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Identification of inter simple sequence repeat (ISSR) markers associated with seed size in wheat

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Abstract The feasibility of identifying inter-simple sequence repeat markers associated with seed weight in hexaploid wheat was tested using 113 recombinant inbred lines developed by the single-seed descent method, from a cross between Rye selection111, an Indian genetic stock obtained through the introgression of genes for bold seed size from rye, and Chinese Spring having small seed size. Three markers were associated with low seed size with gene effects of 14.8%, 9.5%, and 6%, while four markers with contributions of 8%, 4.66%, 2.92% and 2.61% were found to be linked to high seed size, together contributing 31% of the phenotypic variance in seed size. Nulli-tetrasomic and di-telosomic analysis revealed the presence of three low seed size QTL-associated markers on three chromosomes, 6BL, 2DL, and 1DS respectively. This study clearly demonstrates that ISSRs are highly useful for finding markers associated with major and minor genes controlling agronomically important traits in wheat.

Keywords Wheat · Thousand kernel weight (TKW) · Quantitative trait loci (QTLs) · Inter simple sequence repeat (ISSR) markers · Recombinant inbred lines (RILs), Nullitetrasomic (NT) lines

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Introduction

Wheat (*Triticum aestivum*, 2n=6*x*=42) next to rice,is one of the major food crops of India, ranking India as the second largest producer of wheat in the world. Although India has achieved self sufficiency in wheat production in recent years, the quality of Indian wheat needs improvement not only for domestic consumption but also for meeting International standards for export purpose (Ranjekar et al. 1998). The end-use quality of wheat is greatly influenced by seed size (Campbell et al. 1999) which can be measured indirectly by 1000-kernel weight (TKW). TKW is positively correlated with agronomic yield (Baril 1992) and flour yield (Chasten et al. 1995). Moreover, the high heritability values of kernel weight in most of the cultivars studied so far have proved that this character is phenotypically the most-stable yield component (Giura and Saulescu 1996). Finally, large seeds usually command consumer preference and thereby represent an important factor in controlling the economic value of wheat. The development of improved seed size-specific cultivars is thus an important breeding objective in wheat agriculture (Campbell et al. 1999). Grain size in wheat is a complex character (Giura and Saulescu 1996) and any information on its genetic control is useful for increasing breeding efficiency. Identifying molecular markers linked to quantitative trait loci (QTLs) controlling seed size would facilitate selection in early generations and may contribute to improved end-use quality in wheat by accumulating such loci into elite backgrounds.

In wheat, several molecular-marker approaches have facilitated the identification of chromosomal regions associated with agronomically important traits like dwarfing and vernalization response (Korzun et al. 1997), leaf rust resistance (Feuillet et al. 1995, 1997; Naik et al. 1998), kernel hardness (Sourdille et al. 1997), cadmium uptake (Penner et al. 1995), pre-harvest sprouting tolerance (Roy et al. 1998), protein content (Prasad et al. 1999; Mesfin et al. 1999; Blanco et al. 1996), resistance to common bunt (Demeke et al 1996), powdery mildew resistance (Qi et al. 1996), kernel traits (Campbell et al.

1999), and flour viscosity (Udall et al. 1999). Mapping agronomically important genes comprising qualitative as well as quantitative traits requires informative markers in an intraspecific context. In such cases, restriction fragment length polymorphism (RFLPs) markers are of very limited use although co-dominant and highly reliable in nature, since less than 10% of RFLP markers are polymorphic in wheat (Roder et al. 1998). Randomly amplified polymorphic DNA markers have become popular in a short time because of their technical simplicity and speed. However, in wheat the level of polymorphism obtained by RAPDs is likely to be as low as with conventional RFLPs, apart from the difficulties of reproducibility and transfer across populations (Devos and Gale 1992). Microsatellites or simple sequence repeat (SSR) markers with tandem repeats of a basic motif of <6 bp are the most polymorphic and hence are highly useful markers in wheat (Roder et al 1998); however, their development is laborious and highly expensive.

Recently, inter simple sequence repeat (ISSR) markers (Zeitkiewicz et al. 1994) have emerged as an alternative system with the reliability and advantages of microsatellites (SSR) along with the broad taxonomic applicability of RAPDs. The technique involves amplification of genomic segments flanked by inversely oriented closely spaced microsatellite sequences by a single primer based on SSRs anchored 5′ or 3′ with 2–4 purine or pyramidine residues. Several studies have indicated that ISSR markers are potentially useful both for cultivar identification and for phylogenetic studies (Wu and Tanksley 1993; Gupta et al. 1994; Zietkiewicz et al. 1994; Kantety et al. 1995; Charters et al. 1996; Fang and Roose 1997; Nagaoka and Ogihara 1997; Parsans et al. 1997; Blair et al. 1999; Joshi et al. 2000; Prevost and Wilkinson 1999). Nagaoka and Ogihara (1997) have shown that ISSRs are more informative compared to RFLP and RAPD markers in wheat. The utility of ISSR markers for tagging agronomically important genes was first demonstrated by Akagi *et al*. (1996) by identifying tight linkage with a nuclear restorer gene in rice. Tsumura *et al*. (1996) have reported the Mendelian inheritance of ISSR markers in Douglas fir and Pine. More recently, Ratnaparkhe *et al*. (1998) have studied the inheritance of ISSR markers in chickpea and established linkage between an ISSR marker and a Fusarium wilt resistance gene.

Here we report the identification of ISSR markers associated with seed size in hexaploid wheat. To the best of our knowledge, this is the first report of the applicability of ISSR markers for tagging agronomically important genes in wheat.

Materials and Methods

Plant material and phenotypic measurements

A recombinant inbred population (RIL) of 113 individual lines was developed by making a cross between Rye Selection 111, an Indian genetic stock obtained through the introgression of genes for bold seed size from rye, and Chinese Spring having small seed size. RILs were advanced by the single-seed descent method to the F_6 generation wherein panicles were bagged in each generation. The progenies in each generation were scored for seed size by recording 1000-grain weight from first two tillers of five plants from each progeny. Rye Selection 111(57.3 g) had twice the seed weight of Chinese Spring (29.16 g). Seeds of nullitetrasomic and ditelosomic lines were procured from Dr.B.S. Gill, Kansas State University, USA.

DNA extraction and procurement of oligonucleotide primers

DNA was extracted from 15-day old seedlings of parents and RILs grown in the glasshouse according to the procedure described by Anderson et al. (1992). One hundred primers of 15–23 nucleotides in length (UBC set #9) were obtained from the Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada.

PCR amplification and electrophoresis

PCR reactions were carried out in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM dNTP, 0.2 μM of primer, 15 ng of genomic DNA per 25-μl reaction volume with 0.8 units of *Taq* DNA polymerase (Perkin Elmer). PCR amplifications were performed in a Perkin Elmer Cetus 9700 thermal cycler, with initial denaturation at 94°C for 5 min, then 94°C for 30 s, annealing at 50°C for 45 s and extension at 72°C for 2 min for 45 cycles, with a 5-min final extension at 72°C. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and scored for the presence or absence of bands.

Data analysis

The genotypic data of ISSR marker loci in the RIL population were analyzed according to the chi-square test for goodness of fit. Linkage relationships among markers were determined by maximum-likelihood analysis of the segregation pattern using the computer software program Mapmaker version 2.0 (Lander et al. 1987). Simple linear regression analysis was performed using the computer program QGENE (Nelson 1997) to determine significant associations between markers and the phenotypic data of each RI line. Only significant LOD scores (>2.0) were interpreted to indicate co-segregation of putative QTLs for seed-weight and genetic markers. A multivariate linear regression model was developed to determine the combined effect of the marker loci to the phenotype.

Chromosome assignment

Nullitetrasomic (NT) (Sears 1966) and ditelosomic (Sears and Sears 1978) lines of Chinese Spring wheat were used to localise ISSR markers showing an association with seed size on wheat chromosomes.

Results

Frequency distribution of seed weight in RI lines

The TKW in the RIL population ranged from 53.38 g to 16.99 g; the parents used in this cross, Rye Selection 111 and Chinese spring, differ significantly in their TKW (57.3 g and 29.16 g) value, indicating a continuous variation which is a characteristic of polygenic nature of the trait. A frequency distribution plot was prepared (Fig. 1)

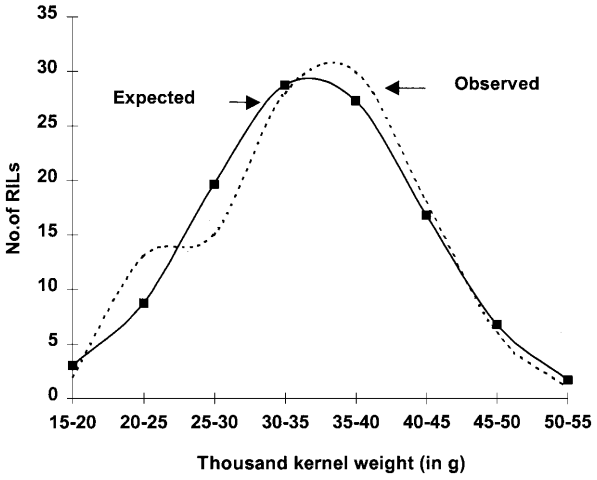

Fig 1 Frequency distribution of TKW in Rye Selection 111 x Chinese Spring RILs

using the TKW data of RILs, and a chi-square analysis was done to test the goodness of fit to a normal distribution. The data suggested a good fit (χ^2=4.3, *P*=0.05–0.07) to a normal distribution. Significant transgressive segregants occurred towards a low TKW parental value whereas transgressive segregation was not significant towards a high TKW parental value. The recovery of progenies having a grain weight as high or low as the parents indicated that the population was useful for the molecular tagging of the trait.

Optimization of PCR conditions for ISSR amplification in wheat

PCR conditions of all ISSR amplifications were optimized in a total reaction volume of 25 µl by using a different concentration of formamide (1% to 2%), primer (0.2 to 0.8 µM) and DNA template (5 to 50 ng), and varying annealing and thermal cycling procedures. Optimal conditions were selected based on the reproducibility and scorability of the PCR products. Finally, 1.5% formamide, 0.2 µM of primer, 15 ng of template DNA (although some of the studies used a higher DNA template) gave clear and intense banding patterns as opposed to Nagoaka and Ogihara (1997) who used much less DNA (5 ng) in their analysis. Although the annealing temperature was based on the melting temperature (T_m) of the primers, three annealing temperatures 45°C, 50°C, and 54°C were examined for all the primers. An annealing temperature of 50°C in 45 cycles of PCR gave clear banding patterns with many primers. $(AT)_n$-rich primers anchored at the 3′ end repeatedly resulted in smears, even after trying an annealing temperature as low as 30°C. Tsumura et al. (1996) and Nagoaka and Ogihara (1997) also experienced a similar problem in the case of Douglas-fir, Sugi and wheat, respectively. This result once again proved the abundance of $(AT)_n$-rich repeats in plant genomes.

Parental screening using ISSR primers

Out of 100 ISSR primers used to detect parental polymorphisms, 55 were successful in amplifying loci from parental DNAs. The bands generated by ISSR-PCR amplifications were consistent in their intensity and band selection was based on their resolution, intensity and reproducibility in three independent replications. A total of 18 primers showed polymorphism (approximately 32.7% out of the 55 primers that gave amplification) among the parents, generating 122 bands, of which 21 were polymorphic. Out of 18 polymorphic primers, the highest number of polymorphic bands were produced by dinucleotide repeats, namely $(CT)_n$, $(GA)_n$ and $(CA)_n$, one trinuclotide repeat, $(GTT)_n$, and three tetranucleotide repeats, $(GATA\ GACA)_2$, $(GACA)_n$, and $(GGAT)_n$, while others were a mixture of different repeat sequence motifs. Most of the primers were anchored the at 3′ end, except for UBC 882, an $(AT)_n$-rich primer having degenerate bases anchored at the 5′ end which gave amplification. But $(AT)_n$ rich primers having the 3′-end anchor did not give amplification profiles. It was, therefore, concluded that anchor nucleotides at the 5′ and 3′ ends of the primers play an important role in detecting polymorphism. Ratnaparkhe et al. (1998) also demonstrated that changes in sequence at the anchoring ends of the primers might give more useful information. In our study, $(CT)_n$ primers were most polymorphic, followed by $(GA)_n$ and $(CA)_n$ repeats. Tetranucleotide repeats, which generated a greater number of bands, might be more useful for fingerprinting studies rather than for inheritance and tagging studies.

Identification of putative ISSR markers through selective genotyping

DNAs from high and low TKW RILs, 13 each from both extreme tails of the population, were amplified using the identified polymorphic primers for selective genotyping (Lander and Botstein 1989). Figure 2 gives a representative photograph of selective genotype analysis using the UBC815 primer. It can be seen from this figure that a band of molecular weight 850 bp, indicated by an arrow, is present in Chinese Spring (lane B) and absent in Rye selection 111 (lane A). It is also present in 10 out of the 13 RILs with extreme low seed size (lanes P-Z″) and is absent in all 13 extreme high seed size RILs (lanes C–O), suggesting its probability as a linked marker. Markers co-segregating in a phenotype-specific fashion were selected for segregation analysis in the whole RIL population. The primers which did not show co-segregation with the phenotype were excluded from this analysis. ISSR markers were scored for their presence or absence, since they are considered to be dominant markers. A total of 15 putative markers identified by selective genotyping, and generated by amplification using nine ISSR primers, were used to screen the 113 RILs and the data was recorded for linkage analysis. The genotypic data of

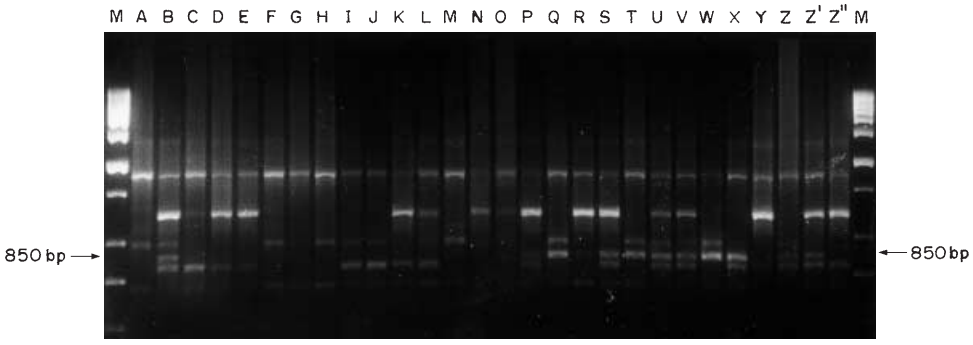

Fig 2 Selective genotyping of RILs representing both extremes of Rye Selection 111 x Chinese Spring with UBC 815. *M*=1 kb λ Ladder; *lane A*: Rye Selection 111, *lane B*: Chinese Spring; *lanes C–O*: high seed size RILs; *lanes P–Z″*: low seed size RILs. An 850-bp band, indicated by an *arrow*, is present in Chinese Spring and low seed size RILs but absent in Rye Selection 111 and high seed RILs

Table 1 Segregation of positive putative ISSR fragments linked to TKW in wheat RILs developed from a cross between Rye selection111 and Chinese spring. a: band present, b: band absent. R: purine, Y: pyrimidine

Primer	Sequence	Fragment size bp	Segregation a:b	χ^2	Probability *P*
UBC815	CTCTCTCTCTCTCTCTT	850	65:47	2.89	0.05–0.10
UBC815	CTCTCTCTCTCTCTCTT	800	52:60	0.57	0.30–0.50
UBC818	CACACACACACACACAG	1000	71:42	10.45	<0.005
UBC812	GAGAGAGAGAGAGAGAA	750	36:74	12.12	<0.005
UBC842	GAGAGAGAGAGAGAGAYG	600	69:44	5.6	<0.02
UBC843	CTCTCTCTCTCTCTCTCTRA	1200	54:56	0.036	0.75–0.80
UBC814	CTCTCTCTCTCTCTCTA	750	56:57	0.008	0.90–0.95
UBC815	CTCTCTCTCTCTCTCTT	1200	30:82	23.92	<0.005
UBC848	CACACACACACACACARG	600	53:58	0.22	0.50–0.75
UBC848	CACACACACACACACARG	1500	36:75	13.7	<0.005
UBC848	CACACACACACACACARG	350	65:44	3.25	0.05–0.10
UBC876	GATAGATAGACAGACA	1100	54:53	0.09	0.75–0.80
UBC840	GAGAGAGAGAGAGAGAYT	1200	30:79	12.0	<0.005
UBC848	CACACACACACACACARG	850	45:65	3.62	0.05–0.10
UBC848	CACACACACACACACARG	900	44:67	5.76	0.01–0.02

Table 2 Percent phenotypic variation in the association of ISSR markers with TKW

Marker	Location[a]	R^2 x 100	*P*
$UBC815_{850}$	6BL	14.8	0
$UBC815_{800}$	2DL	9.49	0.001
$UBC812_{750}$	1DS	6.36	0.007
$UBC818_{1000}$	–[b]	8.0	0.002
$UBC842_{600}$	–	4.66	0.002
$UBC843_{1200}$	–	2.92	0.07
$UBC814_{750}$	–	2.61	0.08

[a] Chromosome localization of markers was based on NT and ditelosomic analyses

[b] Chromosomal localization was not possible using NT analysis, since these marker alleles represented parent Rye Selection111 and were absent in Chinese Spring

ISSR scores were analyzed according to chi-square tests for goodness of fit. In RI lines developed by the single-seed descent method, the expected segregation ratio for the two types of parental alleles was 1:1. A majority of markers in the analysis segregated in the expected ratio of 1:1 while few markers showed skewing towards either of the parental alleles (Table 1).

Determination of the association of ISSR markers with TKW

Single-marker analysis using simple linear regression was done using QGENE software to determine first the association between the markers and QTLs controlling the trait and secondly to explore the genetic basis of TKW (Table 2). Three markers, viz. $UBC815_{850}$, $UBC815_{800}$ and $UBC812_{750}$, were found to be associated with a low TKW. The R^2 values of these markers were 0.148 (*P*=0), 0.0949 (*P*<0.001) and 0.0636 (*P*<0.007), respectively, indicating that these markers are significantly associated with a low TKW. An additional four markers, viz. $UBC818_{1000}$, $UBC842_{600}$, $UBC843_{1200}$ and $UBC814_{750}$, showed a significant association with R^2 values of 0.08 (*P*<0.002), 0.046 (*P*<0.002), 0.0292 (*P*<0.07) and 0.026 (*P*<0.08), respectively, indicating that these markers contribute 8%, 4.6%, 2.9% and 2.6%, respectively, to the trait. A multiple linear regression model with these seven marker loci explained approximately 31% of the total phenotypic variance. The remaining eight markers which were also used to study the complete set of RILs did not show a significant association or make any contribution to the trait.

The Mapmaker version 2.0 software was used to construct linkage groups of the above identified markers at LOD values above 3. All of the markers except $UBC848_{850}$ and $UBC848_{900}$ showed no linkage within themselves. However, though found to be linked, these two markers did not contribute to the trait. The fact that all the markers contributing to the phenotype were unlinked indicated that the QTLs controlling TKW might be associated with different chromosomal segments.

Localization of the ISSR markers on the chromosomes

After identification of the association of specific DNA markers with TKW, the next objective was to localize them on the wheat chromosomes. A complete set of nullitetrasomic (NT) lines in the background of Chinese Spring was used to localize the markers linked to a low TKW. Analysis with NT lines indicated that $UBC815_{850}$ was present on chromosome 6B, $UBC815_{800}$ on chromosome 2D and $UBC812_{700}$ on chromosome 1D. Marker analysis with ditelosomics further indicated that $UBC815_{850}$ was present on the long arm of 6B, $UBC815_{800}$ on the long arm of 2D and $UBC812_{750}$ on the short arm of 1D. We could not localize markers linked to a high TKW using NT lines due to their absence in Chinese Spring.

Discussion

Usefulness of ISSR primers in gene-tagging studies in wheat

Our objective in the present work was to provide information about the genetic control of seed size in wheat and to identify molecular markers associated with this trait as well as to test the usefulness of ISSR markers for gene-tagging studies in wheat. The degree of polymorphism exhibited by ISSR markers in our study is relatively high (32.7%) compared to that of the RFLPs and RAPDs reported by Nagoaka and Ogihara (1997). The data generated during the present work has clearly demonstrated normal Mendelian inheritance and the distrubution of ISSR markers throughout the wheat chromosomes. It was also observed that these markers, in conjunction with selective genotyping, are highly useful as genetic markers for genome mapping and gene tagging. We further speculate that because of their longer primer length and higher annealing temperature, technical simplicity and speed, and Mendelian behavior, ISSR markers may rapidly become invaluable tools for wheat genome analysis in the near future.

Implications of our findings for wheat improvement

During the last two decades, several attempts have been made to understand the genetic basis of seed size in wheat. Guira and Saulescu (1996) have reported that chromosomes 6D and 4A are associated with a high TKW while 5B and 5D are associated with a low TKW. Petrovic and Worland (1988) have identified that TKW is associated with chromosome 5D while Halloran (1976) has reported that chromosome 4B is associated with seed size. Recently Campbell *et al.* (1999) have shown that chromosomes 1A, 1B, 3B and 7A have loci which control TKW, while Gupta et al. (1999) have found chromosome 1AS to be associated with TKW in the same cross as ours using STMS markers. Monosomic analysis of seed weight in the same cross, Rye Selection 111 x Chinese Spring, has indicated the presence of at least two QTLs on 1A and 7A for a high TKW, and two QTLs for a low TKW on 6B and 7D (Singh and Dhaliwal, unpublished).

In our analysis, we could identify only one marker on chromosome 6B for low TKW but could not identify any markers on 7D for a low TKW. Similarly, two QTLs for the high TKW present on 1 A and 7 A also could not be identified. Four markers contributing to the high TKW analyzed in our study may be associated with these chromosomes; however, it can not be claimed conclusively since these markers were absent in the low TKW parent Chinese Spring used for developing nullitetrasomic and ditelosomic lines. A minimum of three chromosomes identified by us to be associated with grain weight, such as 2DL, 6BL and 1DS, have previously not been reported to carry genes controlling grain size in wheat and are reported here for the first time. The remaining four markers, which did not show any linkage with either of these three or with themselves in Mapmaker linkage analysis, suggest the involvement of additional chromosomal segments contributing to TKW. Polygenes controlling important metric traits such as seed size are usually distributed among several quantitative trait loci (QTLs) which may not be linked to one another (Law and Worland 1973; Fatokun et al. 1992). The low level of contribution to the phenotypic variation explained by individual markers in our data confirms the quantitative nature of seed-weight inheritance and implies that transfer of the grain size trait can not be approached by any easy way through conventional breeding programs. Moreover, the identification of QTLs contributing to 31% of the total phenotypic variation indicates that more than half of the phenotypic variance has yet to be tapped. However, the magnitude of seed-size variation explained by these markers is substantial in view of the quantitative nature of the trait.

The identification of markers associated with high seed-size QTLs would help breeders to construct beneficial allelic combinations and accelerate breeding programs for the development of large-seeded cultivars. The markers linked to low seed-size QTLs can also be used as indirect selection tools to eliminate the transfer of these alleles into elite breeding lines. The untapped variation could be due to various factors such as incomplete genome coverage, loose linkages between the marker loci and QTLs for seed-size, interaction between seed size

QTLs which are too small to detect, and environmental factors. Further analysis of this population under different agroclimatic conditions to find out stable QTLs as well as to identify additional QTLs (genes) for this trait using an interval mapping approach, an ultimate aim of our study, is our present effort. It is also possible to establish an ensemble of different QTLs linked to seed weight for improved seed size in standard varieties in wheat.

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