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Development of PCR-based markers linked to dominant genes for male-fertility restoration in Pampa CMS of rye (*Secale cereale* L.)

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Abstract Cytoplasmatic male sterility (CMS) is the basis for commercial hybrid seed production of rye. Nuclear restorer genes are indispensable for a complete restoration of fertility of the CMS lines. The drawbacks of current European restorer lines require the utilisation of new genetic resources that have been recently detected in an Iranian primitive rye population (IRAN IX) and an Argentinean landrace (Pico Gentario). The introgression of these effective restorer genes (*Rfp1* and *Rfp2*, respectively) into breeding material can be facilitated by marker-assisted selection. Using two F_2 populations based on crosses between the non-restorer inbred line Lo6 and the restorer IRAN IX, as well as Pico Gentario, RAPDs and AFLPs were screened and led to a closely linked marker set for each of these genes. The conversion of the closest markers into fragment-specific sequence-characterised amplified region (SCAR) markers resulted in flanking ranges of 2.9 cM (*Rfp1*) and 5.2 cM (*Rfp2*). The application of these markers in backcross programmes is discussed.

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Introduction

Hybrid rye breeding and seed production require a cytoplasmatic male sterility (CMS) system as a hybridisation mechanism. Various sources of CMS have been described, the Pampa (P)-cytoplasm, discovered in the late sixties in an Argentinean landrace (Geiger and Schnell 1970), is most widely used. For the complete restoration of pollen fertility, effective, nuclear-encoded restorer genes for this CMS-inducing cytoplasm are indispensable. The frequency of effective male fertility restorer genes for the P-cytoplasm in European material, however, is lower than 5% (Yuan 1995), and the efficiency of the restoration is strongly subjected to environmental effects (Geiger and Miedaner 1996). Partial restoration of male fertility causes a reduction in the amount of viable pollen, thus facilitating infection by the ergot fungus (*Claviceps purpurea)*. Ergot infection leads to a contamination of the rye grains with sclerotia containing toxic alkaloids. In order to reduce this risk, rye hybrids need effective restorer genes. Out of the generally inferior European materials, the German inbred lines L18 and L161 provided still the best fertility restoration. However, because of their poor agronomic value they cannot be utilised in commercial hybrid breeding programmes. No complete restoration has been achieved in commercial hybrids to-date. In L18 a major restorer locus on chromosome 1R was already detected by an isozyme marker (Wricke et al. 1993) and confirmed by RFLP markers (Miedaner et al. 2000). For the alternative CMS-inducing G-cytoplasm of rye, a major restorer gene (*Rfg 1*) was found on chromosome 4R (Börner et al. 1998). Curtis and Lukaszewski (1993) also identified a gene on chromosome 4RL (*Rfc4*) that restored the CMS-inducing *Triticum timopheevi* cytoplasm in hexaploid wheat using wheat-rye addition lines. By the same authors, another rye restorer gene for the *T. timopheevi* cytoplasm was de-

scribed on chromosome 6RL. Recently, additional restorer sources were detected in IRAN IX, an Iranian primitive rye population, and Pico Gentario, an Argentinean landrace at Hohenheim (Geiger and Miedaner 1996). These exotic materials displayed a significantly higher level of restoration than the currently used European lines and a high environmental stability (Geiger and Miedaner 1996). RFLP mapping studies of both exotic sources each revealed a single major gene for restoration. In both cases the gene, *Rfp1* and *Rfp2*, respectively, is localised on the long arm of chromosome 4R (Dreyer 2000; Miedaner et al. 2000). Marker brackets of approximately 5 to 6 cM were achieved. From marker data, it is not possible to conclude whether the restorer genes are different alleles of the same gene or genes at different loci. Phenotypic data from a segregation study, however, showed that both the two genes are very likely to be allelic or even identical (Miedaner et al. 2000). The objectives of the presented study were to identify additional markers more closely linked to the restorer gene(s) on chromosome 4RL and to develop specific PCR-based assays for easy detection of the fertility restorer genes in commercial backcross programmes.

Materials and methods

Plant material

Inbred lines from two self-incompatible rye populations from Argentina and Iran, Pico Gentario and IRAN IX, were used as male fertility restoration sources (Miedaner et al. 1997). Their production was described in detail elsewhere (Miedaner et al. 2000). For this study, new crosses between the non-restorer inbred line Lo6 (Lochow-Petkus GmbH) in the CMS-inducing P-cytoplasm and inbred lines derived from Pico Gentario and IRAN IX (the latter provided by Lochow-Petkus GmbH), respectively, were used for generating the F_2 mapping populations. The population resulting from the cross Lo6 × Pico Gentario included 651 F_2 individuals and the population of the cross Lo6 × IRAN IX 489 F_2 individuals. For bulk segregant analysis and a rough estimation of the chromosomal location, 96 F_2 individuals per population were selected according to their anther score with 48 individuals being fully male-sterile (anther score 1–2, see below) and 48 being fully male-fertile (anther score 8–9). The genetic distances between the restorer gene and new developed PCR markers were estimated in 258 (Lo6 × IRAN IX) and 272 (Lo6 × Pico Gentario) randomly chosen F_2 individuals.

Male fertility assessment

Male fertility assessment was carried out in each of two environments per population in 1997. The F_2 population Lo6 × Pico Gentario was assessed at Bad Schönborn near Heidelberg, in South Germany and at Rieste near Lüneburg in North Germany; the F_2 population Lo6 × IRAN IX was assessed at Bergen near Celle, and Petkus near Berlin in North Germany by the co-operating private plant breeding companies. Each F_2 individual was cloned to produce three plants per clone. Two plants were used for the evaluation of male fertility and grown as replicates in a randomised complete block design at the respective locations.

A third clone was used for cutting leaf material for DNA extraction. The level of restoration was estimated by visually scoring anthers on a 1 to 9 scale according to Geiger and Morgenstern (1975). Scores from 1 to 3 refer to non-dehiscent, empty anthers with decreasing levels of degeneration; scores from 4 to 6 refer to partial male sterility with <10% to >50% fertile anthers; scores from 7 to 8 refer to pollen-shedding anthers of increasing anther size; score 9 corresponding to fully male-fertile plants in N-cytoplasm. On average, two to three main tiller spikes per plant were scored at middle and late flowering.

Analyses of variance of the field data were computed for each location and combined across locations using standard procedures (Snedecor and Cochran 1989). Since the male fertility data significantly deviated from normality, all statistical tests are only approximate for this trait. Data transformation did not lead to a more favourable distribution. All statistical analyses were performed with the computer package PLABSTAT (Utz 2000). The effects of replicates, genotypes and locations were assumed to be random variables.

Molecular analyses

Total genomic DNA was extracted from 250 mg of ground leaf material as described by Hoisington et al. (1994). For molecular analyses, the RFLP, RAPD and AFLP marker systems were employed.

RFLP analysis

RFLP markers were used as anchors for localising RAPD and AFLP markers on the relevant chromosome segments. RFLP profiles were identified according to the protocol described by Devos et al. (1992), using non-radioactive signal detection. DNA was digested with the restriction enzymes *Dra*I, *Eco*RI and *Eco*RV and hybridised with the probes psr899, psr167 (Devos et al. 1993) and mwg59 (Graner et al. 1991). These probes detected flanking markers to the restorer genes in previous mapping studies (Dreyer 2000; Miedaner et al. 2000).

RAPD analysis

The RAPD analysis was carried out by the laboratories of the co-operating breeding companies, namely PLANTA GmbH, Einbeck, and Resistenzlabor SAATEN-UNION GmbH, Hovedissen. For the marker identification, bulk segregant analysis (BSA, Michelmore et al. 1991) was employed. Each bulk included ten F_2 genotypes selected by phenotypic scores and RFLP data. In both populations, 700 RAPD primers, including OPERON (Operon Technologies) and UBC (University of British Columbia, Vancouver, BC, Canada) primer sets, were screened using the standard PCR method (2 min at 95 °C, followed by 35 cycles with 30 s at 95 °C, 30 s at 42 °C and 1 min at 72 °C, and a final elongation step of 5 min at 72 °C).

Polymorphic marker bands were excised from agarose gels and extracted using the DNA purification kit ‘Easy Pure’ (Biozym Diagnostik GmbH, Hess. Oldendorf, Germany). The development of specific markers was carried out as described in the subsequent paragraph ‘AFLP analysis’.

AFLP analysis

In the AFLP analysis (Vos et al. 1995) 164 (Iran IX) and 152 (Pico Gentario) AFLP enzyme primer combinations (EPC) were employed, including mainly *Pst*I + 2/*Mse*I + 3 and *Pst*I + 3/*Mse*I + 3 as well as some of *Eco*RI + 3/*Mse*I + 3 combinations.

Pre-selection of feasible enzyme primer combinations and screening for putative marker fragments were carried out by Keygene N.V. (Wageningen, The Netherlands) using radioactively labelled AFLP primers. The AFLP reactions for confirmation of linkage and for the mapping studies, however, were based on the fluorescence-based AFLP protocol described by Dehmer (2001), employing the ABI system (ABI PRISM 377 DNA, PE Biosys-

tems). Selected AFLP marker fragments were separated on 6% polyacrylamide gels and stained with silver nitrate according to Budowle et al. (1991). Marker fragments were directly excised from the dried gel and subsequently amplified without additional pre-treatment by PCR applying an initially long denaturation step (15 min at 95 °C followed by 35 cycles with 30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C, using Hot-*Taq* polymerase, Qiagen). Amplified fragments were cloned into the pGEM-T vector (Promega) according to the manufacturer's instructions. For each isolated fragment, ten random clones were screened by PCR with the corresponding AFLP primers. The size of the resulting amplified products was verified on a 6% polyacrylamide gel. Three to five clones with the correct insert size were sequenced.

Sequence-specific PCR analysis

Based on the marker fragment sequences, specific primer pairs were designed (Vector Nti Suite, InforMax, Incorporated) and SCAR (Sequence Characterised Amplified Region; Paran and Michelmore 1993) as well as CAPS (Cleaved Amplified Polymorphic Sequences; Konieczny and Ausubel 1993) markers were developed. The sequence-specific PCR was performed using 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 2.5 pmol of sense and antisense primer, respectively, 0.5 to 0.25 U of Hot-*Taq* polymerase (Qiagen) and 5 ng of template DNA per 25-µl reaction. The basic PCR profile started with 15 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 30 s annealing and 1 min at 72 °C. Annealing temperature was varied from 49 °C to 66 °C depending on the primer pair utilised. Converted dominant markers were assessed on agarose gels, while for the co-dominant marker a 5% polyacrylamide gel was employed.

Statistical analysis

At each marker locus deviation from expected Mendelian segregation ratios (1:2:1, 3:1) and from an allele frequency of 0.5 were tested by the standard χ^2 method (Snedecor and Cochran 1989). The software package MAPMAKER V3.0 (Lincoln et al. 1993) which utilised the mapping function of Haldane (1919) was employed to construct the maps.

Results and discussion

The anther scores for population Lo6 × Pico Gentario, including 651 individuals, were highly correlated (r = 0.94, P = 0.01) between the locations Bad Schönborn and Rieste. A similarly tight correlation existed between Bergen and Petkus, the test sites for 498 individuals of the population Lo6 × IRAN IX (r = 0.92, P = 0.01). In both populations the distribution of the mean anther scores clearly revealed two peaks (Fig. 1), one in the class of male-sterile genotypes (N = 176 and 114 individuals, respectively) and the second in the male-fertile class (N = 475 and 384 individuals, respectively). In this calculation, the partially fertile plants were included into the fertile class. The observed frequencies are in accordance with a 3:1 segregation ratio for both populations (χ^2 test = 0.97 and 1.44, respectively) indicating a monogenic dominant inheritance of fertility restoration. When comparing the anther score distributions of the two populations, it has to be considered that the scores were taken at different test sites and by different persons. Independent samples tested one year later in a balanced design did not yield significant differences in the efficacy of the two fertility restoration sources (unpublished data).

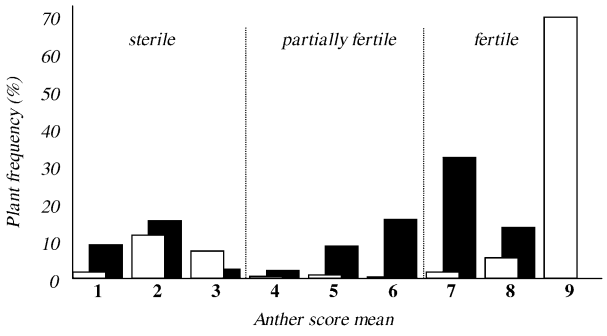

Fig. 1 Frequency distributions of anther score (*1–3* = male sterile, *4–6* = partially male fertile, *7–9* = male fertile, for further details see 'Material and methods') in the F_2 mapping populations Lo6 × PG (*black bars*) with 651 individuals and Lo6 × IRAN IX with 498 individuals (*white bars*)

As recently reported by Dreyer (2000) and Miedaner et al. (2000) a major restorer gene in Pico Gentario as well as in IRAN IX was located on chromosome 4RL. A QTL analysis across all chromosomes showed that the respective genomic region on 4 RL explained 59 and 68% of the phenotypic variation, respectively (Miedaner et al. 2000). The partially fertile individuals (anther scores 4 to 6) possessed the alleles of the fertile parent at the loci of the flanking markers, with the exception of the expected recombinants (Dreyer 2000). Therefore, it was justified to include partially fertile individuals into the fertile class for segregation analysis. According to Dreyer (2000) the RFLP markers Xmwg59 and Xpsr899 flanked the *Rfp2* gene in Pico Gentario, whereas in IRAN IX the RFLP marker Xpsr167 was most closely linked to *Rfp1*. By using these three markers the location of the major restorer gene on the long arm of chromosome 4R was confirmed in both the independent mapping populations of this paper. The study by Dreyer (2000) already showed that the RFLP marker Xpsr167 had a significantly (P = 0.01) distorted segregation in population Lo6 × IRAN IX, with a lower proportion of genotypes being homozygous for the non-restorer allele at the marker locus than expected. In rye, segregation distortions have been frequently published in mapping studies (Plaschke et al. 1993; Philip et al. 1994; Loarce et al. 1996). In particular, Korzun et al. (1998) also observed a region of distorted segregation on the chromosome 4RL.

Based on the above RFLP data, ten genotypes of the phenotypic classes 1 to 2 (sterile) and 8 to 9 (fertile), respectively, were selected to be used as bulks for RAPD and AFLP bulked segregant analysis.

Out of 700 screened RAPD primers three showed an association with the male fertility in IRAN IX (OP-XX04, OP-AJ04 and OP-Y09) and one in Pico Gentario (OP-Y03). The conversion of these marker fragments into PCR-based sequence-specific markers was successful in three out of the four cases, namely for

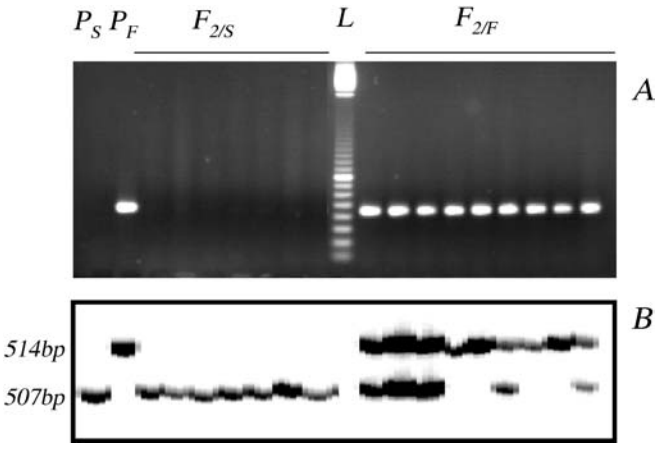

Fig. 2 Converted RAPD markers SCY09d (*A*) and SCY09cd (*B*) for the population Lo6 × IRAN IX, separated on agarose gel (*A*) and polyacrylamide gel (*B*), respectively. P_S = male-sterile parental line Lo6, P_F = male-fertile parental restorer line from IRAN IX, $F_{2/S}$ = male-sterile F_2 individuals, $F_{2/F}$ = male-fertile F_2 individuals, all entries in P-cytoplasm, *L* = DNA ladder (100 bp, Pharmacia)

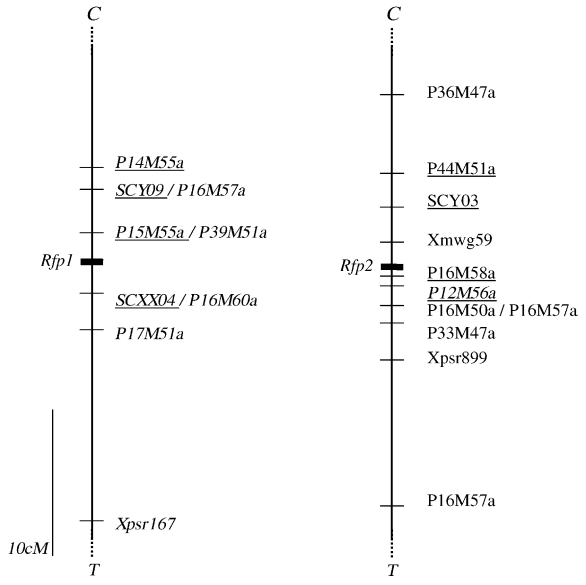

Fig. 3 Partial map of chromosome 4RL showing the localisation of the restorer genes in population Lo6 × Pico Gentario (*Rfp2*) and in population Lo6 × IRAN IX (*Rfp1*) by RFLP markers (X…), converted RAPD markers (S…) and AFLP markers (P…), based on 96 plants per population. For *underlined markers* specific primer pairs were generated (see Table 1). *C* = centromer *T* = telomer

OP-XX04, OP-Y09 and OP-Y03. In PCR reactions with a sequence-specific primer pair for marker OP-XX04 (IRAN IX), one monomorphic product was generated in both the sterile and the fertile genotypes. A sequence comparison indicated an additional restriction site for *Nco*I in the fertile genotype based on a single A/T nucleotide substitution. Therefore, a CAPS marker was developed, which generally leads to a co-dominant marker. Cleavage of the monomorphic fragments by *Nco*I allowed us to differentiate between fertile and sterile genotypes; however, only a dominant marker (SCXX04) was obtained (data not shown).

In the case of RAPD marker OP-Y09, the derived primer pair amplified monomorphic fragments, as well. A sequence comparison revealed a 7-bp insertion of the fragment from the restorer parent. Based on this insertion, primer pairs for a dominant SCAR marker (SCY09d) and a co-dominant SCAR marker (SCY09cd) were designed. The presence (fertile genotypes) or absence (sterile genotypes) of the primer-binding site caused the dominant marker, while the co-dominant is based on the 7-bp-length polymorphism (Fig. 2).

For Pico Gentario the specific primer pair designed for the RAPD fragment OP-Y03 yielded a dominant SCAR marker (SCY03) with an amplified fragment in the case of the fertile genotype.

The screening of 164 (IRAN IX) and 152 (Pico Gentario) AFLP enzyme primer combinations was performed using radioactive and fluorescent-labelled primers. The fragments were selected in a range of 200 bp to 500 bp. In this range, AFLP assays indicated polymorphism between the respective crossing parents of 35 to 40%.

Based on the radioactive BSA, ten AFLP fragments in population Pico Gentario and 12 in population IRAN IX were associated with male fertility restoration. Using the corresponding fluorescent-labelled AFLP primers, the linkage to the restorer loci was confirmed in eight (population Lo6 × Pico Gentario) and six (population Lo6 × IRAN IX) out of these marker fragments.

In Fig. 3 the maps give an approximate estimation of the locations of the linked AFLP markers and of the converted RAPD markers, with the limitation that the calculations were based on the phenotypically most-extreme 96 F_2 genotypes, respectively. Minor distance changes are to be expected if larger and random samples of the populations were analysed (see below). Yet the map provided useful information on the positions of the two types of markers. The distal or proximal chromosome positions of the markers were analysed relative to the positions of the RFLP markers Xpsr167, Xmwg59 and Xpsr899, as published in the mapping analyses by Dreyer (2000). Since the marker sets in IRAN IX and Pico Gentario are completely different from each other, it is not possible to draw final conclusions on the identity or non-identity of the restorer loci in the two populations. However, Miedaner et al. (2000) observed in a large F_2 population, derived from a cross between a Pico Gentario and a IRAN IX line, only restorer plants, indicating that the two restorer genes are identical.

Confirmation at the molecular level would require further comprehensive sequence comparisons of the specific marker fragments and, possibly, an extension of these fragments to find useful polymorphisms.

For conversion of the AFLP markers into SCARs, silver-stained gels were used. This allowed the accurate cutting of the corresponding fragments. Nevertheless,

Table 1 List of developed sequence specific primers

Restorer source	Primer	Marker type	Primer sequence 5′-3′	
PG	SCY03	Dominant	Sense	ACA GCC TGC TTG ACA CGT GT
			Antisense	CCA ATG CAA CCC CAT AGA CA
	SCP12M56	Dominant	Sense	ACG CAA TAA TAA TGG GCC AC
			Antisense	GTA GCA TGG GCG GAT CAT AG
	SCP16M58	Dominant	Sense	ACC CCC TCT TTC TAG CCT GT
			Antisense	ACG TCT ACT CAA CTC ATG CAC C
	SCP44M51	Dominant	Sense	CAG CTC AAC ATC ACC AGT GAG
			Antisense	TGA GTA ACC ACC ACT CGG AA
IRAN IX	SCY09d	Dominant	Sense	TGG GCA TCA GCA CGC TTG
			Antisense	AAT GGC CCG GTC CGA GTG
	SCY09cd	Co-dominant	Sense	AGC AGC GCA CTA GAC AGT ACA A
			Antisense	AGT CCA CGA GGT AAC GAA GTT C
	SCP15M55	Dominant	Sense	TCC TGA GTA ACG AGC TGC TG
			Antisense	GTA CAT GCA GCA CCC TTT TG
	SCP14M55	Dominant	Sense	AAC GAC ATT TTC CTG GGA GC
			Antisense	ACG ACA GAG TGA CGT GCT GA
	SCXX04	Dominant	Sense	[a]
			Antisense	[a]

[a] Primer information is available for non-commercial use on request by Dr. V. Korzun, Lochow-Petkus GmbH, c/o PLANTA GmbH, Grimsehlstrasse 31, D-37574 Einbeck, Germany; e-mail: korzun@lochow-petkus.de

background contaminations could not be completely avoided. In some cases, different inserts in colonies from a single transformation event were observed. For selecting the correct fragments, amplified inserts were separated on a polyacrylamide gel for the screening of small size-differences. Up to ten inserts with same fragment size were sequenced.

In the Pico Gentario population, the marker fragments P12M56a and P16M58a were converted into PCR-based dominant specific markers. The conversion of P44M51a, however, resulted only in indistinguishably monomorphic fragments. For the population IRAN IX, five AFLP fragments were isolated and sequenced. Two of them (P15M55a and P14M55a) were converted into dominant specific markers SCP15M55 and SCP14M55, respectively. No specificity was obtained for the sequenced marker fragments P16M60a and P39M51a.

All converted markers and the corresponding specific primer sequences are summarised in Table 1.

For the converted markers the map positions were estimated in samples of 258 (population Lo6 × IRAN IX) and 272 (population Lo6 × Pico Gentario) randomly chosen F_2 individuals. In IRAN IX, the closest linked markers, SCAD04 and SCP15M55, flanked the restorer gene at distances of 1.2 cM and 1.7 cM, respectively. In Pico Gentario, the markers SCY03 and SCP16M58 flanked the locus at distances of 3.0 cM and 2.2 cM, respectively.

All closely linked markers are dominant. This does not affect their usefulness in backcross programmes, since only homozygous recessives and heterozygotes need to be distinguished. However, co-dominant markers would enable the identification of homozygous restorer genotypes among individuals obtained from selfing highly backcrossed heterozygotes as a final step of a backcross programme. For that reason, an extension of this study could be worthwhile. The genome walking strategy (Siebert et al. 1995; Clontech Laboratories) or the inverse PCR method (Ochsman et al. 1988; Triglia et al. 1988) could be useful for extension of the marker fragments. Therefore, providing additional sequence information would allow screening for polymorphisms in restriction sites, which generally enable co-dominant scoring.

The practical application of the developed markers in the breeding process requires a pre-screening of all available elite lines for polymorphism at the corresponding marker locus. If no polymorphisms can be found, the available fragment sequence information can be used for fragment extension strategies to screen for line-specific polymorphisms. Thus, comprehensive and time-consuming searches for new AFLP markers and their conversion to PCR-based markers would not be necessary.

A frequent problem in backcross programmes are negative effects of the “linkage drag” (Brinkman and Frey 1977; Tanksley et al. 1989). The average length of the introduced non-adapted donor segment can be estimated according to Stam and Zeven (1981). For the present study we can assume that: (1) the length of chromosome 4R is about 180 cM (Dreyer 2000), (2) the target gene position is near to the centromere, and (3) the marker-assisted backcross selection is based on two markers which flank an interval of 2.9 cM (IRAN IX) and 5.2 cM (Pico Gentario), respectively. In this case the estimated length of the donor chromosome segment surrounding the flanking markers is on average 45 cM in BC4 and about 32 cM in BC6. This implies that a large number of non-adapted donor genes will be introduced into the genetic background of a recipient elite line. So

far, nothing is known about the functions of the genes located in the vicinity of the male fertility restorer genes. But the unintentional selection of undesirable alleles additional to the restorer gene cannot be ruled out, if selection is based on the flanking markers only. For instance, Börner et al. (2000) reported a QTL for spikes per plant on 4RL in the region between Xmwg539 and Xpsr119. Unfavourable dominant alleles, influencing the number of spikes per plant, might therefore reduce the yield of hybrids based on the converted restorer lines. However, linkage drag can be significantly reduced by inclusion of additional markers in the selection process, as shown by Frisch et al. (1999, 2001).

The application of additional markers is crucial, not only for reducing linkage drag, but also for speeding up the backcrossing process in general. In classical backcross programmes six backcross generations are generally recommended when selection for the desired phenotype is carried out (Fehr 1987). Marker-assisted backcrossing using markers covering the entire genome in addition to the flanking markers of the target region, is a powerful strategy to reduce the donor background more efficiently as shown by Frisch et al. (1999). These simulation studies revealed that selection based on two flanking target markers and a set of markers distributed over the entire genome in an average density of 20 cM, results in the BC_3 generation at the same proportion of the recurrent parent genome as reached in BC_7 without molecular-marker applications. Different maps (Korzun et al. 1998, 2001; Dreyer 2000; Ma et al. 2001) including RFLP markers are available in rye and in combination with the developed flanking markers for the male fertility restoration gene(s), these are useful tools for an efficient marker-assisted backcross programme.

A specific advantage in using marker-based backcrossing for the transfer of exotic restorer genes to pollinator lines allows one to perform selection and backcrossing in the same season, since no test crosses need to be established and evaluated. Combined with the aforementioned marker-based background selection, this leads to an enormous acceleration of the development of hybrids with full restoration.

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