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Constructing a Preliminary Wheat Genetic Map Using RGA and AFLP Markers

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ABSTRACT

Scientists are constructing genetic linkage maps composed of DNA markers for a wide range of plant species. In this work, use of Resistance gene analogue (RGA) and Amplified fragment length polymorphism (AFLP) markers in a doubled haploid population derived from a cross between two parents named O734 and F1054 (with corn & wheat crossing method) has been investigated in order to construct a primary genetic map. In total 107 individuals were selected from a population. Mean percentage of RGA, AFLP and both of two markers polymorphism was obtained at the rate of 26.8%, 28.6% and 26.8%, respectively. Data indicated that %56.8 of markers (54 markers) could be placed in 16 linkage groups, whereas 43.2% of them (41 markers) remained un-linked.

Key Words: Genetic map; Linkage groups; Wheat; Marker; AFLP; RGA

INTRODUCTION

Genetic maps provide a powerful research tool in pure and applied genetic studies on many organisms. A complete linkage map is essential for efficiently carrying out molecular analyses such as molecular marker assisted selection, quantitative/qualitative loci mapping and tagging, heterosis prediction and investigation of genetic evolutionary processes among lineages. In wheat, gene mapping is difficult due to polyploidy and low polymorphism. Bread wheat is an allohexaploid species (AABBDD, 2n = 6x = 42) and its haploid genome length is 1.6×10^{10} bps (Maleki *et al.*, 2003). As with numerous other technological advances, many insights have emerged from application of the new tools of molecular biology. RFLP method was first used as a DNA marker to construct genetic maps by Botstein *et al.* (1980). But some practical limitations such as high DNA concentration requirements, complementary analyses of marker (Southern blotting), low polymorphism and little numbers of loci per each analysis made PCR-based markers application more common in genetic mapping. Thus markers like Random amplified polymorphism polymorphic DNA (RAPD), AFLP, Simple sequence repeat (SSR), RGA etc., have become the most common mapping methods in many studies.

A molecular marker has its own advantages and disadvantages. For example, non-replicable RAPD results have decreased its use (Khlestkina *et al.*, 2002). Now-a-days, AFLP, SSR and RGA have been widely used to construct linkage genetic maps rather than other methods. SSR markers have been used more for constructing of basic genetic maps (Raman *et al.*, 1999; Toojinda *et al.*, 2000; Young, 2000; Cervera *et al.*, 2001; Somers *et al.*, 2004); but AFLP and RGA markers have been used to saturate genetic maps (Raman *et al.*, 2000; Kriegner *et al.*, 2000; Cervera *et al.*, 2001; Zhang *et al.*, 2002; Maleki *et al.*, 2003). Less-known RGA marker has significant abilities for quantitative and qualitative loci mapping. The aim of this study was evaluation of AFLP and RGA marker techniques for constructing of preliminary genetic map in wheat.

MATERIALS AND METHODS

Plant material and DNA extraction. A double haploid (DH) population developed from a cross between two wheat parents namely F1054 and O734 (from eastern & western Asia, respectively) through wheat and corn cross. In order to construct a genetic map, a population with 107 individuals, were selected. DNA extraction was carried out according to Dellaporta *et al.* (1983).

RGA Analysis

Selection of primers. RGA reaction was performed according to Chen *et al.* (1997) with little modifications. Eighteen primers (9 pairs) were used in this study, which were previously designed based on conserved leucine rich repeat (LRR), nucleotide binding site (NBS) and serine (threonine) protein kinases (PtoKin) motifs of several known resistance genes (Table I).

PCR amplification. Each RGA-PCR reaction was performed in a 25 µL reaction consisting of 37.5 ng of template DNA, 0.4 mm of all four dNTPs mixture, 2 mm MgCl2, 6 mm of reverse and forward primers (Table II), 1 U of *Taq* DNA polymerase, and 1 X PCR buffer. Amplification was carried out in a BioRad thermocycler with the following cycle profile: 94ºC for 5 min (primary denaturation) and 36 cycles including 94ºC (1 min.), 45ºC

(1 min.), 72ºC (2 min.) and 72ºC (7 min.) for final extension.

Gel electrophoresis, silver staining and photography. After amplification a 5 µL PCR amplicon was electrophoresed on 1% agarose gel. Detection of a low-molecular-weight (30 bp to 2 kbp) smear indicated a successful amplification. To the remaining 20 µL solution, 7 µL of formamide loading buffer [98% formamide, 10 mm EDTA, pH 8, 0.5% (w/v) bromophenol blue & 0.5% (w/v) xylene cyanol] was added to prepare samples for polyacrylamide gel electrophoresis (PAGE). Denaturing PAGE was used to separate amplicons. A 6% polyacrylamide gel (398mm x 338mm x 0.4 mm) was prepared using Bind Silane (Promega). After polymerization, gel was pre-run in 1 X TBE buffer for 40 min at 120 W to reach a gel temperature of 50ºC. Prior to loading the samples and ladder DNA in formamide loading buffer were denatured for 5 min at 94ºC and immediately put on ice. Five to ten µL of each sample was loaded on the gel. Each gel was run at 120 W and 50ºC for 2.5 - 3 h. After electrophoresis the gel was silver stained and photographed.

AFLP analysis. AFLP reaction was performed (with little modifications) according to Vos *et al.* (1995). Genomic DNA (0.5 µg) was incubated for 3 h at 37ºC with 5 U each of *Eco*RI and *Mse*I in 40 µL of 1 X OPA buffer. Next, 10 µL of a solution containing 5 pMmol *Eco*RI adaptors, 50 pMol *Mse*I adaptors, 1 U T4 DNA- ligase, 10 mm ATP and 1 X OPA buffer was added and incubated for 3 h at 37ºC. AFLP-PCR amplification was carried out in two steps: a pre-amplification PCR reaction with *Eco*RI primer sets (*E*) and *Mse*I primers sets (*M*). All *E* primer sets contained the core sequence 5'.GACTGCGTACCAATTC, while all *M* primers had sequence 5' GATGAGTCCTGAGTAA in common. Subsequently, the product of the preamplification was used as template for selective amplification using *E* + NNN in combination with *M* + NNN primers (Table II). Preamplification was carried out in a 25 µL reaction including digested/ligated DNA (6 µL of DNA from previous steps diluted at 1:5 ratios with dd.H_2O), 50 ng *Mse*I and *Eco*RI core primers, 0.2 mm dNTPs, 1 X PCR buffer, 1 U *Taq* polymerase and ddH2o. AFLP reaction with primers having no selective nucleotide performed for 30 cycles with the following cycles profile: a 30 s DNA denaturation step at 94ºC, a 30 s annealing step at 60ºC and a 60 s extension step at 72ºC. Selective amplification was performed using combination of AFLP primers and a 20 µL reaction solution including pre-amplified DNA, as a template (diluted at the 1:5 ratio with dd.H2O) 37.5 ng *Mse*I and *Eco*RI primers, 0.2 mm of all four dNTPs, 1 X PCR buffer, *Taq* polymerase (0.75 U) and dd.H2O. PCR were performed for 36 cycles with the following cycle profile: a 30s DNA denaturation step at 94ºC, a 30ºC annealing step and a 60 s extension step at 72ºC. The annealing temperature in the first cycle was 65ºC, was subsequently reduced each cycle by 0.7ºC for the remaining 23 cycles.

Statistical analysis and scoring. Segregation of marker loci in the population was tested for significant deviation from the expected Mendelian ratio of 1:1, using chi-square analysis (α = 5%). Bands obtained were manually scored in order to perform statistical evaluations. The presence of a

Table I. Primers used for RGA reaction

primer	Sequence ($5'-3'$)
Pto kin 1	GCATTGGAACAAGGTGAA
Pto kin 2	AGGGGGACCACCACGTAG
RLRR for	CGCAACCACTAGAGTAAC
RLRR rev	ACACTGGTCCATGAGGTT
S2	GGIGGIGTIGGIAAIACIAC
AS3	IAGIGCIAGIGGIAGICC
S2	GGIGGIGTIGGIAAIACIAC
AS1	CAACGCTAGTGGCAATCC
XLRR for	CCGTTGGACAGGAAGGAG
XLRR rev	CCCATAGACCGGACTGTT
RLK for	GAYGTNAARCCIGARAA
RLK rev	TCYGGYGCRATRTANCCNGGITGICC
NLRR for	TAGGGCCTCTTGCATCGT
NLRR rev	TATAAAAAGTGCCGGACT
NBS-F1	GGAATGGGNGGNGTNGGNAARAC
NBS-R1	YCTAGTTGTRAYDATDAYYYTRC
CLRR for	TTTTCGTGTTCAACGACG
CLRR rev	TAACGTCTATCGACTTCT

Y=C/T , N=A/G/C/T , R=A/G , D=A/G/T and I=inosine

Table II. Universal and selective primers used for AFLP reaction

Primer codes	EcoRI and MseI selective primers
$E\,37\,,M\,62$	*E*ACG/*M*CTT
$E\,35\,,M\,49$	*E*ACA/*M*CAG
$E\,33\,,M\,50$	*E*AAG/*M*CAT
$E\,38\,,M\,50$	*E*ACT/*M*CAT
$E\,35\,,M\,48$	*E*ACA/*M*CAC
$E\,37\,,M\,50$	*E*ACG/*M*CAT

Table III. Genomic coverage by linkage groups with more than two markers (cM)

RGA+AFLP	RGA
49.7	49.7
194.4	170.7
61.8	61.8
45.8	45.8
49	49
20.3	20.3
111.3	
91.6	

Table IV. Advanced data analysis

Marker	Marker specific primer/polymorphic band score	Chromosome	LOD
AFLP-64	$E_{37}-M_{62}-11$	1a1	6.3
RGA-12	AS_3-S_2-12	3b	3.2
RGA-17	$XLRR-5$	1a1	4.8
RGA-18	$XLRR-6$	1a1	5.8
RGA-45	$RLRR-6$	6u*	3.4
RGA-46	$RLRR-7$	6u	3.2

*u=undefined chromosome

band was marked with A and its absence was considers as H letters. Linkage relationship among markers was derived based on Mapmaker 3.3 (Lander *et al.,* 1987) and drawmap 1.1. software and genetic linkage map were constructed. Linked and unlinked groups were first determined with Mapmaker software with "group" command. "Compare" command was then applied to determine the most appropriate markers with the maximum distance (50 cM) and the most Likelihood of odds (LOD) value (LOD > 2.5) and linkage distances were calculated for each linked group with "map" command. Data obtained were finally used to construct chromosomal map of linkage groups using Drawmap software.

RESULTS

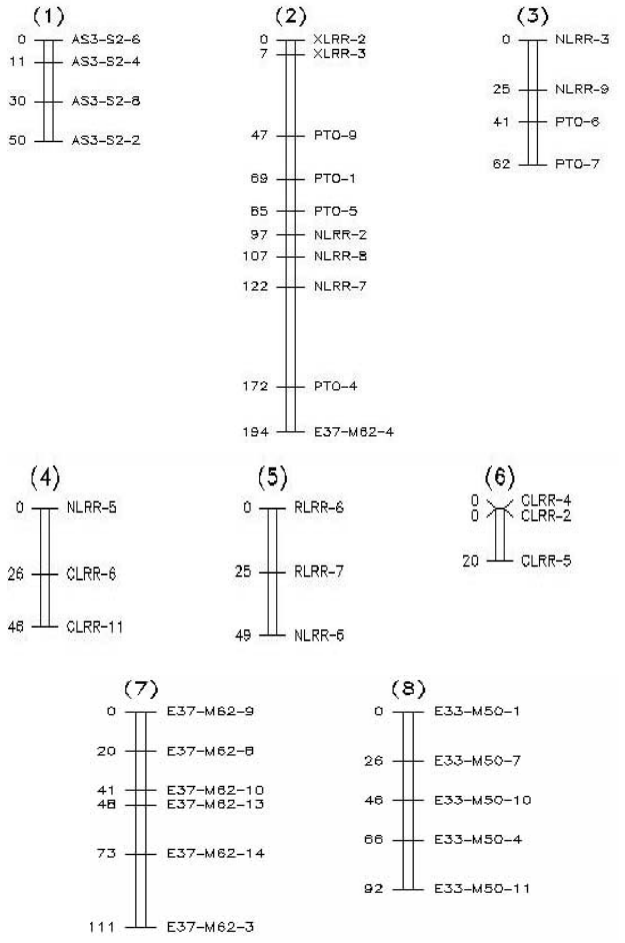
AS1/S2 and NBS (F1/R1) RGA pair primers produced no band, but other seven primers produced many polymorphic bands. Among AFLP primers, three pair primers (*E*33-*M*50, *E*35-*M*49 & *E*37-*M*62) were selected due to their frequent polymorphic bands. A total of 210 - 260 bands were observed among the parents using seven RGA pair primers of which 26.8% showed polymorphism. Using three AFLP pair primers a total of 119 bands were observed of which 28.57% were polymorphic. Deviation from the expected Mendelian ratio within RGA and AFLP markers were 25.4% and 32.4%, respectively (total deviation was 28.4%). About 60.3% of RGA markers were clustered in 12 linkage groups, whereas 39.7% remained un-linked. A 56.8% of total markers (AFLP + RGA) was clustered in 16 linkage groups and 43.2% were un-linked (Fig. 1). More than two markers were observed in 6 RGA linkage groups and total 8 linked marker groups; genomic coverage was calculated for each group (Table III).

With advanced data analysis marker chromosomal loci could be characterized through linkage map construction. Generally in order to achieve the aim, framework maps with known specific marker loci (*i.e.* SSR) were used. One AFLP marker (*E*32- *M*62-11) was mapped in chromosome 1a1. Other remained unlinked and was not assigned to chromosomal loci. Among RGA markers, AS3-S2-12, XLRR-5, XLRR-6; RLRR-6 and RLRR-7 were mapped on chromosomes 1b, 1a3, 1a1, 6 un-defined (u) and 6u, respectively; other remained un-linked and their chromosomal loci were not characterized (Table IV).

DISCUSSION

RGA and AFLP markers were used to construct a preliminary genetic map of wheat. Nine RGA and several AFLP marker pairs were studied and assessed. Among 9 RGA marker pairs, NBS (F1 & R1) and (AS1 & S2) markers produced no band, which is consistent with Chen *et al.* (1997) for wheat, whereas other primers produced many bands most of which were polymorphic. In this study RGA, which produced as many bands as AFLP, proved as a

Fig. 1. Linkage map of wheat (LOD≥2.5); map distances based on cM (numbers on left) and markers (codes on right)



powerful tools for constructing linkage maps (Chen *et al.,* 1997; Kanazin *et al.,* 1997; Raman *et al.,* 1999; Gowda *et al.,* 2002; Maleki *et al.,* 2003).

Three AFLP marker pairs were selected based on their sharpness and scorable bands. Average RGA polymorphism was 26.8%, which is approximate to previous report of 21.3% (Chen *et al.*, 1997). Average polymorphism was almost the same for both RGA (26.8%) and AFLP (28.6%) markers.

With chi-square test (α = 5%), 25.4%, 32.4% and 28.4% of RGA, AFLP and combined markers, respectively deviated from Mandelian segregatation ratio (1:1), which is consistent with other reports (Blanco *et al.*, 1998; Thoquet, 2002; Yuan- De Tan, 2001). With extension in population size, it becomes clear that probability of segregate deviation tends to the standard value. There are various reasons for occurrence of segregate deviation in each experiment such as scoring error, technical problems, small population size, occurrence of mutations in a single locus and artifact bands. Likewise, segregate deviation could be explained through incompatible loci linkage or lethal allele's linkage in

gametes, lethal loci occurrence, interspecific crosses (due to decrease of recombination rate) and finally sampling deviation (Lynch & Walsh, 1998). In this study segregate deviation is more likely result of insufficient markers and consequently from biased sampling. However, heterogeny of parents, selection during doubled haploids population production, non-inbred crosses, introduction of foreign seeds production process and finally band scoring error are major reasons of segregate deviation within DH populations.

Various linkage groups were characterized with RGA and AFLP methods (LOD ≥ 2.5 & maximum genetic distance = 50 cm) and some markers remained un-linked. Altering of LOD values change probability level; the less LOD level, the more markers will be assigned to linkage groups and vice versa. LOD levels are usually applied at 2 or more values. LOD ≥ 3 for durum wheat (Blanco *et al.*, 1998), LOD ≥ 2 for DH population of bread wheat (Somers *et al.*, 2004), LOD ≥ 3.8 for barley DH population (Toojinda *et al.*, 2000), LOD ≥ 2 for tomato BC1 population (Zhang *et al.*, 2002), LOD ≥ 5 for sweet potato (Kriegner *et al.*, 2000), LOD ≥ 2 for Acer (Cervera *et al.*, 2001) and LOD ≥ 2 for wheat genetic mapping (Maleki *et al.*, 2003) are good examples. Un-linked markers are also useful; they could be assigned to linkage groups when used with other frequent linked markers to saturate genetic maps. XLRR-5 and XLRR-6 markers occupying the same linkage group were assigned to chromosome 1a1 through complementary analyses. Likewise, other un-linked markers, were linked when analyzed based on basic marker map and along with the other markers.

RLRR-6 and RLRR-7 markers were classified in a linkage group with another marker (NLRR-6) at LOD ≥ 2, but were un-linked at LOD ≥ 3 and assigned to the wheat chromosome through complementary analyses (along with the other marker within basic map) at LOD ≥ 3. The results indicated that LOD level and presence of other markers in basic map are important factors in marker chromosome mapping. Therefore, values of obtained markers could be argued based on above factors.

Although total marker genomic coverage has been calculated in other works (Blanco *et al.*, 1998; Gowda *et al.*, 2002; Paillard *et al.*, 2003), in this study genomic coverage was separately calculated for each linkage group since limited markers and individuals were available.

There are different marker coverage rates among three genomes of wheat. A and B genomes often show the highest and D genome shows the least coverage rate. AFLP markers often are assigned to A and B and D genome occupied by a few AFLP markers (Somers *et al.,* 2004). In this study, only one AFLP marker was assigned to the A genome. In wheat genetic map constructed by Somers *et al.* (2004) the most microsatellite loci belonged to B genome and the least ones located on D genome. Specific markers could be used for covering D genome due to its low polymorphism. RGA genetic mapping of wheat by Maleki *et al.* (2003) indicated that about a half of markers belonged to B (48%) and other markers were assigned to D (28%) and A (24%). In this study, three markers to A, one marker to B and two others were assigned to un-defined chromosomes; most markers were not assigned to any genomic group. A high Frequency of non-assigned RGA markers in our research is consistence with the results of Maleki *et al.* (2003). The reason of such observation could originate due to lack of proper polymorphism among genomes and coincident migration of fragments.

CONCLUSION

This work has allowed primary wheat genetic map to be constructed using RGA and AFLP markers. RGA and AFLP markers were appropriate for linkage map construction rather than other markers such as SSR, RAPD, RFLP etc., due to more frequent bands, high polymorphism and informative data. RGA has potential for characterizing beneficial genes is its advantage compared with AFLP markers and could be of significant help with genetic map constructing/saturating of crops, quantitative gene tagging, particularly disease resistance breeding, finger printing and biodiversity investigating for basic studies. The represented genetic map is still passing its saturating stages; use of various frequent markers would be beneficial for map saturation.

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