

## Identification of an AFLP marker linked with yellow rust resistance in wheat (*Triticum aestivum* L.)

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**Abstract:** An important application of molecular markers in plant systems involves improvement in the efficiency of conventional plant breeding by carrying out indirect selection through molecular markers linked to the traits of interest. AFLP analysis was used to identify molecular markers associated with yellow rust resistance in wheat (*Triticum aestivum* L.) in this study. DNA isolated from the selected yellow rust tolerant and susceptible F<sub>2</sub> individuals derived from a cross between Izgi2001 (resistant) and ES14 (susceptible) at seedling and adult stage was used for bulked segregant analysis combined with AFLP. From the screening of 34 *Pst*I/*Mse*I AFLP primer combinations, the AFLP marker *P-GAC/M-ACG* (133 bp) was identified and presented in the resistant parent and resistant F<sub>2</sub> individuals but not in the susceptible ones. Future research will obtain more adjacent sequences associated with the polymorphic *M-ACG/P-GAC*<sub>133bp</sub> marker by the PCR walking method to design SCAR markers for wheat breeding programs.

**Key words:** AFLP, bread wheat, disease resistance, SCAR, yellow rust

### 1. Introduction

Wheat (*Triticum aestivum* L.) is the staple food for a large part of the world's population including that of Turkey; enormous population growth and changing lifestyles have posed challenges to wheat breeders to develop newer wheat varieties with high yielding performance, high quality seed, and resistance to pests and stress conditions (Motawei et al., 2007). A diverse group of pathogens including fungi, viruses, bacteria, and nematodes may attack wheat and cause significant yield losses. Among the fungal diseases of wheat, rusts are major disease-causing pathogens. Yellow rust, also known as stripe rust, caused by *Puccinia striiformis* f. sp. *tritici*, is a major disease-causing agent that hampers the production of wheat in many parts of the world (Moldenhauer et al., 2006). Although fungicide applications may dramatically reduce crop losses, high cost, potential environmental problems as a result of fungicide applications, and increased fungicide tolerance through selective pressure on *P. striiformis* create

major drawbacks. Therefore, the deployment of disease-resistant varieties is still considered the most economically effective and environmentally safe method for controlling yellow rust (Line, 2002; Chen, 2005). Seedling resistance to yellow rust is mostly race-specific and is conditioned by the interaction of resistance alleles in the host and avirulence alleles in the pathogen (Torabi and Nazari, 1998). Adult plant resistance, which has been reported to be more durable than seedling resistance (Dyck and Kerber, 1985), can also be race-specific (McIntosh et al., 1995). Genes for adult plant resistance that are effective against a wide spectrum of rust pathotypes can provide horizontal resistance (Robinson, 1976).

The development and use of molecular marker technologies has also facilitated the subsequent cloning and characterization of disease-, insect-, and pest-resistance genes from a variety of plant species including wheat (Hammond-Kosack and Jones, 1997; Meyers et al., 1999). Polymerase chain reaction (PCR)-based assays for

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plant DNA fingerprinting (Ateş Sönmezoğlu et al., 2012; Göçmen Taşkın et al., 2012; Surgun et al., 2012; György et al., 2013; Uslu et al., 2013), amplified fragment length polymorphism (AFLP), has been developed, which reveals significant levels of DNA polymorphism (Vos et al., 1995). The combination of bulk segregant analysis (Michelmore et al., 1991) and high polymorphic PCR-based markers permit the identification and mapping of useful molecular markers for breeding programs. The AFLP technique, with its high multiplex ratio and general robustness, has found various applications in plant genetics (Kiers et al., 2000; Quagliaro et al., 2001; Zhang et al., 2006; Karakas Metin et al., 2013). This technology has been successfully used in several applications such as marker-assisted breeding, construction of high-density molecular linkage maps, and for positional cloning of genes of interest (Breyne et al., 1997; Sorkheh et al., 2007). AFLP markers have been reported for different rust diseases in several plant species, including wheat for leaf rust (Prins et al., 2001; Jia et al., 2004; Li et al., 2007) and for yellow rust (Shao et al., 2001; Imtiaz et al., 2004; Cao et al., 2005; Li et al., 2009). Although the AFLP technique can be used to identify a large number of markers rapidly, AFLP analysis is relatively costly and the method is technologically demanding, which limits its application in breeding programs. Thus, it is important to convert AFLP markers into convenient and inexpensive PCR-based markers such as sequence characterized amplified region (SCAR) markers for marker-assisted selection (MAS) (Wang et al., 2011), which is a powerful tool for breeding programs, since it provides significant advantages to traditional phenotypic screening. MAS is rapid and relatively inexpensive, and it is not hampered by pathogen unavailability.

The objective of this study was to identify AFLP-based markers for yellow rust disease resistance using the bulk segregant analysis approach, which can be beneficial in selecting resistant genotypes in wheat breeding programs.

## 2. Materials and methods

### 2.1. Plant material and yellow rust scores

A cross between the yellow rust resistant and susceptible winter-type Turkish wheat cultivars, Izgi2001 and ES14, respectively, was made in the wheat breeding program of the Anatolian Agricultural Research Institute (AARI). The aim of using these cultivars was to improve the competitiveness of public wheat-breeding programs through the intensive use of modern, particularly marker-assisted, selection technologies. The seedling resistance of grown Turkish wheat parental cultivars and  $F_2$  generations covering 500 individuals was tested in the greenhouse using pathogen races originating from the local Turkish-wheat yellow rust populations. The most resistant and susceptible  $F_2$  progenies at the seedling and adult stage were selected

after 15–20 days following inoculation. The infection types were recorded by using the 0–9 scale of McNeal et al. (1971) at the seedling stage and the 0–100 scale of Cobb at the adult stage (Peterson et al., 1948). The uredospores collection of the Central Research Institute of Field Crops (CRIFC) were dried in silica gels for 8–12 h and stored in glass tubes in liquid nitrogen prior to inoculation. The yellow rust spore population used in inoculations contains *Yr2*, *Yr6*, *Yr7*, *Yr8*, *Yr9*, *Yr11*, *Yr12*, *Yr17*, *Yr18*, *Yr27*, and *YrA+* virulence, and is avirulent for *Yr1*, *Yr5*, *Yr10*, *Yr15*, *Yr24*, *YrSP*, and *YrCV* genes. Following spore inoculations, the most resistant and the most susceptible  $F_2$  progenies at the seedling and adult stages were collected at 15–20 days.

### 2.2. DNA isolation and setting the bulk materials

DNA extraction was performed according to Weining and Langridge's (1991) method for young leaf tissue with modifications. Leaf tissues were collected and frozen in liquid nitrogen before being ground to a powder using the Retsch MM301 system. For bulk segregant analysis, equivalent amounts of genomic DNA from 30 resistant  $F_2$  plants and 30 susceptible  $F_2$  plants were pooled to form resistant and susceptible bulks respectively.

### 2.3. AFLP analysis

AFLP analysis was established according to Vos et al. (1995). A total of 34 *PstI*/*MseI* primer combinations were used for AFLP analysis (Table 1). For each genotype, 500 ng of genomic DNA was digested with *MseI* and *PstI* restriction endonucleases (5 U of each enzyme) for 2–3 h at 37 °C in 10X Buffer Tango at 40 µL final volume. The enzyme activities were terminated by incubating at +80 °C for 15 min. After controlling for complete digestion, double stranded adapters were ligated to the fragment ends. Next, 10 µL of an adapter–ligation mixture (50 pmol *MseI* adapter, 5 pmol *PstI* adapter, 10X T4 DNA ligase buffer, and 1 U of T4 DNA ligase [MBI Fermentas, Germany]) was added to each sample, followed by incubation for 2 h at 22 °C. The adapter–ligation reaction was terminated by incubating at 65 °C for 10 min. The initial PCR reaction was prepared in a 25-µL total volume containing 2 µL of template DNA (100 ng), 50 ng of *MseI* and *PstI* primers, 0.1 mM dNTPs, 1X PCR buffer, 2 mM  $MgCl_2$ , and 1.25 U of Taq polymerase (MBI Fermentas, Germany). The initial 19 cycles of PCR amplification were performed as follows: 94 °C denaturation for 30 s, 56 °C annealing for 30 s, and 72 °C polymerization for 1 min. The pre-amplified DNA was diluted (1:5) and used for selective amplification. Selective amplifications were performed in a 25-µL reaction containing 5 µL of the diluted DNA, 50 ng of *MseI* and *PstI* primers each having 3 selective nucleotides, 0.2 mM dNTPs, 1X PCR buffer, 2 mM  $MgCl_2$ , and 1.5 U of Taq polymerase (MBI Fermentas, Germany). Samples were subjected to the selective amplification as indicated: [(94 °C, 60 s; 65 °C, 60 s; 72 °C, 60 s) × 1 cycle;

**Table 1.** Primer combinations used in the study and polymorphic bands rates (%).

M/P	Primer combination		Polymorphic bands	
	<i>MseI</i> -	<i>PstI</i> -	Number	%
1B	CCT	GTT	3	1.95
1C	CCT	GAC	5	3.24
2B	CAC	GTT	3	1.95
2C	CAC	GAC	2	1.30
2D	CAC	TGG	2	1.30
3A	ACC	CCA	2	1.30
3B	ACC	GTT	3	1.95
3C	ACC	GAC	5	3.24
3D	ACC	TGG	4	2.60
4A	CCA	CCA	-	0.00
4B	CCA	GTT	4	2.60
4C	CCA	GAC	4	2.60
4D	CCA	TGG	3	1.95
5A	CAA	CCA	5	3.24
5B	CAA	GTT	4	2.60
5C	CAA	GAC	9	5.84
5D	CAA	TGG	4	2.60
6A	ACG	CCA	2	1.30
6B	ACG	GTT	1	0.65
6C	ACG	GAC	10	6.49
6D	ACG	TGG	7	4.54
7B	CGT	GTT	15	9.74
7C	CGT	GAC	10	6.49
7D	CGT	TGG	-	0.00
8B	CGA	GTT	8	5.19
8C	CGA	GAC	8	5.19
8D	CGA	TGG	8	5.19
9A	CAT	CCA	-	0.00
9B	CAT	GTT	3	1.95
9C	CAT	GAC	6	3.90
9D	CAT	TGG	4	2.60
10B	CAG	GTT	3	1.95
10C	CAG	GAC	3	1.95
10D	CAG	TGG	4	2.60
<i>PstI</i> adapter <i>MseI</i> adapter	Ligation		5'-CTC GTA GAC TGC GTA CAT GCA-3' 5'-TGT ACG CAG TCT AC-3' 5'-GAC GAT GAG TCC TGA G-3' 5'-TAC TCA GGA CTC AT-3'	
Primers <i>PstI</i> <i>MseI</i>	Pre-amplification		5'-GAC GAT GAG TCC TGA GTA A-3' 5'-CTC GTA GAC TGC GTA CAT GCA-3'	

(94 °C, 60 s; 65–56 °C, decrease of 1 °C each cycle, 60 s; 72 °C, 90 s) × 10 cycle], (94 °C, 60 s; 56 °C, 60 s; 72 °C, 60 s) × 22 cycle; +4 °C (∞). All PCR amplifications were carried out using a Gene Amp PCR System 9700 thermocycler (Applied Biosystems). The selective amplification products were denatured and resolved in 6% polyacrylamide gel electrophoresis at 70 W for 3 h using a Sequi-Gen GT Sequencing Cell system (Bio-Rad), and the products were visualized by silver staining. For confirmation of putative polymorphisms between parental samples and bulk segregants as well as the members of the respective pooled groups, AFLP reactions were triplicated for each primer combination.

#### 2.4. Cloning DNA sequencing of AFLP marker and SCAR primer design

The selected polymorphic bands from yellow rust resistant genotypes were cut out from the polyacrylamide gels and re-amplified using the same primer combinations to be purified by Wizard SV Gel and PCR Clean-Up System (Promega). The amplified polymorphic fragments were cloned using pGEMT-Easy Vector systems (Promega). Following the blue–white colony screening of the positive transformants, the cloned fragments from positive colonies were verified by PCR amplification using T7 and SP6 universal primers. Sequence analyses of the recombinant plasmids were performed using M13-47 sequencing primers and the GenomeLab GeXP Genetic Analysis System (Beckman Coulter).

The sequence data of the cloned polymorphic AFLP fragments were used to design SCAR primers with Primer Premier 5.0 software. A total of 4 primer combinations including 2 forward and 3 reverse primers were generated as SCAR markers (Table 2); these markers were tested on the parental varieties (Izgi2001, ES14) as well as yellow rust resistant  $F_2$  individuals to confirm their specificity in selection of resistant genotypes. The PCR reactions were carried out in 25- $\mu$ L volumes containing 50 ng of template DNA, 2.5  $\mu$ L of 10X PCR reaction buffer, 2.5 U of Taq DNA polymerase, 1.5  $\mu$ L of 25 mM  $MgCl_2$ , 0.5  $\mu$ L of 2 mM dNTPs, and 5 pmol each of forward and reverse primers. The annealing temperature of the SCAR primers was optimized first, using the following cycling parameters: 1

cycle of 2 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at an annealing temperature, and 80 s at 72 °C. Then 5  $\mu$ L of PCR products from each sample were examined in a 1.5% agarose gel to confirm whether the SCAR primers were amplified successfully.

### 3. Results

#### 3.1. Yellow rust scoring

In order to establish resistant and susceptible bulks that can be used in BSA for tagging yellow rust resistance in wheat, we first undertook disease inoculation assays on the parental genotypes. Infection type of selected resistant (R)  $F_2$  individuals was 0–1, while it was 8–9 in susceptible (S)  $F_2$  individuals. The score of Izgi2001 (resistant parent) was 0, while that of ES14 (susceptible parent) was 8 in greenhouse assays. In the field assays, the coefficient of infection (CI) value of Izgi2001 was 0, while that of ES14 was 80. For BSA, 30 resistant and 30 susceptible  $F_2$  seedlings underwent investigation.

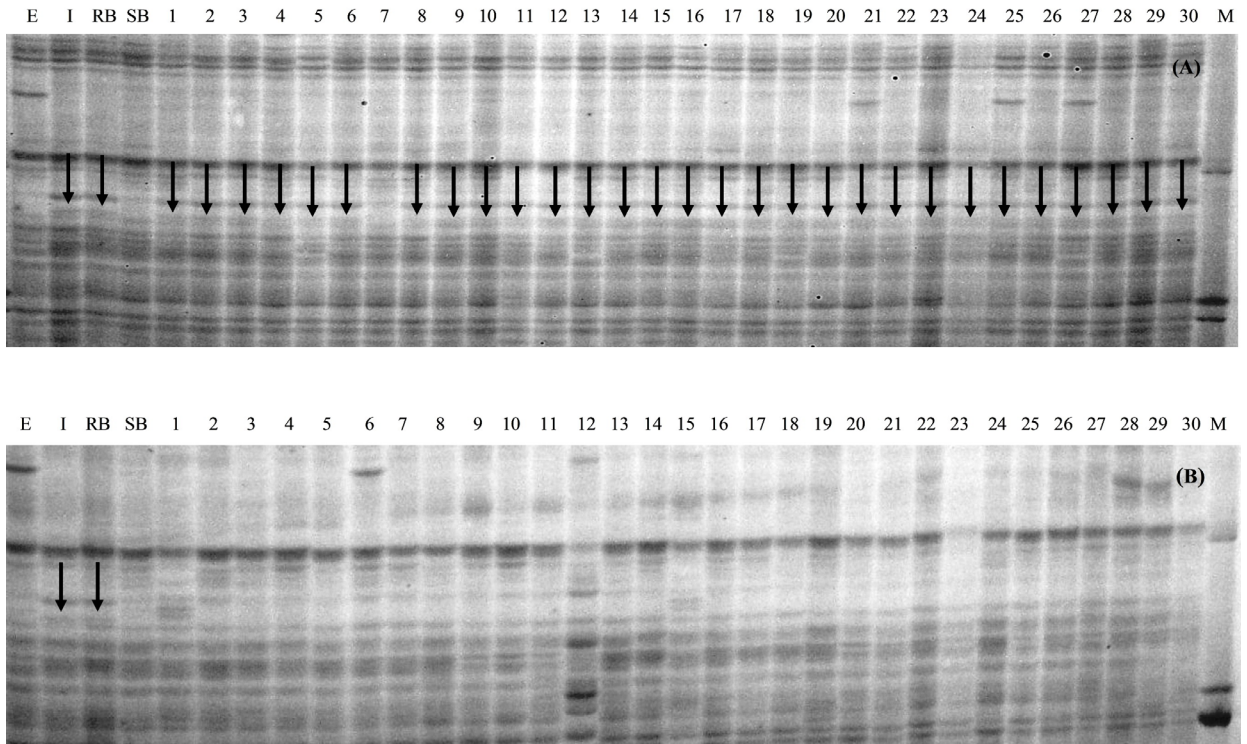
#### 3.2. AFLP analysis

Thirty-four AFLP primer pairs were initially tested to check whether they revealed polymorphic bands between the resistant (Izgi2001) and susceptible (ES14) parents (Table 1). Thirty-one primer pairs (91%) amplified 154 polymorphic fragments in parents, and the remaining 3 primer pairs (9%) amplified monomorphic fragments. These polymorphic primers were also screened against resistant and susceptible bulks of seedling and adult plant stages. The *M*-ACG/*P*-GAC primer combination amplified a DNA fragment of 133 bp that was present in the resistant parent (Izgi2001) and resistant  $F_2$  individuals, but not in the susceptible individuals. The 133-bp fragment was present in 29 out of 30 individuals in resistant bulk at the seedling plant stage and 26 out of 30 individuals in resistant bulk at the adult plant stage, but it was absent in the susceptible individuals (Figures 1 and 2).

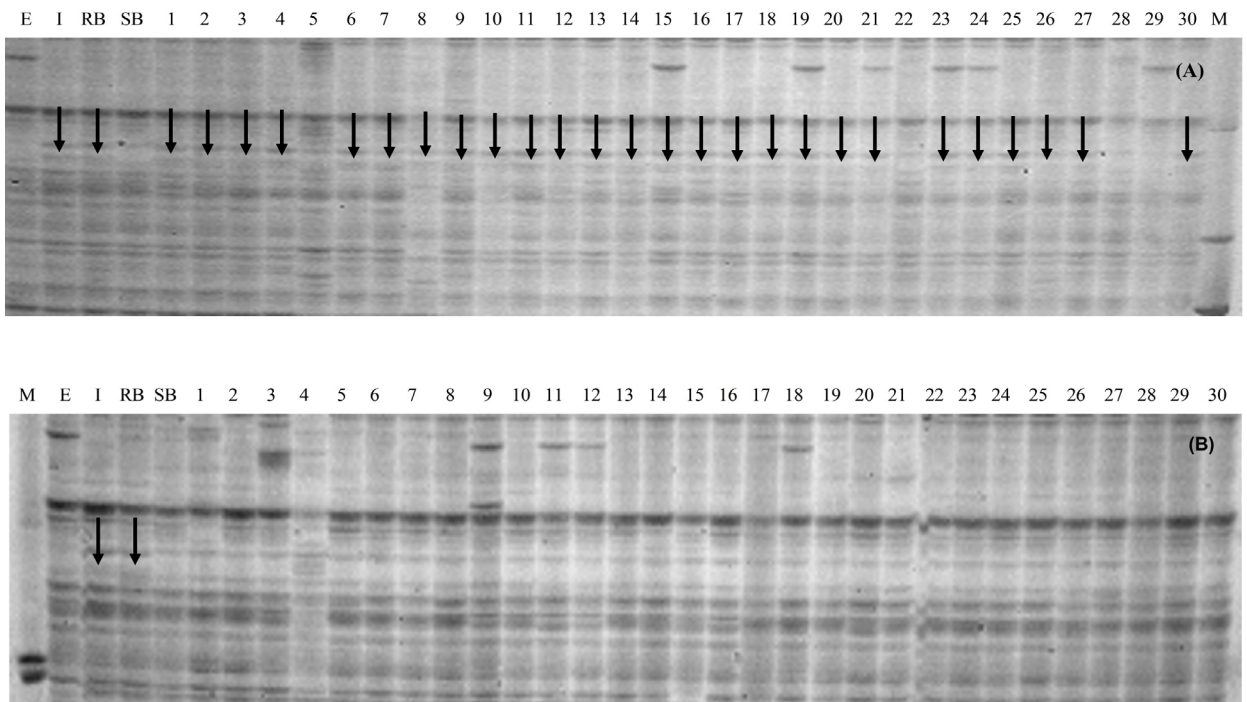
For the purpose of utilizing the markers for yellow rust resistance for fast selection in breeding programs, it is important to convert AFLP markers to SCAR markers. The polymorphic AFLP marker (*M*-ACG/*P*-GAC<sub>133bp</sub>) from Izgi2001 (resistant line) and 55 resistant  $F_2$  individuals (29 from seedling stage and 26 from adult stage) in the resistant

**Table 2.** Primer combinations for SCAR markers.

Primer name	Forward primer (5'–3')	Reverse primer (5'–3')	Tm (°C)
SM1	CAA ACC ACC ACC AAC TAC A	ACG GGG CAT TGA CAA CAC T	66
SM2	ACC ACC AAC TAC AAC ACC C	GCC ATC GAG AAT CAG TTA GAC	66
SM3	CAA ACC ACC ACC AAC TAC A	GCC ATC GAG AAT CAG TTA GAC	57
SM4	CAA ACC ACC ACC AAC TAC A	GGA CGG GGC ATT GAC A	57



**Figure 1.** Bulk segregant analyses of *M*-ACG/*P*-GAC marker at seedling stage in a) resistant F<sub>2</sub> individuals; b) susceptible F<sub>2</sub> individuals. M: 50-bp DNA ladder, E: ES14, I: Izgi2001, RB: Resistant Bulk, SB: Susceptible Bulk, 1–30: F<sub>2</sub> individuals.



**Figure 2.** Bulk segregant analyses of *M*-ACG/*P*-GAC marker at adult stage in a) resistant F<sub>2</sub> individuals; b) susceptible F<sub>2</sub> individuals. M: 50-bp DNA ladder, E: ES14, I: Izgi2001, RB: Resistant Bulk, SB: Susceptible Bulk, 1–30: F<sub>2</sub> individuals.



bulks for both seedling and adult stages was cloned and sequenced. Figure 3 indicates that sequence of the target band amplified by *M*-ACG/*P*-GAC in Izgi2001. Based on the sequence, the 4 combinations of SCAR markers including 2 forward and 3 reverse primers were designated using Primer Premier 5.0 software; their sequences are shown in Table 2. The results with 4 primer combinations derived from the AFLP marker sequence showed that there is no polymorphic band between resistant parent and susceptible parent for the SCAR marker. Thus, the AFLP fragment (133 bp) was not successfully converted into a dominant SCAR marker.

#### 4. Discussion

Bulked segregant analysis (BSA) is a time- and labor-saving approach for QTL-MAS analysis to eliminate the screening of a relatively large number of individuals in the population without compromising the represented marker diversity within the population. Although BSA was first applied to the identification of qualitative markers linked to single-gene-controlled traits such as disease resistance (Michelmoore et al., 1991), it has also recently been applied for quantitative traits (QTLs) such as heat tolerance (Zhang et al., 2009), salt tolerance (El-Kadi et al., 2006), and drought tolerance (Venuprasad et al., 2009; Kanagaraj et al., 2010; Vikram et al., 2011). Despite the fact that the BSA approach may not consider all recombination options in the population for molecular markers, it has still been proven to be powerful enough to identify major qualitative traits that are useful for molecular marker screening.

AFLP is a very powerful tool for generating markers for genetic mapping and for generating markers around a specific locus of interest (Thomas et al., 1995; Vos et al., 1995). AFLP markers have been reported for yellow rust diseases in several plant species including wheat (Imtiaz et al., 2004; Li et al., 2009). In our work, 34 AFLP primer combinations were screened, and 31 primer combinations displayed polymorphism in a Izgi2001 × ES14  $F_2$  segregated population against central Anatolian *Yr* strains (*Yr1*, *Yr5*, *Yr10*, *Yr15*, *Yr24*, *YrSP*, and *YrCV*) at both adult and seedling stages.

One AFLP marker linked to yellow rust was acquired: *M*-ACG/*P*-GAC<sub>133bp</sub>, which was found to be linked to yellow rust resistance. However, it is difficult to employ the AFLP technique directly in a MAS program or for map-based gene cloning because of its high cost and complicated

methodology. Converting AFLP markers into easy-to-use markers such as SCAR is critical. The converted SCARs are highly reliable, relatively inexpensive, and can be easily manipulated. Thus, they are valuable in practical breeding programs where large numbers of individuals need to be genotyped at low cost (Kelly et al., 2003), and for map-based gene cloning (Bradeen and Simon, 1998; Qu et al., 1998; Xu et al., 2001). In the literature, there are examples of converting AFLP markers into SCAR markers (Shan et al., 1999; Xu et al., 2001; Boukar et al., 2004; Shirasawa et al., 2004). Li et al. (2009) used AFLP technology in combination with BSA for mapping the yellow rust resistance gene *YrC591* in wheat, and a SCAR marker (*SC-P35M48*) derived from an AFLP marker (*P35M48373*) was identified as being closely linked to the resistance gene. In contrast to these studies, several studies in the literature revealed the inability to convert AFLP fragments with sizes <200 bp (De Jong et al., 1997; Negi et al., 2000). According to Horn et al. (2003), the possibility of converting AFLP markers into sequence-specific markers is often restricted because of the markers being too short, and the fact that most AFLP polymorphisms seem to originate in differences within the restriction sites. Bradeen and Simon (1998) pointed out that the AFLP fragment is too short for designing an appropriate PCR primer to amplify a polymorphic band, while Prins et al. (2001) reported that different AFLP fragments of the same size may migrate together in the gel, and a target polymorphic band may contain contaminating fragments from adjacent bands. This is not the case for *M*-ACG/*P*-GAC<sub>133bp</sub> marker in our study, because of the uniform sequence data of the marker from different lines including resistant parent and resistant  $F_2$  individuals from both stages. Shan et al. (1999) found that the restoration of the original polymorphism remains difficult since the cloning procedure required for the AFLP conversion often contributes to the loss of the original polymorphism (Wei et al., 1999).

In our study, the amplified AFLP fragment was cloned and sequenced and then used to produce extended primers. The *P*-GAC/*M*-ACG<sub>133bp</sub> marker fragment was too short and so, based on its sequence, we were only able to design a maximum of 4 primer combinations including 2 forward and 3 reverse primers to try to convert it to fast PCR-based markers; we did not succeed in converting the AFLP marker into a SCAR marker, which may be attributed to the following reasons: (1) the fragment was too short to have a suitable primer combination site within

GACTGCGTACATGCAGACAAACACAATTTAGACTATAGTAAGGTACACGTGCAT  
TGCACGCATGAGATTTGGCAACCAAATTATGAATAAATACCACATTATAGCATG  
CAAGCCTTGAGTCATATATGCATGATGTAGATGTTTCGTTTACTCAGGACTCATC

**Figure 3.** Sequence of target band amplified by *M*-ACG/*P*-GAC in Izgi2001. Italic letters indicate the primer sequence; letters with underlining indicate the open reading frame (ORF).

it; (2) the presence of mutations or deletions at the primer binding sites; (3) the large genome size of wheat ( $1.6 \times 10^9$  bp). There is 3500 kb average in 1 cM of the wheat genome; the fragment may be located outside the resistance sequence but be the same as in the susceptibility sequence. As in our work, Zhang et al. (2006) also did not succeed in converting our AFLP marker, P-AGG/M-GAG<sub>261bp</sub>, into a stable STS marker during several attempts in their study.

Usually, random amplified polymorphic DNA (RAPD) markers are easily converted to either SCARs or cleaved amplified polymorphic sequences (CAPSs) for rapid detection, as RAPD fragments are generally in the size range of 500–1500 bp. However, in the case of AFLP markers, which are from 100 to 300 bp in size, successful conversion is not easy. It has been reported that AFLP markers are almost impossible to convert directly to other types due to their short sequence (<250 bp) (Negi et al., 2000). In our work, the resulting DNA sequence was too short (133 bp) to optimally design SCAR primers, and too short to expect internal polymorphisms, which can be used to differentiate between alleles. This probably severely reduced the efficiency of the marker conversion.

One of the limiting steps in this procedure was the fragment size of the AFLP markers, which are recommended to be greater than 100 bp in length and have a minimum G/C content of 50% (Bradeen and Simon, 1998). In our case, G/C content of the *M*-ACG/*P*-GAC<sub>133bp</sub> marker was about 30%. Moreover, in some cases, because of the predominantly homologous sequences of the different

genotypes and 5'-ends tolerable of mismatches at primer-binding sites, specific primers designed from this sequence will yield monomorphic amplification products (Plieske and Struss, 2001). Several procedures have been proposed to solve the problems mentioned above. In the case of too-short AFLP markers, Negi et al. (2000) pointed out that is essential to isolate the flanking regions for the conversion to SCAR markers. These authors suggested the use of a PCR walking approach to isolate fragments adjacent to the AFLP markers. Our next objective will be to obtain more adjacent sequences associated with the polymorphic *M*-ACG/*P*-GAC<sub>133bp</sub> marker by the PCR walking method. After obtaining target fragments by PCR walking, they will then be purified, cloned, and sequenced in the same way as the *M*-ACG/*P*-GAC<sub>133bp</sub> marker. Based on the flanking sequences, SCAR primers will be expected to be obtained, and these SCAR markers will provide more effective selection for yellow rust resistant genotypes in wheat breeding programs. In addition, more markers are needed for identifying and mapping the genes. Therefore, we expect that the AFLP marker identified in this study, *M*-ACG/*P*-GAC<sub>133bp</sub>, will contribute to meeting this requirement.

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