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AFLP and STS tagging of *Lr19*, a gene conferring resistance to leaf rust in wheat

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Abstract Amplified fragment length polymorphism (AFLP) markers were used to enrich the map of the wheat chromosomal region containing the *Thinopyrum*-derived *Lr19* leaf rust resistance gene. The region closest to *Lr19* was targeted through the use of deletion and recombinant lines of the translocated segment. One of the AFLP bands thus identified was converted into a sequence-tagged-site (STS) marker. This assay generated a 130-bp PCR fragment in all *Lr19*-carrying lines tested, except for one deletion mutant, while non-carrier template failed to amplify any product. This sequence represents the first marker to map on the distal side of *Lr19* on chromosome $7el_1$. The conversion process of AFLP fragments to STS markers was technically difficult, mainly because of the presence of contaminating fragments. Various approaches were taken to reduce the frequency of false positives and to identify the correct clone. We were able to formulate a general verification strategy prior to clone sequencing. Various other factors causing problems with converting AFLP bands to an STS assays are also discussed.

Keywords AFLP · Leaf rust · *Lr19* · STS

Introduction

Resistance to the leaf rust pathogen (*Puccinia recondita* Rob. Ex. Desm. f.sp. *tritici*) is of major importance to many wheat breeding programmes. Forty-seven major genes for resistance have been catalogued to date (McIntosh et al. 1995; http://www.crl.umn.edu/res_gene/wlr.html), almost half of which derive from rye and wild relatives of wheat. Perhaps the most important of these 'alien' genes is *Lr19*, which was first introgressed into wheat from *Thinopyrum* sp. in the form of the substitution line 'Agrus' [Caldwell et al., cited in Sharma and Knott (1966)]. Virulence to wheats carrying *Lr19* was first reported by Huerta-Espino and Singh (1994) in Mexico, but *Lr19* still provides effective resistance against all leaf rust pathotypes in South Africa (Prins et al. 1996, 1997).

A number of modified forms of the *Lr19* translocation (deletion mutants and recombinants), including several lacking the undesirable linked yellow pigment gene, have been derived (Sears 1972a, b; Marais 1992a, b). From these, a restriction fragment length polymorphism (RFLP)-based map of the *Lr19* translocation of wheat was constructed and used to identify *Lr19*-149, the white endosperm recombinant that carries the smallest *Thinopyrum* segment identified to date (Prins et al. 1997; Prins and Marais 1998). In contrast to the preferential transmission of the translocation from which it was derived, the alien segment in *Lr19*-149 self-eliminates in translocation heterozygotes at a rate which is dependent on the genetic background (Prins et al. 1997; Prins and Marais 1999). This characteristic complicates the use of *Lr19*-149 in a conventional breeding program, and has driven efforts to further reduce the alien content in this recombinant via induced homoeologous pairing, which is required because the *Lr19* translocated segment does not recombine spontaneously with its wheat homoeologue (Marais and Marais 1998). For this purpose, additional genetic markers, more closely linked to *Lr19* than any of the available RFLP loci, are required.

The AFLP (amplified fragment length polymorphism) technique (Vos et al. 1995), with its high multiplex ratio and general robustness, has found various applications in plant genetics, such as in the assessment of genetic diversity, the construction of high-density genetic maps

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and as a resource in marker-enrichment strategies for target loci, as discussed in detail by Shan et al. (1999). However, due to its technical complexity and associated high cost, AFLP markers are not as well suited to high-throughput marker-assisted selection situations as are sequence-tagged (STS) markers. AFLPs can be converted to STS, but the process has been shown to be non-trivial in wheat and barley (Shan et al. 1999). The aim of the study reported here was to identify AFLP markers that map close to *Lr19* and to reduce these to the format of a simple, breeder-friendly STS assay to test for its presence.

Materials and methods

AFLP analysis and mapping of *Lr19* mutants

Sixteen lines of the deletion set of the Indis *Lr19* translocated segment described by Marais (1992a) were used for the identification of informative AFLP markers. DNA was extracted from fresh seedling leaf material following the technique of Doyle and Doyle (1990), with minor modifications. Two DNA bulks were generated by combining equimolar amounts of DNA of, respectively, the ten lines lacking *Lr19* (*–Lr19* bulk), and the six carrying *Lr19* (+*Lr19* bulk). In addition, separate DNAs of *Lr19*-149 and Transfer 10 (Sears 1972a, b), which have retained the shortest *Thinopyrum* segments, were used to enable the identification, among the selected AFLP markers, of those most closely linked to *Lr19.* In both recombinants, a large proportion of the alien segment proximal to *Lr19* has been replaced with wheat chromatin; in addition, for the critical chromosome in *Lr19*-149, most of the region distal to *Lr19* is also of wheat origin (Prins and Marais 1998).

The AFLP protocol of Donini et al. (1997) was followed to generate *Sse*I+2/*Mse*I+2 profiles of the *–Lr19* and +*Lr19* bulks, as well as of the control cultivars Inia 66 (susceptible) and Indis (*Lr19* carrier). Primer combinations amplifying AFLP fragments only in the positive bulk and the resistant control were then applied to templates of *Lr19*-149 and Transfer 10, and to the individual templates constituting the +*Lr19* bulk, to select for those fragments mapping closest to *Lr19.*

Cloning of AFLP fragments

Chosen bands (+) were excised by superimposing the autoradiograph over the dried gel. A piece of dried gel (no visible AFLP band) was also taken from the corresponding site in the non-*Lr19* lane to be used as negative control (–). The fragment of gel and backing paper was incubated in 50 µl 1× $TE_{0.1}$ (10 m*M* Tris-Cl, 0.1 m*M* EDTA, pH 8.0) to elute the DNA, and 1 µl of a 1:10 dilution of the eluate was used as template in a polymerase chain reaction (PCR) with the relevant AFLP primer combination to amplify sufficient DNA to allow blunt-ended cloning into the plasmid vector pMos*Blue* (Amersham), following the supplier's protocol. Blue/white selection was used to discriminate empty clones from clones with inserts. A major complicating factor in cloning DNA fragments from AFLP gels, particularly when derived from large genome templates such as wheat, is the high frequency of false positives, which arise from co-migration of multiple PCR products. These 'hidden' bands represent products which are either amplified below the level of sensitivity for visualization (Koebner et al. 1998), or are unlabeled (and are therefore invisible in autoradiographic assays) (Bachem et al. 1998). Thus, when an AFLP band is excised and cloned, there is a high probability of heterogeneity among the resulting clones. A number of approaches were taken to reduce the frequency of false positives or to identify the correct clone. These included:

1) Increasing the number of selective bases in the AFLP primers.
2) Comparison of the sizes of clones on 3% agarose gels, following amplification with either the M13 forward and reverse primers or the relevant selective AFLP primers.
3) Comparison of the *Alu*I, *Rsa*I and *Hae*III restriction digest profiles of amplified inserts on 3.5% agarose gels.
4) Probing dot blots containing selective AFLP reactions of the +*Lr19* and *–Lr19* lines with non-radioactively alkaline phosphatase-labeled (Gene Images™ Alkphos Direct™ labeling and detection system, Amersham) candidate clones, chosen on the basis of restriction digest profile. Two microliters of each amplification reaction was spotted onto Hybond N+ membrane (Amersham), which was immersed in 0.4 *M* NaOH for 10 min, and then neutralised in 2× SSC buffer. Hybridization temperatures of 55°C, 60°C and 65°C were tested. Two primary and secondary washes each were performed at the same temperature that the hybridisation was performed. Chemiluminescent signal generation and detection were obtained with CDP-*Star*™. Autoradiography film was exposed for between 20 s and a few hours.
5) Size comparison of cloned inserts on sequencing gels. Clones ('+'and '–') were amplified with the appropriate [^{33}P]-labeled selective AFLP primers, and the products were separated on gels similar to those used for the original AFLP screening. For comparison, the original selected AFLP product was included as a marker track.
6) Exclusion of *Mse*I/*Mse*I (or *Sse*I/*Sse*I) clones by testing for amplifiability using only a single AFLP primer. *Mse*I/*Sse*I clones only amplify successfully when both AFLP primers are included in the PCR reaction, but *Mse*I/*Mse*I clones amplify in the absence of the *Sse*I primer.

STS design and analysis

Inserts of candidate clones were sequenced in both directions on an ABI automatic sequencer, and primer sets were designed, internal to the AFLP selective primers, from these sequences. The primers were tested for specificity in PCR reactions from templates of the susceptible cultivar Inia 66 and the *Lr19* carrier Indis in 25 µl PCR reactions containing 50 ng genomic DNA, 12.5 pmol each primer, 0.2 m*M* each dNTP, 0.625 U *Taq* polymerase (Bioline), 1× PCR buffer and 2 m*M* $MgCl_2$. The amplification protocol was: 4 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C and a final extension for 5 min at 72°C. The PCR products were separated on a 3% w/v agarose gel (0.5× TBE buffer, 2 h at 80 V).

Results

AFLP analysis and physical mapping

Seventy-five primer combinations were tested, of which 15 identified 21 PCR products (AFLP bands) present in the profiles of the +*Lr19* bulk and Indis, but absent in those of both the *–Lr19* bulk and Inia 66. Analysis of the individual components of the +*Lr19* bulks and the two *Lr19* recombinants showed which of these fragments were present in both (or at least one) of the recombinants and how they segregated among the mutant lines (Table 1), presumably reflecting differences in the size and physical location of the relevant deletion. From this analysis, we conclude that fragments A, C, F and G are located between *Lr19* and *Wsp-D1c* [water-soluble protein, Liu et al. (1989)]; fragments B, D and I are present in all the +*Lr19* mutants, and are therefore probably closely located (proximal or distal) to *Lr19*; while frag-

Table 1 Physical mapping of AFLP fragments

Deletion mutants	*Lr19*	Fragment[a] B-P20/M18 D-S12/M14 I-S13/M13	Fragment A P20/M16[b]	Fragment C P20/M21[b]	Fragment F P22/M11[b]	Fragment G S13/M12[b]	*Wsp-D1c*	Fragment H S13/M12	Fragment E S12/M15[c]	Fragment J S12/M20[c]	*Sr25*
87M23–103	+[e]	+	–	–	–	–	–	–	+	+	+
89M2–245	+	+	+	+	+	+	–	–	–	–	–
89M2–426	+	+	+	+	+	+	+	–	–	–	–
87M23–225	+	+	+	+	+	+	+	+	+	+	–
89M1–18	+	+	+	+	+	+	+	+	+	+	+
87M23–266	+	+	+	+	+	+	+	+	+	+	+
Recombinants											
Lr19-149	+	+	+	+	+	+	+	+	–	–	–
Transfer 10	+	+	+	+	+	+	+[d]	+	+	+	+[d]

[a] Proximal or distal to *Lr19*
[b] Mapped in the same region
[c] Mapped in the same region
[d] Not determined in this study, but distal portion of *Thinopyrum* chromatin has been retained (Zhang and Dvorák 1990; Prins and Marais 1998)
[e] +, Present; –, absent

Table 2 Sequences of primers used for amplification of products in each of the five groups of sequences derived from fragment G

Sequence	Number of clones	Primer identifier	Sequence of primer (5′–3′)	Primer pair polymorphic
Group Ga	4	Ga1F	CGA TCT GGA CCC AAC CCG TCA	No
		Ga1R	CGC ACG CGA CCG AGC AG	
		Ga2F	ATC TGG ACC CAA CCC GTC AAC	No
		Ga2R	GCA CGC GAC CGA GCA G	
		Ga3F	ACC CAA CCC GTC AAC GTC CTT	No
		Ga3R	CGC ACG CGA CCG AGC AG	
		Ga4F	TAC TAA CCT GGC CCT TGC	No
		Ga4R	CCG ATC TGG ACC CAA CCC	
Group Gb	2	GbF	CAT CCT TGG GGA CCT C	Yes
		GbR	CCA GCT CGC ATA CAT CCA	
Group Gc	2	GcF	GGA GCA GGT ATG GTA GGC	No
		GcR	CAC TAA TGG CGT GAC TGG	
Group Gd	1	GdF	GGA CCA CAC TCT TCG GTC	No
		GdR	CAA AAC AGC CAA GAA GCA	
Group Ge	3	GeF	AGG AGC GCC TCA TCG AGC	No
		GeR	CTC TGC CGC TCC ACA CGA	

ments H, E and J map distal to *Wsp-D1c*. Fragments E and J are present in Transfer 10 (which retains the distal *Thinopyrum* region) and in the three deletion lines that have retained the largest *Thinopyrum* segments. However, it is absent in *Lr19*-149, which indicates that it is located distally to fragment H. Fragments E and J are also present in mutant 87M23–103, which expresses a stem rust resistance similar to that produced by *Sr25* and which has been shown to have lost *Thinopyrum* chromatin on both sides of this gene (Prins et al. 1996). Although it has been suggested that an unknown *Sr* gene in the background, or a mutated suppressor of resistance, could have produced the resistance of this mutant (Prins et al. 1996), the present genotyping supports the model that this line has an intercalary deletion and has indeed retained *Sr25*. Because the two recombinants Transfer 10 and *Lr19*-149 have retained relatively small segments of *Thinopyrum* chromatin proximal to *Lr19* (Prins and Marais 1998), those AFLP fragments present in both lines provided a suitable criterion to identify markers closely linked with *Lr19*.

Cloning

As a pre-cloning step, we attempted to reduce the number of false positives by generating AFLP reactions using the original *Sse*I+2 primer in combination with each of the four +3 extensions to the relevant *Mse*I+2 primer. However, no unequivocal identification of the target fragments was achieved in this way. Following band excision from the +2/+2 profiles and cloning, we picked 40 clones from each ligation reaction of both the target band (+) and its negative control (–). Only fragments G (<200 bp) and I (<500 bp) were targeted for cloning as they were present in both recombinant forms

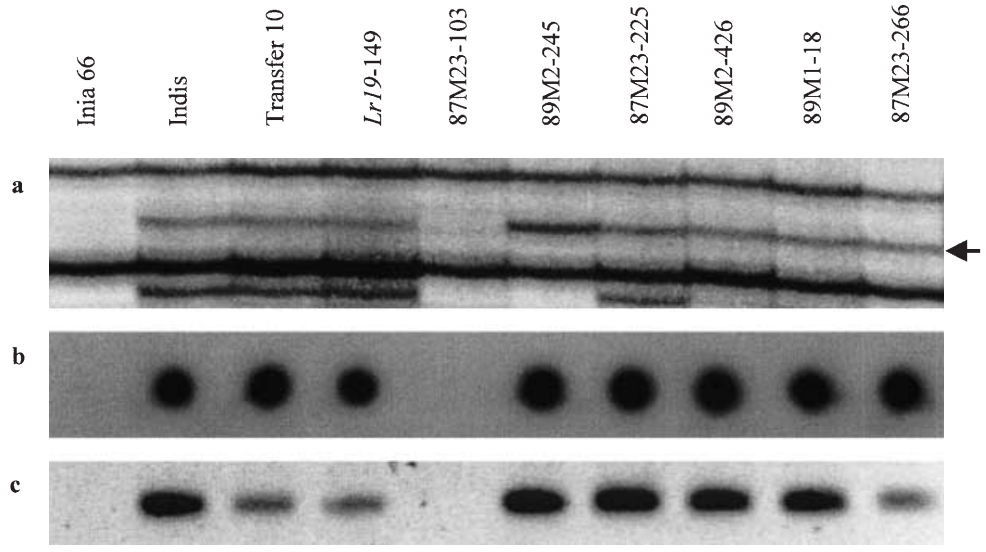

Fig. 1a Partial AFLP profile using the *Sse*I primer S13 and the *Mse*I primer M12, illustrating the segregation of marker fragment G (indicated by the *arrow*). Dot blot (**b**) of a fragment G clone from group B, hybridized with cold selective amplification reactions and segregation (**c**) of fragment G-derived marker, $STSLr19_{130}$, amplification products on an agarose gel. The same wheat lines were used in all three figures

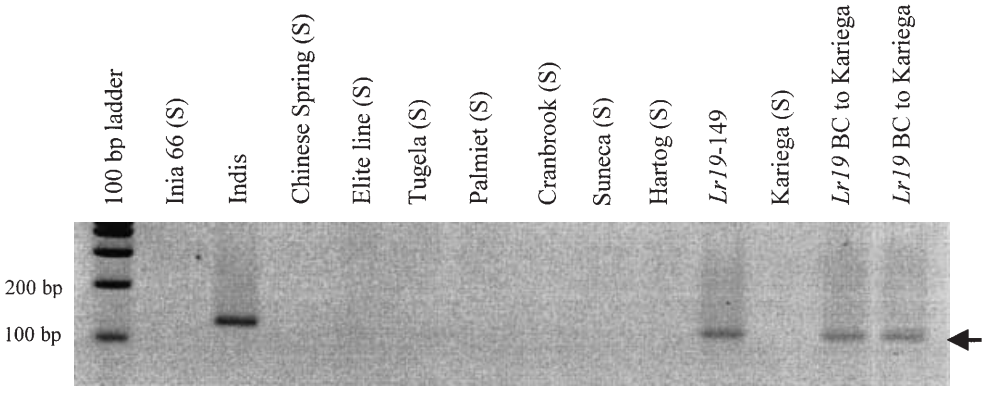

Fig. 2 Correlation of the presence of $STSLr19_{130}$ with *Lr19* among a range of wheat lines

and were of the desired size for primer design purposes. Fragment I was regarded as the optimal candidate for cloning, since fragment G was absent in mutant 87M23–103 and thus is probably further from *Lr19* than fragment I. Both the + and the – clones were clearly heterogeneous, showing clear size variation between the clones, even at the relatively poor resolution achievable in agarose gels. The spectrum of insert size was similar among clones from both templates. Those + clones with insert sizes clearly different from the AFLP target band were immediately discarded.

Fragment G

Clones from this fragment were screened by comparing insert size on sequencing gels following labeled amplification with the original AFLP primers. As a result of this screen, 12 clones were selected for sequencing. The AFLP primer sequences were recovered in all 12 clones. Five distinct sequences were identified (Ga to Ge), containing four, two, two, one and three clones, respectively (Table 2).

Primer sets specific to each of the five groups of sequences were designed (Table 2). When used to amplify genomic DNA of Inia 66 and Indis, four of the five primer sets (Ga, Gc, Gd and Ge) yielded identical PCR products from each template. However, set Gb generated an amplification product of 130 bp in Indis, but no product was amplified from the Inia 66 template. When tested on the individual *Lr19*-carrying mutants and recombinant lines, the results were in complete accordance with the AFLP genotyping with respect to fragment G (Fig. 1). Mirroring the AFLP profiles which led to the identification of fragment G, the clone from which the set Gb primers were obtained was also able to identify seven of the eight *Lr19*-carrying selective AFLP products in a dot blot assay (Fig. 1). The usefulness of this primer set was also confirmed as it clearly distinguishes lines carrying *Lr19* from those lacking it (Fig. 2).

Fragment I

Four different types of profiles were generated after restriction digestion of 18 of the M13 amplified inserts from similar-sized + clones, but it was not possible to distinguish a predominant restriction profile, as was generally found by Shan et al. (1999). Strong hybridization signals were obtained when each of these four classes of insert was used to probe dot blots consisting of unlabeled

selective AFLP reactions of +*Lr19* and –*Lr19* lines. The relevant *Sse*I and *Mse*I primers were then used individually and together to amplify each of the + clones. A similar-sized PCR product was obtained with the *Sse*I/*Mse*I combination and with *Mse*I alone, but no amplification occurred when the *Sse*I primer was used on its own. This test demonstrated that all four of these clone classes contained *Mse*I/*Mse*I inserts, and, therefore, that none could have represented the target AFLP band.

Discussion

The combination of AFLP technology and useful modified genetic forms of the *Lr19* translocation has facilitated the identification of new DNA markers that map in close proximity to the *Lr19* gene, a region that is poor in markers. One of the two fragments targeted for conversion was successfully converted to a dominant STS marker and was named $STSLr19_{130}$. This sequence was found to be present in all the *Lr19* genotypes tested, except for the irradiation-induced mutant 87M23–103, which indicates that its chromosomal location is distal to *Lr19*. This lack of association between $STSLr19_{130}$ and *Lr19* does not pose any problems for its use for marker-assisted selection, since the alien segment (both the original and recombined forms) does not normally recombine with its wheat homoeologue in the presence of the homoeologous pairing gene *Ph1*.

The AFLP conversion process was found to be technically difficult, mainly due to the presence of contaminating fragments. In our experiments the dominant clone was not necessarily the one carrying the correct insert, which suggests that a plausible reason for the failure of a number of efforts to convert AFLPs to STS may be that selecting the most frequently represented clone as the candidate for conversion unwittingly reduces the chance of success. In our case, the conversion attempt of fragment I failed, because all 40 clones contained a *Mse*I-*Mse*I (<500 bp) insert(s), which co-migrated with the target fragment. Vos et al. (1995) have suggested that larger *Mse*I-*Mse*I fragments amplify more easily than smaller ones as they are less likely to form the stem-loop structures that will compete with primer binding. This may explain why we did not encounter the same problem in the cloning of the smaller fragment G (<200 bp).

Based on our experience in cloning AFLP fragments of alien segments in wheat, we would propose the following verification strategy prior to clone sequencing:

1) Reject clones that are amplifiable by the *Mse*I primers alone.
2) Select clones on the basis of equal mobility on sequencing gels of PCR products amplified with AFLP primers and the original AFLP band.
3) Sequence remaining clones and check specificity by PCR for all the different groups of sequences.

Not all AFLP products will be readily convertible to an STS assay. Clearly, repetitive sequences are not suitable, as primers directed to such sequences will simultaneously assay many loci in the genome. Although the use of *Sse*I for the initial restriction of the wheat genomic DNA lessens the representation of repetitive DNA in the AFLP template by disciminating against methylated restriction sites, it does not eliminate it altogether, as demonstrated by the large number of wheat AFLP bands that cannot be assigned a chromosomal location when fingerprints from aneuploid wheat are compared with those of euploid wheat (R. Koebner, unpublished data). When *Eco*RI and *Mse*I are used to generate the initial template (neither of which discriminate against methylated restriction sites), this number is much higher than the approximately 60% reported by Shan et al. (1999) and Huang et al. (2000), since both of these studies assigned chromosome specificity on the basis of absence of the band in only one of the two possible nullisomic-tetrasomic lines for each wheat chromosome. Tellingly, when ditelosomic lines were tested, the latter authors report that under 6% of the total number of bands in the AFLP profiles of Chinese Spring can be assigned a chromosomal arm location. Short, low-copy sequences appeared to represent the optimum targets for conversion in *Asparagus* (Reamon-Büttner and Jung 2000). Where the AFLP is too short to leave sufficient sequence to design primers against, inverse PCR can be resorted to in order to target the regions flanking the AFLP (Brigneti et al. 1997; Bradeen and Simon 1998).

A more serious drawback arises where the assumption fails that a differential AFLP band in a genotypic contrast represents a sequence unique to the + genotype. This will occur whenever one of the restriction sites is deleted in the contrasting template due to a single nucleotide polymorphism. Although the AFLP fragment is absent from this profile, nevertheless, the allelic sequence is largely intact in the genomic template. This excludes using the STS product as a hybridization probe in a dot blot assay, and the PCR STS assay can only work if the primer specifically targets the polymorphic base at its 3′ end. Our assumption is that the most common source of sequence polymorphism leading to AFLP is through insertion/deletion (indel) events, just as it is in the case of RFLPs (Sharp et al. 1988). A combination of point mutations and small indels has been found in the small number of cases where allelic sequences derived from a converted AFLP have been compared (Bradeen and Simon 1998; Paltridge et al. 1998; Parker and Langridge 2000). The size and position of the indel relative to the STS sequence will determine whether a dot blot can be used, and the STS assay may generate a codominant pattern if the size of the indel is small relative to the size of the clone. For a hybridization strategy to be generally feasible, the sequence corresponding to the differential AFLP band must be absent altogether in the – genotypes. In wheat, and probably in most highly bred species, this scenario is only likely where an alien translocation, such as *Lr19*, is the target, or in certain special cases such as sexual dimorphism, where the structural gene is only present in one of the two contrasting genotypes. These

considerations may well explain why only a small number of workers have reported the successful conversion of AFLP products to STS assays in a variety of plants (Meksem et al. 1995; Brigneti et al. 1997; Qu et al. 1998; Shan et al. 1999; Paltridge et al. 1998; Parker and Langridge 2000; Reamon-Büttner and Jung 2000).

Apart from being a useful marker for marker-assisted selection, the original AFLP fragment and $STSLr19_{130}$ is also present in the secondary recombinants of *Lr19*-149 (Marais and Marais 1998), which emphasizes its close linkage with the gene (G.F. Marais, unpublished data). It therefore can serve as a useful tool both in attempts to further shorten the alien segment and also as a molecular landmark for a map-based isolation of *Lr19*, should this prove to be a worthwhile undertaking. For application in a breeding context, the STS that we have described needs be used in a PCR format, although we are currently determining the conditions under which the STS product could be used as a dot blot probe. Developments in marker technology promise formats, that are more easily scaled up to levels of throughput which are realistic in plant breeding (Koebner et al. 2001), but these all require access to a DNA sequence closely linked to the target.

Since the first published application of AFLP as a technique to generate DNA fingerprinting in wheat (Donini et al. 1997), there have been only three examples to date of its exploitation as a source of STS. In the cases of Qu et al. (1998) and Shan et al. (1999), the AFLPs converted were identified by comparing euploid wheat profiles with, respectively, those of a deletion mutant and a nullisomic line. In contrast, Parker and Langridge (2000) isolated allele-specific AFLPs identified from a segregation analysis. A poor conversion success rate of just 3 out of 16 led Shan et al. (1999) to conclude that STS derivation from AFLP is an inefficient process. However, under current conditions, since AFLP offers the best available means of uncovering linked DNA sequences, efforts to improve its convertibility will be worthwhile.

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