Fingerprinting of common wheat cultivars with an Alw44I-based AFLP method

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Abstract. A simplified AFLP method, based on methylation-sensitive Alw44I restriction endonuclease, has been developed and evaluated for fingerprinting 15 wheat cultivars. The selected germplasms represented groups of spring and winter wheats with and without the 1BL.1RS translocation. Ten selective primers yielded 57 markers, including 19 polymorphic bands. Three markers (15.8%) were specific to wheat carrying the 1BL.1RS translocation, thus conflicting with the frequency expected by random marker distribution (2.4%), and suggesting qualitative differences in DNA methylation among winter wheat cultivars with the 1BL.1RS translocation. Mean Dice’s similarities ranged from 0.85 to 0.99, thus all cultivars could be identified by the banding profile. Winter wheat cultivars, with and without the 1BL.1RS chromosome, were slightly more similar to one another (0.959) than spring wheat cultivars (0.952). Five (9%) specific markers were obtained from cultivars Sicco, Cheyenne, Fenman, Disponent and Chinese Spring.

Key words: AFLP, 1BL.1RS translocation, genetic diversity, Triticum aestivum, wheat.

Introduction

Common wheat, Triticum aestivum (2n = 6x = AABBDD), is one of the most important crops for the global economy. Many genetic maps of wheat have been developed with available marker systems (Devos and Gale 1996; Röder et al. 1998; Gupta et al. 2002). These maps can serve as a starting point for the detection of gross rearrangements in the genome and subsequent monitoring of alien introgression during the breeding process. At present, projects are running for SNP (Single Nucleotide Polymorphism) identification and validation (http://wheat.pw.usda.gov/ITMI/WheatSNP/), which can contribute to the creation of new high-density genetic maps. Maps can be exploited for accelerated identification of markers linked with a trait of interest and sometimes genes (Yan et al. 2003). Many quantitative trait loci (QTL) have been mapped on wheat chromosomes, including disease resistance (Waldron et al. 1999; Anderson et al. 2001), quality parameters (Blanco et al. 1998; Prasad et al. 1999; Rousset et al. 2001; Batey et al. 2002; Blanco et al. 2002), yield-affecting traits (Shah et al. 1999; Börner et al. 2002), lodging (Keller et al. 1999), preharvest sprouting, and seed dormancy (Zanetti et al. 2000; Kato et al. 2001; Groos et al. 2002). Application of molecular markers in wheat breeding has been reviewed by Gupta et al. (1999) and updated in the Wheat Gene Catalogue (McIntosh et al. 2003).

Modern wheat cultivars display a low level of gene pool variation, which can result from different breeding traditions and specific environmental conditions (Metakovsky and Branlard 1998). Many DNA marker systems have been used for estimation of polymorphism in common wheat. RFLP and STS-PCR generated from 1.2 to 3.3 analyses (Kim and Ward 1997; Paull et al. 1998) and from 1.9 to 4.3 polymorphic fragments (Chen et al. 1994; Burkhamer et al. 1998). The number of alleles per locus in wheat for microsatellite markers varied from 3.7 (Nagaoka
and Ogihara 1997) to 10.5 (Röder et al. 2002). The AFLP method proved to be a robust marker system and has been widely used, mainly for mapping purposes and diversity analysis.

However, many recent modifications of AFLP have been developed. They aim at detection of changes in DNA methylation (Knox and Ellis 2001), merging with primers recognizing a given gene or gene family, and application of different resolution power. Wong et al. (2000) proposed the use of HPLC in place of gel electrophoresis as a reliable and efficient purification step for resolving the multicomponent PCR because the process can be readily automated and the collected fractions can be directly sequenced after vacuum drying. Silver staining of AFLP products has been described and can substitute for the isotope method (Chalhoub et al. 1997). Lan and Reeves (2000) designed modified adapter primers to enhance the frequency of MseI-EcoRI fragments in AFLP banding patterns. The addition of phosphorylated amino groups prevented the formation of MseI-MseI fragments and left the amplification of EcoRI-EcoRI bands linear.

For each two-cutters-based AFLP employing methylation-sensitive endonuclease, a methylation-insensitive assay can be developed by preamplification of a partially digested template before the use of the methylation-sensitive cutter as in SDAFLP (secondary digest AFLP) (Knox and Ellis 2001). A simplified AFLP method has also been described. It exploits a single cutting enzyme (EcoRI) and the resolution of amplified fragments on agarose (Suazo and Hall 1999). Potentially, AFLP methods can be developed for any restriction enzyme (RE) that leaves a 5’ or 3’ overhang. There is a need for development of such methods to detect differences among closely related clones or specimens. In the case of bacterial genomes, selective primers with one selective nucleotide are often used. This restricts the number of primer combinations to 16, which may not be sufficient to generate differential fingerprints or markers for homogenous clones or species and thus require the use of alternative restriction enzymes (Lan and Reeves 2000). Combinations with alternative enzymes (XbaI, BglIII), without selective nucleotides, have been employed in AFLP for high-density mapping of the Mlo locus in barley (Simons et al. 1997). Another promising endonuclease is PsiI, which recognizes the site 5’-CTGCAG-3’, and up to 8% of clones generated with this enzyme in barley are significantly similar to coding regions (Michalek et al. 1999). *Alw44I* shares some features of PsiI, like sensitivity to methylation, and recognizes similar sequences (5’-GTGCAC-3’), which are the core of the G-box (Bága et al. 1995). Cutting by *Alw44I* and *PstI* is prevented by cytozyme methylation. *Alw44I* is not sensitive to adenine methylation in homozgyous GTGCA\(^{m5C}\) form (Roberts and Macelis 2001). The use of another RE opens the possibility of methylation assessment in different DNA motifs.

The objectives of this study were: (1) to develop a simplified AFLP protocol based on the *Alw44I* enzyme, and (2) to determine the extent of DNA polymorphism and the genetic relatedness among selected common wheat cultivars.

### Materials and methods

#### Plant material

The 15 wheat cultivars used in the study were randomly selected to represent three groups: (a) spring wheat: Frontana (CGN12668), Era (CGN08469), Marquis (CGN12699), Timentin (CGN10464), Chinese Spring (CGN12743), Sicco (CGN16084), (b) winter wheat: Fenman (CGN05450), Sava (CGN09043), Cheyenne (CGN09215), Kharkof (CGN04370), and (c) winter wheat with 1BL/1RS translocation: Disponent (CGN16117), Aurora (CGN09021), Hornt (CGN16125), Solaris (CGN14985), and Clement (CGN08570). The seeds were provided by Dr. LJM van Soest (Plant Genetic Resources Centre, Wageningen, The Netherlands). The presence of chromosome arm 1RS was verified by using Iqbal and Rayburn’s (1995) method.

#### Template DNA preparation

DNA was extracted from 2 different bulk samples, made of 5 leaves collected from plants germinated on Petri dishes for each variety. Milligan’s (1992) DNA extraction method was used with final RNAse treatment. The method of template preparation was the same as in Suazo and Hall (1999) with minor modifications.

Two \(\mu\)g of DNA were digested with 5 U of *Alw44I* (Fermentas, Lithuania) for 18 hours at 37°C in 1X Buffer Y\(^+\) (33 mM Tris-acetate, pH 7.9, 10 mM MgCl\(_2\), 66 mM potassium acetate 0.1 mg mL\(^{-1}\) BSA) in a total volume of 20 \(\mu\)L. Five \(\mu\)M concentrations of double-stranded *Alw44I* adaptors were prepared after renaturation of equimolar volumes of AL(AF) and AL(AR) primers (Table 1). Ten \(\mu\)L of ligation mix (500 nM *Alw44I* adaptor, 1 U ligase, 120 mM
Tris-HCl, 30 mM MgCl₂, 30 mM DTT, 1.5 mM ATP, pH 7.8 at 25 °C) were added to the digested sample for a final volume of 30 μL, and the samples were incubated for 4 hours at 37 °C. Subsequently ligated DNA was precipitated for 2 hours at –20 °C with 90 μL of EtOH:AcNa (3 M, pH 5.2) mix (24:1), centrifuged for 20 min at 14 000 rpm and rinsed twice with 70% ethanol. DNA was dissolved in 50 μL of water and stored at –20 °C until required.

Selective amplification

PCR reactions were carried out in 20 μL volume containing: 2 μL of 10-fold diluted ligation mix, 200 μM of each dNTP, 1.5 mM MgCl₂, 0.4 U Taq DNA Polymerase (Fermentas), 1 X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40), 750 nM selective primer (Table 1). Amplifications were carried out in a PE 480 thermal cycler under the following programme conditions: initial denaturation for 2.5 min at 95 °C, 7 cycles with 30 s at 94 °C, 30 s at 67-61 °C (decreasing 1 °C per cycle) and 30 s at 72 °C, followed by 33 cycles with 45 s at 94 °C, 45 s at 60 °C, 45 s at 72 °C, and a final extension of 10 min at 72 °C. Products of amplification were resolved in a PE 480 thermal cycler under the following programme conditions: initial denaturation for 2.5 min at 95 °C, 7 cycles with 30 s at 94 °C, 30 s at 67-61 °C (decreasing 1 °C per cycle) and 30 s at 72 °C, followed by 33 cycles with 45 s at 94 °C, 45 s at 60 °C, 45 s at 72 °C, and a final extension of 10 min at 72 °C. Products of amplification were resolved on a 1% agarose gel containing 0.1% EtBr in 1 X TBE buffer (89 mM Tris-borate, 2.5 mM EDTA). Fragments were examined under UV transilluminator and photographed. The Gene Ruler® 100 bp DNA Ladder Plus was used to determine the size of the products.

Data analysis

Photographs of the gels were analysed by using Scion Image Beta 2 software. Presence or lack of bands was considered as a single trait and values 1 or 0 were assigned respectively. Genetic pairwise similarities between analysed materials were estimated with a formula developed by Dice, as in Nei and Li (1979):

\[ F = \frac{2N_{XY}}{N_X + N_Y}, \]

where \( N_{XY} \) is the number of bands shared by two compared genotypes (X and Y), while \( N_X \) and \( N_Y \) are total numbers of bands observed in genotypes X and Y, respectively. Basing on the matrix of genetic similarity, cluster analysis was performed. The UPGMA method was used for clustering, employing the NTSYS-pc program (Rohlf 2001). Bootstrap analysis was performed with WinBoot software (Yap and Nelson 1995).

**Results and discussion**

The total number of 57 scorable products were produced by 10 selective primers (range 1–8). The average number of polymorphisms generated by a selective primer was 1.9 and varied from 0 to 5. In a similar study with the Mph1103I (AvaIII) restriction enzyme, only monomorphic fragments were obtained (not shown). Interestingly, 3 out of 19 polymorphic bands were specifically amplified in wheat samples with the 1BL.1RS translocation with AL3 and AL5 primers (Figure 1 A,B). Moreover, the DNA pattern obtained with these primers was of ‘smear’ shape, thus suggesting that additional multiple cutting events occurred in selected motifs of wheat carrying 1RS. This can be explained by the qualitative differences in the DNA methylation pattern associated with the presence of the 1RS chromosome arm in the winter wheat background. Castilho et al. (1999) found no quantitative differences in methylation pattern of R and AB genomes in triticale Lasko. Assuming random distribution of DNA markers along wheat chromosome arms, it can be expected to find one chromosome-arm-specific marker for 42 bands, so the frequency of 1RS-specific markers found in the present study is 6.6 times higher, thus supporting the hypothesis of qualitative differences in DNA methylation.

Mean Dice’s similarities within winter wheat cultivars with and without the 1RS chromosome arm were 0.959, compared to 0.952 in the case of spring wheat. Mean Dice’s similarity between the groups of winter and spring wheat was 0.952, while larger distances were observed for these groups in relation to translocated winter wheats.

**Table 1. Oligonucleotides used in Alw44I-AFLP**

<table>
<thead>
<tr>
<th>Primer symbol</th>
<th>Sequence 5′–3′</th>
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<tbody>
<tr>
<td>AL(AF)</td>
<td>CTG gTA gAC TgC gTA C</td>
</tr>
<tr>
<td>AL(AR)</td>
<td>TgC Agg TAC gCA gTC</td>
</tr>
<tr>
<td>Core sequence:</td>
<td>gAC TgC gTA CCT gCA CNNN</td>
</tr>
<tr>
<td>Selective bases</td>
<td></td>
</tr>
<tr>
<td>AL1</td>
<td>Ag</td>
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<tr>
<td>AL2</td>
<td>AT</td>
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<td>AL4</td>
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<td>AL8</td>
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</tr>
<tr>
<td>AL9</td>
<td>gAA</td>
</tr>
<tr>
<td>AL10</td>
<td>gAg</td>
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These values are well represented on the UPGMA dendrogram (Figure 2). Wheat cultivars with 1BL.1RS translocation are well separated from the other germplasm intermixed in the main cluster. In the previously described simplified AFLP method that uses the single PstI endonuclease, 4.4 polymorphisms per primer on average were detected on a similar set of wheat cultivars (Tyrka 2002). However, PstI with respective selective nucleotides did not enable group-specific discrimination of cultivars with better separation of spring wheat. Five (9%) cultivar-specific markers were obtained for Sicco, Cheyenne, Fenman, Disponent and Chinese Spring. No cultivar was identical according to values of similarity, thus...
suggesting that the extent of analyses performed is sufficient to discern all genotypes analysed.

Conclusions

The unexpectedly high frequency of 1RS-specific bands suggests qualitative differences in DNA methylation pattern among 1BL.1RS winter wheat cultivars. The Alw44I-based AFLP method has a relatively low efficiency (1.9) for generating DNA polymorphisms, but it is sufficient for fingerprinting of the cultivars analysed.

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REFERENCES


