+	−	−	+	4	5	3	5	7	
	gwm513	4BS	+	−	−	+	4	6	1	4	5	1
	gwm192	4DL	+	−	+/−	+/−	2	7	3	3	8	
	gwm165	4DL	+	−	+/−	+/−	2	6	4	4	6	

BS = Bulk sensitive, BT = Bulk tolerant; P1 and P2 = Parent 1 and parent 2 each cross; + = presented as homozygous efficient, − = presented as homozygous inefficient, +/− = presented as both parents (heterozygote).

gwm513 (chromosome 4B), gwm192 and gwm165 (chromosome 4D). The segregation of gwm192 and gwm165 deviated from the expected 1:2:1 ratio in the FB-F_2 population. In the BSW-F_2, the segregation of only two markers, gwm192 and gwm165, was consistently with a 1:2:1 ratio. PCR product of bulk and individuals of BSW cross amplified with gwm192 and gwn165 are shown in Fig. 2.

QTL analysis

ANOVA on each marker (gwm192 and gwm165) in Table 3 was performed to identify the association between markers and GSI of F_2-derived F_3 families. GSI and marker genotypes (homozygous P1 type, heterozygous and homozygous P2 type) were compared. A significant difference in GSI between marker genotypes was found in BSW for both gwm192 and gwm165. In this cross, Bonza marker alleles (P1) at both loci were associated with high GSI and significantly higher than SW 41 marker alleles (P2), while heterozygotes were closest to the high GSI type.

By QTL analysis, the QTL for B efficiency was found to be flanked by two SSR markers, gwm192 and gwm165 in BSW cross (Table 4). Twenty one percent of the total variation in GSI for B efficiency was explained by this QTL region with a dominance and additive effect of 5.6 and 9.5, respectively. The ratio of dominance effect was relatively high (0.6) for the QTL in this region which indicated partially dominant gene action. This QTL is located on the

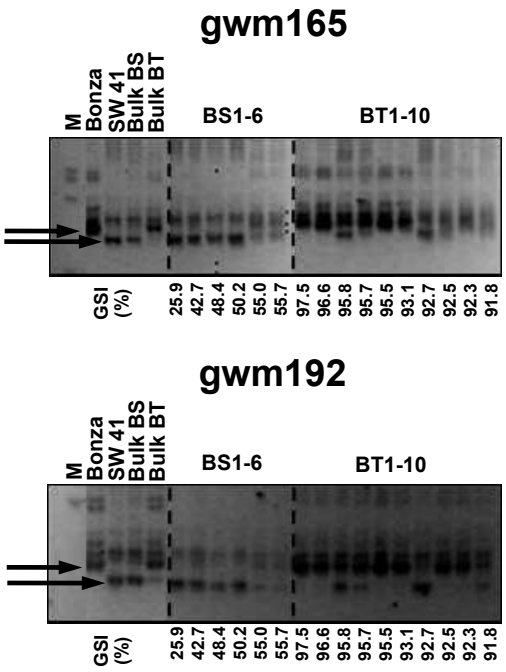

Fig. 2 PCR product of DNA amplified with linked markers, gwm165 and gwm192, in F_2 bulks of Bonza × SW 41 population. The first lane (M) was 100-bp ladder followed by parents; Bonza (inefficient), SW 41 (moderately inefficient), bulk sensitive (BS) and bulk tolerant (BT). PCR product was run on 8% PAGE, 80 V, 400 mA, 999 minutes, run overnight.

Table 3 Mean effect of the presence of either the P1 or P2 marker allele on GSI (%) of (Bonza × SW 41) F_3 and (Fang 60 × Bonza) F_3 mapping populations grown at low B.

P1 × P2 / SSR marker	Marker allele	GSI (%)	Number of lines within class	SE	P	$LSD_{0.05}$
(Bonza × SW 41) BC_2F_3						
gwm192	P1	85.0[a]	25	2.6	0.013	4.4
	H	79.9[ab]	26	2.5		
	P2	73.2[b]	19	2.9		
gwm165	P1	83.8[a]	23	2.7	0.019	4.3
	H	81.0[a]	30	2.4		
	P2	71.8[b]	16	3.2		
(Fang 60 × Bonza) F_3						
gwm192	P1	82.8	20	3.1	0.372	
	H	81.1	79	1.6		
	P2	77.2	23	2.9		
gwm165	P1	83.3	23	2.9	0.176	
	H	75.7	82	1.5		
	P2	80.1	21	3.0		

P1 = Parent 1, H = Heterozygous, P2 = Parent 2; SE = Standard error of mean from individuals in each class. Different letter separated significant difference between mean GSI in each row.

Table 4 QTL analysis for chromosome 4D associated with GSI (%) of (Bonza × SW 41) BC_2F_3 populations by MAP MANAGER QTX b20 using LOD threshold of 3.0 (P = 0.001). Phenotypic variation explained by marker interval for GSI presented in R^2 (%).

Marker interval	Distance (cM)	LOD	R^2	a[†]	d[†]	[a/d][*]
(Bonza × SW 41) BC_2F_3						
gwm192–gwm165	7.6	3.54	21	9.5	5.6	0.6
(Fang 60 × Bonza) F_3						
gwm192–gwm165	4.3	0.98	4	−4.9	−0.2	24.5

[†] a and d represent additive effect and dominance effect of main effect QTL.
QTL was classified according to their [a/d] (degree of dominance).

long arm of chromosome 4D between gwm165 and gwm192, 6 cM from gwm192 with 3.54 of LOD. A partial linkage map for *Bo_d2* was constructed from this backcross population (Fig. 3). The chromosome length of 4D on which the QTL for *Bo_d2* was located on was 145.1 cM. No QTL was detected in the F_3-FB cross.

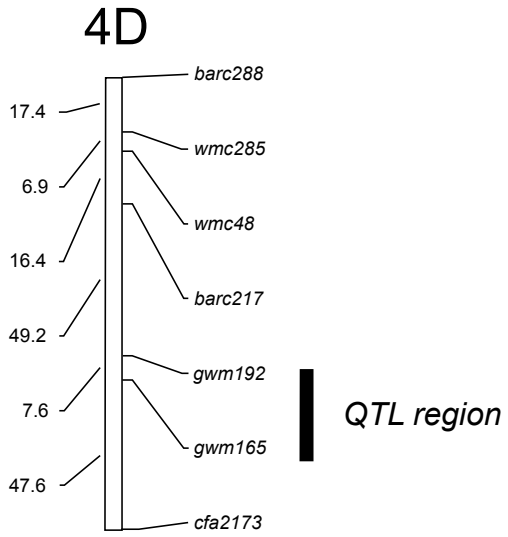

Fig. 3 A partial map and the position of QTL associated with *Bo_d2* on chromosome 4D based on GSI (%) of (Bonza × SW 41) BC_2F_3. Marker names are given on the right side. The left side indicates distance between markers in centimorgan (cM).

DISCUSSION

Genetic control of B efficiency

It has been observed and confirmed that reproductive process is extremely sensitive to boron (B) deficiency, which limits grain set and grain yield in wheat. Incorporation of B efficiency gene(s) in wheat breeding would be highly desirable in regions with low B soils[6]. This study has again demonstrated that reproductive process exhibited in terms of GSI (%) was efficient to use as criteria index for B efficiency as previously reported[6,7,20]. In this study we showed that genetic variation in B efficiency, quantified in GSI (%), existed in all mapping populations. The distribution of the mean GSI of F_3 families for the two crosses was skewed in the direction of the more efficient parent, indicative of efficiency being dominant. The GSI was depressed in B inefficient progenies, whereas efficient progenies set grain normally. Response to B deficiency in F_3 of Bonza × SW 41 differed at one B efficient locus, whereas F_3 of Fang 60 × Bonza differed at two B efficient loci in the same way as indicated by Jamjod et al[7]. However, segregation of GSI (%) in Bonza × SW 41 was smaller than in Jamjod et al[7] but with continuous distribution. Response of GSI (%) of progenies varies according to B treatment, the parental combination and environment factors[7,28].

Boron efficiency has been reported to be qualitatively inherited and under the control of a single

gene for a number of species including celery, tomato, and red beet[11–13]. Jamjod et al[29] reported that both additive and dominant gene effects were involved in controlling B efficiency in bread wheat but more complicated genetics of B efficiency than a single gene was suggested for wheat[30].

QTL analysis

One QTL for B efficiency in wheat was identified and mapped in the Bonza × SW 41 cross in this study. The long arm of chromosome 4D was identified as the region associated with the *Bo$_d$2* gene from bulked segregant analysis through QTL analysis. That region accounted for 21% of phenotypic variance explained in the population. This QTL was associated with improved grain set in terms of GSI on B-deficient soil for progenies which were as efficient as the SW 41 parent. However, expression of the trait of progenies from only one cross (Bonza × SW 41) was found to have strong association with the marker, whereas in Fang 60 × Bonza, there was a weak association shown by QTL analysis.

A region of chromosome 4D in wheat appears to be involved in B efficiency and uptake and tolerance to other toxicities; for example, K^+/Na^+ discrimination[31,32], salt tolerance[33], and aluminium tolerance[34,35]. Miftahudin et al[36] reported that the polymorphism rate for 4DL is extremely low, making further analysis in wheat difficult. Thus they exploited the rice/rye syntenic relationship for Al tolerance to develop polymorphic markers to be used in segregating F_2 populations in wheat. Although the D genome contained a number of functional genes in wheat[37], low marker polymorphism has impeded genetic studies for the 4D chromosome[38,39]. Thus further markers such as single nucleotide polymorphism should be used in order to locate other QTL for B efficiency genes including *Bo$_d$1*. Previous research also demonstrated that other related species such as rye contain the genes responsible for nutrient efficiency, as in the case of Cu (4B/5R wheat-rye translocation)[40]. Furthermore, in barley, chromosome 4H was associated with Mn efficiency[41] and barley-4H also compensates for wheat chromosome 4D for drought stress[42].

Recently, the QTL for B efficiency were examined in *Arabidopsis*[17] and *Brassica*[18,19] with suggested benefits in selection of Cruciferae species. In *B. napus*, B efficiency is expressed as a dominant trait and controlled by a major gene in F_2 segregating populations[15] and in $F_{2:3}$ populations[43] in terms of seed yield which is tightly linked to growth period (bolting dates and maturity dates). Xu et al[18] also identified a B-efficient gene in *B. napus*, *BE1*, on linkage group 9 that was found to be linked with one QTL for bolting date, *bd9b*, by using RFLP and AFLP markers. After that, Shi et al[44] verified that *BE1* is the major gene controlling B efficiency by QTL mapping. Moreover, Zhao et al[19] found new B-efficient locus, *BnBE2*, located on linkage group N14, which is associated with seedling growth. In *Arabidopsis thaliana*, B uptake efficiency, revealed by *BOR1* gene transporter, contributed to higher efficiency in xylem loading in low B stress[45].

Mechanisms involved in B efficiency

In genetic studies of tolerance to mineral nutrient deficiencies, selection criteria may have important implications. Different selection criteria may identify different mechanisms. In case of B efficiency, the extent of variation for each genotype or plant species was considered with respect to the physiological nature of the efficiency mechanisms, the genetic basis of inheritance, screening techniques and the practical implications of the genotypic variations[30].

Mechanisms for B efficiency differ with the genotypes being compared and with the intensity of deficiency. Generally, B uptake and use in plants plays a major role in overall B efficiency. In case of GSI, the mechanisms involved include greater B uptake and distribution. Nachiangmai[46] reported that Fang 60 had a greater ability to distribute B into the developing ear under low B during the critical stage of microsporogenesis than SW 41 and suggested that, this is the main mechanism for B efficiency in wheat. Furthermore, a study with ^{10}B found that Fang 60 was able to retranslocate B from older to younger tissues after B supply to the roots was withdrawn, whereas Bonza did not have this ability[47]. Mechanisms controlling B efficiency in wheat in terms of grain set was suggested to be related to the ability to supply B for reproduction, other mechanisms and genes may be responsible for other phenotypic responses. For example, in cereals such as wheat, grain set is the key trait involved in, so the GSI is the phenotypic expression of B efficiency. In *B. napus*, seed yield, maturity and bolting date are the key traits that are used for screening B efficient genotype[18]. It is as yet unclear which mechanism is conferring B efficiency as expressed by high GSI and used as trait for genetic studies described here.

SSR polymorphism

Overall, SSR analysis in this study showed high polymorphisms between three parents (43%). The highest polymorphisms existed between Fang 60 and Bonza

parents and between Bonza and SW 41 parents. The levels of polymorphism reflected genetic similarity of the parental lines and their origins. Boron efficient genotypes are more frequent in wheat germplasm from Thailand[48], Nepal[49], and India[50] where B deficiency in wheat has been identified and active selection for B efficiency has been conducted. Fang 60 (efficient) and SW 41 (moderately inefficient) both originated from CIMMYT, were selected and released in Thailand, whereas Bonza was from Colombia.

In this study, progress has been made in developing markers for B efficiency associated with Bo_d2. However, linkage analysis could not locate the other B efficient gene (Bo_d1). Additional markers should be examined. Further study of Bo_d1 will enhance selection and breeding of genotypes with B efficiency in the same range as Fang 60. Improvement of agronomic performance of crop varieties is influenced mainly by complex quantitative traits for yield and quality. Molecular marker technology has made it feasible to identify and localize the contributing genetic factors as quantitative trait loci (QTLs) and to use these QTLs for crop improvement[51]. QTL associated with the B efficiency gene identified in this study should bring us closer to breeding wheat for B efficiency on low B soils, although the controlling mechanism for B efficiency is still to be identified.

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