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RAPDs as molecular markers for the detection of *Aegilops markgrafii* chromatin in addition and euploid introgression lines of hexaploid wheat

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Abstract *Aegilops markgrafii* contains resistance genes to powdery mildew, leaf rust and stripe rust, and also has high crude protein and lysine contents, which can be useful for wheat improvement. These important traits are localized on different chromosomes. Disomic *Triticum aestivum-Ae. markgrafii* addition lines and euploid introgression lines showing leaf-rust and powdery mildew resistance were screened with RAPDs to detect chromosome-specific markers which can accelerate the breeding process. RAPD markers for all six available disomic addition lines were obtained. The additional chromosomes B, C, D, E, F and G were identified by three, three, three, two, one and seven primers, respectively. All three chromosome-B-specific RAPD markers demonstrated the presence of alien chromatin in the leaf-rust-resistant 42-chromosome introgression lines as well as in the segregating progeny. The three chromosome-C-identifying primers also demonstrated the presence of that chromosome in powdery mildew-resistant euploid introgression lines. The substitution lines (5A)5C and (5D)5C with different genetic backgrounds for both parents, in comparison to the lines mentioned above, showed the chromosome C-specific band with only two of the three primers. The chromosome F-specific primer and a primer evident on all the *Ae. markgrafii* chromosomes analysed did not generate the expected fragments on the chromosome F_{del} addition line, indicating that the markers are located on the deleted part of chromosome F.

Key words *Aegilops markgrafii* · *Triticum aestivum* · RAPD · Addition lines · Leaf rust · Powdery mildew

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Introduction

Aegilops markgrafii (Greuter) Hammer (synonym *Ae. caudata* L., 2n = 2x = 14, genome CC) is of value for wheat improvement because of its resistance genes to powdery mildew, leaf rust and stripe rust (Valkoun et al. 1985; Schubert et al. 1995). Therefore, a set of disomic *Triticum aestivum-Ae. markgrafii* addition lines was established to provide the basis for alien gene transfer into wheat and for gene localization on the added chromosomes. During this process, spontaneously occurring genetic rearrangements produced different 42-chromosome introgression lines with powdery mildew and leaf-rust resistance and these were selected.

The alien chromosomes can be identified by C-banding (Friebe et al. 1992), morphological characters, isozymes, seed storage proteins and resistance reactions (Schubert and Blüthner 1992, 1995; Schmidt et al. 1993). Schubert et al. (1993) identified alien chromosome-B chromatin in euploid introgression lines on the basis of their resistance to leaf rust found in the disomic addition line B. Furthermore, tightly-linked molecular markers are necessary to monitor the introduction of valuable characters into wheat.

RAPDs were chosen as molecular markers because they are easy to use and cost little. RAPDs have been described as molecular markers for alien chromosomes in wheat addition lines containing *Hordeum vulgare*, *H. chilense* and *Thinopyrum bessarabicum* chromosomes, respectively (Devos and Gale 1992; King et al. 1993; Hernández et al. 1995). They were also successfully used to analyse genetic variability between wheat varieties (Devos and Gale 1992; He et al. 1992) and for the development of closely linked markers to important characters. RAPD markers linked to important resistance genes in wheat were described by Qi et al. (1996) and Demeke et al. (1996). Schachermayr et al. (1995) established a sequence-tagged-site (STS) marker

derived from RAPD analysis in common wheat which was completely linked to the leaf-rust resistance gene *Lr24* from *Agropyron elongatum*. An STS-based marker for assisted selection of stem-rust resistance in barley was produced by Horvath et al. (1995). In the present paper we describe RAPD markers useful for identifying *Ae. markgrafii* chromatin in disomic wheat addition lines and euploid introgression lines.

Materials and methods

Plant materials

RAPD analysis was performed on *T. aestivum* L. cv 'Alcedo', *Ae. markgrafii* (Greuter) Hammer var. *markgrafii* (accession 'S740-69'), the amphiploid *T. aestivum* cv 'Alcedo'-*Ae. markgrafii* (acc. 'S740-69'), and six derived disomic addition lines carrying the *Ae. markgrafii* chromosomes B, C, D, E, F and G, respectively. In addition, one line containing a deleted F-chromosome pair was tested. During the production of the addition lines, 42-chromosome introgression lines showing powdery mildew (Eg lines) and leaf-rust (Pr lines) resistance were obtained by spontaneous genetic rearrangements. The substitution line SII contains the *Ae. markgrafii* chromosome C. The production of this material has been described by Schubert (1989), Schubert and Blüthner (1992, 1995) and Schubert et al. (1993). Resistant and susceptible plants were obtained from a segregating progeny of selfed leaf-rust-resistant wheat-like plants.

The (1D)1C *T. aestivum-Ae markgrafii* substitution line produced by Kihara (1958) was supplied by F. Zeller (Technical University Munich-Weihenstephan, Germany). This 1C chromosome should be homologous to chromosome A of accession 'S740-69' which has not yet been added to wheat. The (5A)5C and (5D)5C *T. aestivum-Ae. markgrafii* substitution lines described by Muramatsu (1973) were obtained from M. Muramatsu (University of Okoyama, Japan). The 5C chromosome should be homologous to chromosome C of 'S740-69'.

In addition, 37 *Ae. markgrafii* accessions of different geographic origin were tested with regard to RAPDs.

DNA extraction and RAPD analysis

DNA extraction from fresh leaves was carried out according to the CTAB procedure outlined by Saghai-Maroof et al. (1984).

PCR amplifications were performed in 25-µl reaction mixtures containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 1 U *Taq*-polymerase (AGS, Heidelberg, Germany), 0.2 µM primer (10-mers from Operon Technologies Inc., Alameda, USA, and Roth, Karlsruhe, Germany; 12-mers and 15-mers were kindly provided by T. Debener, BAZ, Ahrensburg, Germany) or 0.5 µM primer (27 10-mers kindly provided by T. Debener, BAZ, Ahrensburg, Germany), respectively, and 20 ng of genomic DNA. The solution was overlayed with 25 µl of paraffin oil (Roth, Karlsruhe, Germany). To check the RAPD marker reproducibility, each primer was tested two or three times on at least two different plants of each introgression line.

Amplifications were performed in a Perkin Elmer Thermal Cycler 1 (Applied Biosystems, Weiterstadt, Germany) as follows: after an initial denaturation of 5 min at 94°C, the reaction was subjected to 45 cycles of 1 min at 92°C, 2 min at 36°C for 10-mer-, 39°C for 12-mer-, 55°C for 15-mer-primers, respectively, and 2 min at 72°C, followed by a final 10-min extension at 72°C. PCR-products were mixed with 0.25 vol of 40% sucrose loading buffer and analysed by electrophoresis in 1.3% agarose gels in 1×TAE-buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) under constant voltage.

Results

The identification of RAPD markers for *Ae. markgrafii* chromosomes in disomic 'Alcedo' addition lines involved two steps of marker screening. In the first marker selection step, we tested 221 single random 10-mer, two 12-mer, and fifteen 15-mer primers with the wheat parent 'Alcedo', the *Ae. markgrafii* parent, and two plants of the 'Alcedo'-*Ae. markgrafii* amphiploid, for the amplification of products polymorphic for the parents and present in the amphiploid. Fiftynine single primers, that is 24.8% of those tested, showed one or more potentially useful polymorphic fragments in the parents and were selected for further screening. In the second step, the selected primers were applied to the parents, the amphiploid, and the seven disomic addition lines, using at least two individuals of each. Fifteen 10-mer and one 15-mer primer produced a distinct reproducible band for only one additional chromosome (Table 1 and Fig. 1). Additional RAPD markers produced by eight different primers showed insufficient reproducibility and they were therefore excluded from further investigations. The two 10-mer primers 3034 and AP-08 showed polymorphic bands for two different added chromosomes. Two clearly distinguishable bands in the *Ae. markgrafii* parent and the amphiploid were also produced by primer L-08 (Fig. 2),

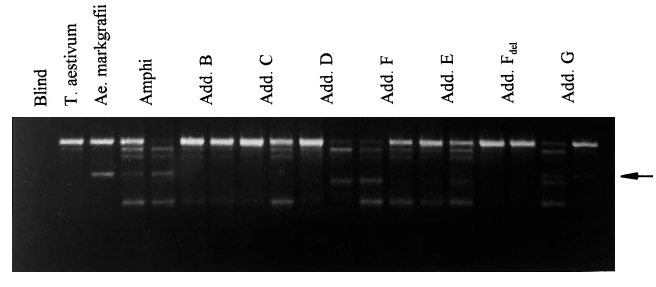

Fig. 1 The chromosome G-specific fragment (primer S-18) evident in *Ae. markgrafii*, the *T. aestivum-Ae. markgrafii* amphiploid, and addition line G (marked by *arrow*)

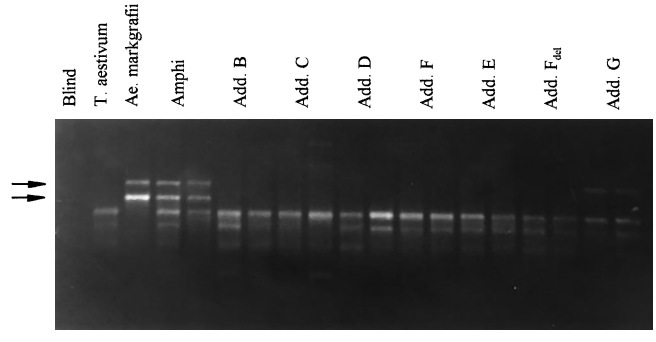

Fig. 2 The chromosome G- and potentially A-specific fragments (primer L-08) evident in *Ae. markgrafii*, the *T. aestivum-Ae. markgrafii* amphiploid and addition line G (marked by *arrows*)

Table 1 *Ae. markgrafii*-specific RAPDs evident in *Ae. markgrafii*, the *T. aestivum-Ae. markgrafii* amphiploid, the derived disomic addition lines (Add), and euploid introgression lines showing leaf rust (Pr lines, Wl res) and powdery mildew (Eg lines) resistance, and the (1D)1C, (5A)5C, (5D)5C substitution lines. x and y: *Ae. markgrafii*-specific bands; -band not evident

Primer	Sequence 5′ → 3′	Alcedo	S740	Amphi	Add B	Add C	Add D	Add E	Add F	Add G	Add F_{del}	Pr 1	Pr 2	Pr 3	Pr 4	Pr 5
2661	CGGCCACTGT	–	x	x	–	x	–	–	–	–	–					
2906	GGTGCACTCC	–	x	x	–	–	–	–	–	x	–					
3034	TCTGTGCTGG	–	xy	xy	x	–	–	–	–	y	–	x	x	x	x	x
3039	GACCGCTTGT	–	x	x	–	–	x	–	–	–	–					
3040	TTCGAGCCAG	–	x	x	–	–	x	–	–	–	–					
3081	TTGGCGGCCT	–	x	x	–	–	–	x	–	–	–					
3221	AAGAGGCCAG	–	x	x	–	–	–	–	–	x	–					
29469	ACCGCGAAGGGGACC	–	x	x	–	–	–	–	–	x	–					
AP-04	CTCTTGGGCT	–	x	x	x	–	–	–	–	–	–	x	x	x	x	x
AP-08	ACCCCCACAC	–	xy	xy	–	x	y	–	–	–	–					
AP-11	CTGGCTTCTG	–	x	x	x	x	x	x	x	x	–					
AP-12	GTCTTACCCC	–	x	x	–	–	–	–	–	–	–					
AV-09	GAGGTCCTAC	–	x	x	–	–	–	–	x	–	–					
AX-01	GTGTGCCGTT	–	x	x	–	–	–	x	–	–	–					
J-10	AAGCCCGAGG	–	x	x	–	x	–	–	–	–	–					
L-02	TGGGCGTCAA	–	x	x	–	–	–	–	–	x	–					
L-08	AGCAGGTGGA	–	xy	xy	–	–	–	–	–	x	–					
S-13	GTCGTTCCTG	–	x	x	x	–	–	–	–	–	–	x	x	x	x	x
S-18	CTGGCGAACT	–	x	x	–	–	–	–	–	x	–					

Primer	Sequence 5′ → 3′	Pr 6	Pr 7	Pr 8	Wl res	Wl sus	S II	Eg I	Eg III	Eg IV	Eg V	Eg VIII	(1D)1C	(5A)5C	(5D)5C
2661	CGGCCACTGT						x	–	–	x	–	–	–	x	x
2906	GGTGCACTCC						–	–	–	–	–	–			
3034	TCTGTGCTGG	x	x	x	x	–									
3039	GACCGCTTGT														
3040	TTCGAGCCAG						–	–	–	x	–	–			
3081	TTGGCGGCCT														
3221	AAGAGGCCAG						–	–	–	–	–	–			
29469	ACCGCGAAGGGGACC						–	–	–	–	–	–			
AP-04	CTCTTGGGCT	x	x	x	x	–	–	–	–	–	–	–	–		
AP-08	ACCCCCACAC						x	–	–	x	–	–	–	–	–
AP-11	CTGGCTTCTG														
AP-12	GTCTTACCCC												–		
AV-09	GAGGTCCTAC						–	–	–	–	–	–	–		
AX-01	GTGTGCCGTT						–	–	–	–	–	–	–		
J-10	AAGCCCGAGG						x	–	–	x	–	–	–	x	x
L-02	TGGGCGTCAA														
L-08	AGCAGGTGGA						–	–	–	–	–	–	y		
S-13	GTCGTTCCTG	x	x	x	x	–									
S-18	CTGGCGAACT														

but only one band was recognized by one of the addition lines (addition G). The other band seems to be a marker for chromosome A, which has not yet been added to 'Alcedo'. Primer AP-12 also produced a common band in *Ae. markgrafii* and the amphiploid but not in any of the six addition lines, indicating it could also be a marker for chromosome A. Primer AP-11 expressed a specific band in all six addition lines but not in the addition line containing the deleted chromosome pair F (Fig. 3). Altogether, the chromosomes B, C, D, E, F and G were identified by three, three, three, two, one and seven primers, respectively.

Three primers specific to *Ae. markgrafii* chromosome B which carries resistance to leaf rust were applied to eight euploid lines with introduced segments of *Ae. markgrafii* chromosome B (Pr lines), five wheat-like and resistant plants (Wl res), and one wheat-like but susceptible plant (Wl sus), as a control, from a segregating progeny (Fig. 4). DNA of all resistant lines and plants, but not the wheat-like susceptible plant, showed the chromosome-B-specific fragment when amplified with primers 3034, AP-04 and S13.

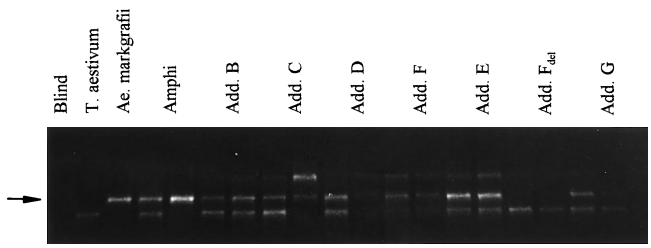

Fig. 3 The *Ae. markgrafii*-specific fragment (primer Ap-11) evident in *Ae. markgrafii*, the *T. aestivum-Ae. markgrafii* amphiploid, and all six addition lines carrying the complete alien chromosome pairs (marked by *arrow*), but absent from the deleted F-chromosome pair

The powdery mildew-resistant euploid introgression lines EgI, III, IV, V and VIII and the substitution line SII containing *Ae. markgrafii* chromatin were tested against primers specific for each of the six added *Ae. markgrafii* chromosomes (chromosome B: AP-04; chromosome C: 2661, AP-08, J-10; chromosome D: 3040, AP-08; chromosome E: AX-01; chromosome F: AV-09; chromosome G: 2906, 3221, 29469, L-08), and against primer L-08, potentially specific for the *Ae. markgrafii* chromosome A. As shown in Table 1, only four primers generated a specific fragment in some of the lines analysed. Primers 2661, AP-08 and J-10 generated the chromosome-C-specific fragment only in lines SII and EgIV, as shown in Fig. 5 for primer 2661. DNA amplification of the Eg lines and the SII substitution line with primer 3040, specific to chromosome D, gave the chromosome-D fragment in line EgIV, whereas amplification with primer AP-08, also specific for chromosome D, did not give the chromosome-D fragment. These results show that line EgIV contains *Ae. markgrafii* chromatin from two different chromosomes, C and D. Substitution line SII contains at least chromatin of the *Ae. markgrafii* chromosome C.

The chromosome-C-specific primers 2661, AP-08 and J-10 were also applied to DNA of six plants of the substitution line (5D)5C and one plant of a (5A)5C

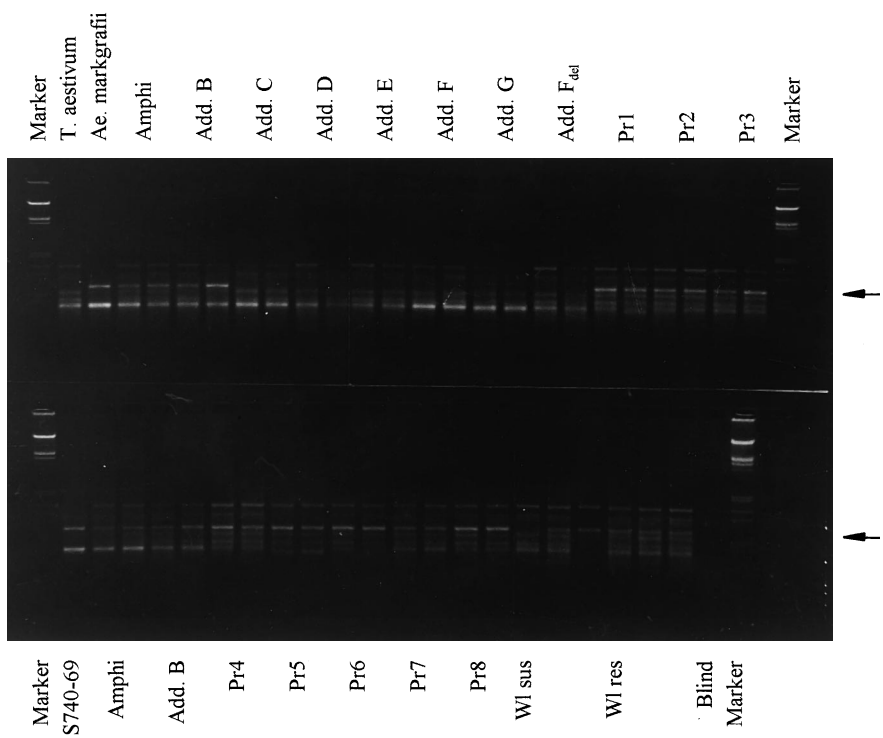

Fig. 4 The chromosome-B-specific fragment (primer AP-04) evident in *Ae. markgrafii*, the *T. aestivum-Ae. markgrafii* amphiploid, addition line B, all Pr lines and the five leaf-rust-resistant wheat-like plants-(*Wl res*), (marked by *arrow*), but not in the susceptible wheat-like plant (*Wl sus*)

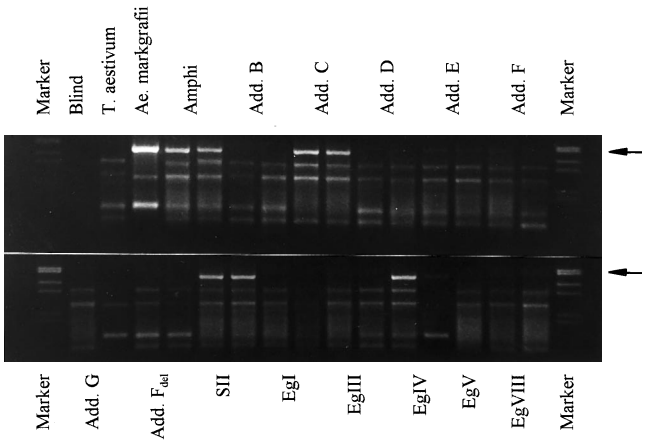

Fig. 5 The chromosome-C-specific fragment (primer 2661) evident in *Ae. markgrafii*, the *T. aestivum-Ae. markgrafii* amphiploid, addition line C, the substitution line SII, and the 42-chromosome powdery mildew-resistant introgression line EgIV (marked by *arrow*)

substitution line. Only primers 2661 and J-10 could identify the alien chromosome. After PCR with primer AP-08 and gel electrophoresis, neither substitution line expressed the expected fragment.

The application of the potentially chromosome-A-specific primers (AP-12, L-08) to substitution line (1D)1C (assumed to be homologous to *Ae. markgrafii* chromosome A) produced the expected fragment with primer L-08 but not with primer AP-12. Primers specific for the other chromosomes, AP-04 (chromsome B), 2661 and J-10 (chromosome C), AP-08 (chromosomes C and D), 3040 (chromosome D), AX-01 (chromosome E), AV-09 (chromosome F) and L-08 (chromosome G), did not result in any specific fragments.

In agreement with the allohexaploid genomic constitution of wheat, 'Alcedo' produced 19.7% more bands with the primers tested than did the diploid *Ae. markgrafii* accession 'S740-69'. A few of the primers produced additional bands and/or band losses with DNA from the amphiploids or the addition lines in comparison to the parents, indicating genomic changes during the selection process. Thirtyseven different *Ae. markgrafii* accessions were tested for polymorphisms with three primers, one chromosome-B-specific primer (3034) and two chromosome-G-specific primers (2906, 3221), but little variability was seen between the different accessions. In further experiments the two different *Ae. markgrafii* accessions 'S740-69' and 'AE110' were tested for polymorphisms with 180 decamer primers. Seventyfour primers were useful for differentiating between these accessions.

Discussion

Several methods are available to determine specific chromosomes and to identify alien chromatin in other genomes. In addition to morphological characters (Schubert and Blüthner 1995), resistance reactions, biochemical markers such as isozymes (Schmidt et al. 1993) and C-banding [Friebe et al. 1992; Zhong et al. 1996 (in combination with GISH)], molecular markers are also useful to determine alien chromatin in addition, substitution or translocation lines (Chen et al. 1994). RFLPs have been used to characterize *H. vulgare-H. bulbosum* chromosome substitution lines (Timmerman and Pickering 1993) or to detect interchromosomal translocations within the *Triticeae* (King et al. 1994). For molecular mapping in wheat, RAPDs seem not to be very useful because of their non-homoeologous, non-dose-responsive and dominant behaviour, but they can be applied in the analysis of genotypes with manipulated chromosomes (Devos and Gale 1992). *Ae. searsii* chromosome-specific RAPDs were identified in wheat-*Ae. searsii* addition lines (Díaz-Salazar and Orellana 1995). Specific RAPDs for each *H. chilense* chromosome and some chromosome arms were determined in *T. aestivum-H. chilense* addition lines (Hernández et al. 1995) and for *H. vulgare* chromosomes added to the wheat cultivar 'Chinese Spring' (Devos and Gale 1992).

In the present paper, RAPD markers for all *Ae. markgrafii* chromosomes were identified. The criterion for accepting a specific band as a marker was the occurrence of that band in the diploid *Ae. markgrafii* acc. 'S740-69' and in the amphiploid, as well as in one (or more) disomic addition line, and their absence in 'Alcedo'. We conclude from this pattern that *Ae. markgrafii* was the donor of this specific band. The probability that another wheat background would generate this specific fragment seems to be very low. If a fragment was only amplified with DNA from 'S740-69' and the amphiploid, but neither from 'Alcedo' nor from any of the six available addition lines, it was an indication of the presence of this fragment on the missing *Ae. markgrafii* chromosome, chromosome A. This assumption needs to be proven and we are currently attempting to isolate the lacking addition line.

Most primers (seven) were specific to chromosome G, although that chromosome is the smallest one (Schubert et al. 1987). Two primers (AP-12, L-08) evident in the amphiploid, but absent in the addition lines, are potential markers to identify the *Ae. markgrafii* chromosome A which has not been added to 'Alcedo'. All three chromosome-B-specific RAPDs were successfully applied to confirm the presence of B-chromosome segments in the 42-chromosome leaf-rust-resistant Pr lines. Chromosome B carries genes for leaf-rust resistance and non-glaucousness which are loosely linked (Schubert et al. 1993). However, all Pr lines analysed are glaucous indicating that the leaf-rust resistance is much more tightly linked to the B-chromosome-specific RAPDs than to the wax inhibitor gene.

The powdery mildew-resistant Eg lines were supplied to ten German breeding companies. Their results

indicated good field performance and a stable resistance. Therefore, the lines have recently been included in practical breeding programmes. Three chromosome-C-specific RAPDs were used to identify the alien *Ae. markgrafii* chromatin in the lines EgIV and SII. In addition to chromosome-C chromatin, EgIV contains chromosome-D chromatin detected by a chromosome-D-specific RAPD marker indicating that there are strong chromosomal rearrangements in that line. These results are in agreement with observations from genomic in situ hybridizations (unpublished data).

Only one primer (AP-11), of all the *Ae. markgrafii*-specific primers applied, generated bands on all six whole chromosomes analysed. If the sequences are dispersed over all the chromosomes, the primer sequence should also be found on the deleted F chromosome which has lost more than half of its long arm (Schubert 1989). But it is obvious that this RAPD is only localized on the missing chromosome part.

The *Ae. markgrafii* chromosome 5C, evident in the (5A)5C and (5D)5C substitution lines established by Muramatsu (1973) using the hexaploid wheat varieties 'Chinese Spring' and 'Konosu25', shows homology to chromosome C of *Ae. markgrafii* accession 'S740-69' (Schmidt et al. 1993). However, only two (primers 2661 and J-10) of the three chromosome-C-specific primers identified the alien chromosome 5C in the substitution lines. This is probably caused by the use of different parental *Ae. markgrafii* accessions. With regard to the (1D)1C substitution line of Kihara (1958) the same explanation may apply because there is homology between chromosome 1C and chromosome A of the *Ae. markgrafii* accession 'S740-69'. But only one of the two presumed chromosome-A-specific RAPD markers was found in that substitution line.

As expected, the DNA band frequency produced by the different primers was higher in *T. aestivum* cv 'Alcedo' than in the *Ae. markgrafii* accession 'S740-69'. Some primers produced additional bands or indicated band losses in the amphiploid, the addition lines and the euploid introgression lines, compared to the parents. These results are in agreement with the observed chromosomal instability of the amphiploid and the spontaneous alien introgression into the euploid segregants (Blüthner et al. 1988). Such genome changes can be explained by the gametocidal action of alien chromosomes from different *Aegilops* species introduced into wheat. The C genomes of *Ae. markgrafii*, *Ae. triuncialis* and *Ae. cylindrica*, especially, are known to induce chromosomal rearrangements in backcrossing and selfing progenies from crosses with common wheat (Endo and Katayama 1978; Endo 1988; Tsujimoto and Noda 1988). The gametocidal effect can also include the preferential transmission of the introduced alien chromosome. That effect has been found for chromosome C during the establishment of our addition lines (Schubert 1989).

The band variability of three primers tested in 37 different *Ae. markgrafii* accessions was limited. However, 41.1% of 180 primers investigated showed distinct differences between two accessions analysed. Therefore RAPDs could be a valuable tool to differentiate between *Aegilops* accessions, as already demonstrated for *Paspalum* (M'Ribu and Hilu 1996), *Dioscorea* (Ramser et al. 1996) and *Linum* (Bergmann and Friedt 1996).

In addition to the possible chromosomal rearrangements mentioned above, band pattern changes may be due to the general problem of RAPD marker reproducibility as also found in our investigations. Using digested template DNA for PCR can reduce the non-specific amplification but can also alter the RAPD pattern in wheat (Riede et al. 1994). Nevertheless, the reproducibility of RAPD fragment patterns, especially in different laboratories, is a significant disadvantage of this method. These problems can be overcome by the development of specific primers for the respective markers.

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