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A new and versatile method for the successful conversion of AFLP markers into simple single locus markers

Bart Brugmans*, Ron G. M. van der Hulst, Richard G. F. Visser, Pim Lindhout and Herman J. van Eck

Graduate School Experimental Plant Sciences, Laboratory of Plant Breeding, Department of Plant Sciences, Wageningen University, PO Box 386, 6700 AJ Wageningen, The Netherlands

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ABSTRACT

Genetic markers can efficiently be obtained by using amplified fragment length polymorphism (AFLP) fingerprinting because no prior information on DNA sequence is required. However, the conversion of AFLP markers from complex fingerprints into simple single locus assays is perceived as problematic because DNA sequence information is required for the design of new locus-specific PCR primers. In addition, single locus polymorphism (SNP) information is required to design an allele-specific assay. This paper describes a new and versatile method for the conversion of AFLP markers into simple assays. The protocol presented in this paper offers solutions for frequently occurring pitfalls and describes a procedure for the identification of the SNP responsible for the AFLP. By following this approach, a high success rate for the conversion of AFLP markers into locus-specific markers was obtained.

INTRODUCTION

Amplified fragment length polymorphism (AFLP™) is a PCR-based multi-locus fingerprinting technique, which efficiently identifies DNA polymorphisms without prior information on the DNA sequence of the organism(s) (1). AFLP relies on the selective amplification of a subset of DNA fragments from a more complex template pool that has been generated by ligation of adapters to restriction fragments. The advantages of AFLP are: high reproducibility (2), high PCR multiplex ratio, amenable at any genome complexity, the possibility to generate a virtually infinite number of markers and the fact that no prior sequence information is required. This is shown by the more than 1200 papers in which AFLP technology is used for all kinds of applications like genetic diversity analysis, local marker saturation, construction of genetic maps and quantitative trait loci (QTL) mapping in fungi (3), insects (4), plants (5–14) and animals (15–18). For single locus assays, AFLP markers are less suitable (e.g. allele frequency studies, marker-assisted selection or map-based cloning). Although AFLP markers can be used for these applications (19), many AFLP markers are redundant and hence too expensive and too laborious for large-scale single locus screenings. Due to this, there is a strong need to convert specific AFLP markers into single locus PCR markers, such as cleaved amplified polymorphic site (CAPS) (20) markers or sequence characterised amplified region (SCAR) (21) markers, for these marker techniques are easy to use, less laborious and inexpensive for simple locus assays.

Therefore, it is very important to have a reliable and efficient protocol for conversion of AFLP markers into high throughput single locus PCR markers. However, in contrast to AFLP, which can be applied immediately in any organism, the design of new PCR primers for a locus-specific assay does require information on the DNA sequence of the AFLP band. Preferably, the conversion of AFLP markers also aims to design a marker that can distinguish between different alleles. Often, sequencing of the existing alleles is required to identify allele-specific single locus polymorphisms (SNPs).

Although marker conversion seems technically easy, some hurdles need to be taken. The first hurdle is the extraction of an AFLP fragment from a polyacrylamide gel. Often, these extracts contain multiple fragments, which are the result of co-isolation of background amplification products of the AFLP fragment of interest (22). The second hurdle is the relative short size of AFLP bands. The resulting DNA sequence is often too short to optimally design PCR primers, and too short to expect internal polymorphisms, which can be used to differentiate between alleles (23–26). These steps during marker conversion severely reduce the efficiency of current protocols in which only a minority of the AFLPs was successfully converted into a locus- and/or allele-specific assay. Here we present a protocol that integrates various strategies in an optimal order. This step by step protocol guarantees successful marker conversion of virtually every AFLP marker.

MATERIALS AND METHODS

A diploid potato mapping population descending from the cross SH83-92-488 × RH89-039-16 (27) was used to confirm that the genetic map position of the single locus assay was

*To whom correspondence should be addressed. Tel: +31 317484165; Fax: +31 317483457; Email bart.brugmans@wur.nl

Table 1. The generalised set of 12 primers for AFLP-mediated mini-sequencing

Primer name	Sequence	
3N+A	GATGAGTCCTGAGTAA NNNA	For determination of the fourth selective nucleotide
3N+C	GATGAGTCCTGAGTAA NNNC	
3N+G	GATGAGTCCTGAGTAA NNNG	
3N+T	GATGAGTCCTGAGTAA NNNT	
4N+A	GATGAGTCCTGAGTAA NNNNA	For determination of the fifth selective nucleotide
4N+C	GATGAGTCCTGAGTAA NNNNC	
4N+G	GATGAGTCCTGAGTAA NNNNG	
4N+T	GATGAGTCCTGAGTAA NNNNT	
5N+A	GATGAGTCCTGAGTAA NNNNNA	For determination of the sixth selective nucleotide
5N+C	GATGAGTCCTGAGTAA NNNNNC	
5N+G	GATGAGTCCTGAGTAA NNNNNG	
5N+T	GATGAGTCCTGAGTAA NNNNNT	

These primers will provide DNA sequence information of three more bases adjacent to the first three selective nucleotides of the *Mse*I primer.

identical to the results obtained for the original AFLP marker. Plant DNA was isolated essentially according to Steward and Via (28), adjusted for 96-well format using 1 ml tubes of Micronics (Micronic BV, Lelystad, The Netherlands). Leaf tissue was ground using a Retsch 300 mm shaker at maximum speed (Retsch BV, Ochten, The Netherlands).

Template preparation and AFLP fingerprinting were essentially performed as described in Vos *et al.* (1). For conversion of AFLP markers into single locus markers, we choose AFLP fragments of different sizes (between 100 and 400 bp) from template prepared with *Eco*RI/*Mse*I as well as with *Pst*I/*Mse*I.

For the determination of the fourth, fifth and sixth selective nucleotide following the AFLP-restriction site (throughout this text referred to as AFLP-mediated mini-sequencing) a generalised set of 12 degenerated primers (Table 1) was used for AFLP fingerprinting using 100 times diluted +3/+3 pre-amplified product as template and the standard primer concentrations of 0.5 pmol ^{33}P or fluorescently (IRDye™ 700) labelled *Eco*RI or *Pst*I primer and 3 pmol *Mse*I primer per reaction.

To excise the ^{33}P-labelled AFLP fragment out of an acrylamide gel, an AFLP fingerprint was generated using an *Eco*RI+3 or *Pst*I+2 in combination with the, by AFLP-mediated mini-sequencing identified, *Mse*I+6 primer. The polyacrylamide gels, dried on Whatmann 3MM paper, were overlaid with autoradiogram images. The pieces of gel/paper were transferred to 200 µl of TE and incubated for 1 h. Five microlitres of supernatant was used to re-amplify the fragment, using a PCR in which the *Eco*RI+0 or *Pst*I+0 in combination with *Mse*I+0 were used as primers. In total, 200 ng of the re-amplified AFLP fragment was used for direct sequencing using the appropriate AFLP+0 primer as sequencing primer (BaseClear, Leiden, The Netherlands). As an alternative for radioactivity, samples generated by using fluorescently (IRDye™ 700) labelled *Eco*RI+3 or *Pst*I+2 primers combined with fragment-specific *Mse*I+6 primers were analysed on a NEN® Global Edition IR^2 DNA Analyzer (LI-COR® Biosciences, Lincoln, NE). After separation, the polyacrylamide gel was scanned on a LI-COR® Biosciences Odyssey® Infrared Imaging System along with a grid pattern to allow careful positioning of bands. Gel plugs containing fragments were excised using a scalpel and successful fragment extraction was verified by re-scanning the gel (29).

Table 2. Set of relatively cheap frequent cutting restriction enzymes for cost-effective detection of internal polymorphisms that can be used as a CAPS marker

Restriction enzyme
*Aci*I
*Alu*I
*Apo*I
*Bfa*I
*Bsa*JI
*Bss*KI
*Bst*UI
*Dde*I
*Dpn*I
*Hae*III
*Hha*I
*Hinf*I
*Hpa*II
*Hpy*188I
*Hpy*CH4III
*Hpy*CH4IV
*Mnl*I
*Mwo*I
*Nla*III
*Nla*IV
*Rsa*I
*Sau*96I
*Taq*I
*Tsp*509I

After excision, gel plugs were placed in 15 µl of 1× TE and frozen at –80°C for ~30 min, followed by one thawing–refreezing step at –20°C. After thawing, samples were centrifuged for 15 min at 15 000 *g* and 4 µl was taken for PCR re-amplification using *Eco*RI+0 or *Pst*I+0 in combination with *Mse*I+0 primers. Fragments were sequenced directly using the same primers as used for re-amplification on a NEN® Global Edition IR^2 DNA Analyzer using IRDye 800 v2 Acycloterminators™.

The DNA sequence of the excised AFLP band was used to design locus-specific primers. The amplification product obtained with such primers was screened for internal polymorphisms with restriction enzymes listed in Table 2. After restriction, the fragments were separated on a 3% agarose gel including ethidiumbromide.

DNA adjacent to the AFLP fragment was obtained by anchor PCR using the Genome Walker Kit (Clontech, Palo

Alto, CA) based on the method of Siebert *et al.* (30). Ten restriction enzymes (*Aat*I, *Alu*I, *Dpn*I, *Dra*I, *Hinc*II, *Pvu*II, *Sma*I, *Xmn*I, *Ase*I, *Mse*I) were individually used for restriction and ligation of the Genome Walker adaptors. PCRs with an internal primer (based on the sequence of the AFLP fragment) in combination with the Genome Walker adapter primer were performed according to the standard AFLP protocol on 10 times diluted restriction–ligation mix. Nested PCRs using a second internal primer (based upon the sequence of the AFLP fragment) and a second adapter primer were performed on 100 times diluted amplification product of the first PCR. Five microlitres of the final PCR product was checked on a 3% agarose gel to verify that a unique fragment was obtained. The remaining 45 µl of PCR product was used for sequencing (BaseClear). The flanking sequence information was used to design a primer that amplified a fragment in combination with the internal primer of the AFLP fragment. The SNP that originally caused the AFLP was included in this fragment and used for the development of a CAPS or dCAPS.

Single locus PCRs for CAPS or dCAPS were performed using 5 µl of DNA (10 ng/µl), 0.6 µl of each primer (50 ng/µl), 0.8 µl of dNTPs (5mM) and 0.08 µl of *Taq* polymerase (5 U/µl) in a total volume of 20 µl.

RESULTS AND DISCUSSION

A flowchart for the procedure to convert AFLP markers into simple single locus PCR assays, or allele-specific PCR markers, is shown in Figure 1. The protocol is comprised of the following steps: (step 1) AFLP-mediated mini-sequencing, (step 2) re-amplification of the AFLP fragment in a less complex fingerprint and excision of the AFLP fragment, (step 3) direct sequencing of the excised and reamplified AFLP fragment, (step 4) design of internal locus-specific primers, (step 5) screening for additional internal polymorphic sites, (step 6) identification of the SNP that originally caused the AFLP, (step 7) identification of flanking DNA and (step 8) exploitation of the SNP that caused the AFLP in a CAPS or dCAPS marker.

In total, 10 randomly chosen AFLP markers (Table 3), containing relatively small (131 bp) and large (359 bp) fragments were converted into simple PCR markers to demonstrate the universal applicability of the procedure.

AFLP-mediated mini-sequencing

The first step of the protocol aims for the determination of the fourth, fifth and sixth selective nucleotides adjacent to the *Mse*I primer of which the first three selective nucleotides are known. A generalised set of 12 degenerated primers was used (Table 1) to analyse the adjacent nucleotides for all our fragments. A typical image obtained by the mini-sequencing primers is shown in Figure 2. For all 10 selected AFLP fragments, the next three selective nucleotides could be determined unambiguously and are listed in Table 3. Remarkably, the total amount of degenerated primer needed for this technique appeared not very critical and may vary between 3 and 10 pmol per 10 µl of PCR volume. It is however more important that the *Eco*RI+3/*Mse*I+3 or *Pst*I+2/*Mse*I+3 pre-amplified product mixture, used as template, is sufficiently diluted (>100 times) in order to avoid that unused *Mse*I+3 primer from previous PCRs disturbs the reaction and the fingerprints resemble the original +3/+3 amplification pattern (data not shown).

Re-amplification of the AFLP fragment in a less complex fingerprint and excision of the AFLP fragment

Information on the fourth, fifth and sixth selective nucleotides allowed PCR amplification using *Mse*I+6 primers. A new AFLP fingerprint of much lower complexity was generated using the *Mse*I+6 primers using diluted +3/+3 AFLP amplification products as template. Two such fingerprints are shown in Figure 3. In rare cases, background products from the +3/+3 AFLP fingerprint appear as very clear bands in the +3/+6 AFLP fingerprint. These fragments did not influence the extraction of the desired fragment from gel. The intensity of the target AFLP fragment and a few other remaining AFLP fragments is much higher and almost always without co-migrating fragments compared to a standard (+3/+3) AFLP. This increases the probability of successful excision of the desired fragments from dried gels. DNA that dissolved from the pieces of gel/paper into TE buffer was re-amplified with AFLP primers without selective nucleotides using 5 µl from this solution. For all 10 AFLP fragments large amounts of re-amplified DNA could be obtained.

The implementation of extra selective nucleotides is also possible at the rare cutter site to further reduce fingerprint complexity, for example, in very dense fingerprints or if two fragments with a very small size difference appear to have the same six selective nucleotides at the *Mse*I site. For these special cases the fourth, fifth and sixth selective nucleotides for the *Eco*RI site (or the third, fourth and fifth for the *Pst*I site) can be determined by using a set of degenerate *Eco*RI (or *Pst*I) primers and labelled *Mse*I primers. In case fluorescently (e.g. IRDye™ 700) labelled *Mse*I primers need to be ordered for this latter step, it is interesting to note that *Mse*I+2 primers work equally well as labelled *Mse*I+3 primers (results not shown). This will considerably reduce the ordering costs of labelled primers when AFLP fragments of different primer combinations need to be converted. The fragment can successively be excised from a fingerprint generated with a *Mse*I+6 and a *EcoR*I+6 or *Pst*I+5 primer.

Direct sequencing of the excised and re-amplified AFLP fragment

DNA of the excised and re-amplified AFLP fragment was sequenced from both ends using the corresponding core primers without selective nucleotides as sequencing primer. Usually, DNA fragments are cloned into *Escherichia coli*, confirmed by PCR or restriction analysis and sequenced (23,24,31). However, the drawback of this method is that other non-specific co-migrating DNA fragments may be cloned and sequenced. When the number of sequenced clones is low or the cloning efficiency of a particular AFLP fragment is low, co-isolated fragments may outnumber the sequence of the correct fragment and hamper the determination of the right sequence (22). In contrast, direct sequencing of a fragment allows the determination of the level of putative impurities in the PCR product. This can be inferred from the trace-file (peak pattern) from the DNA sequencer, where clear peaks should be prominent against little background.

Out of the 10 double-stranded sequences that were obtained, eight had no ambiguities and two had <5% ambiguous

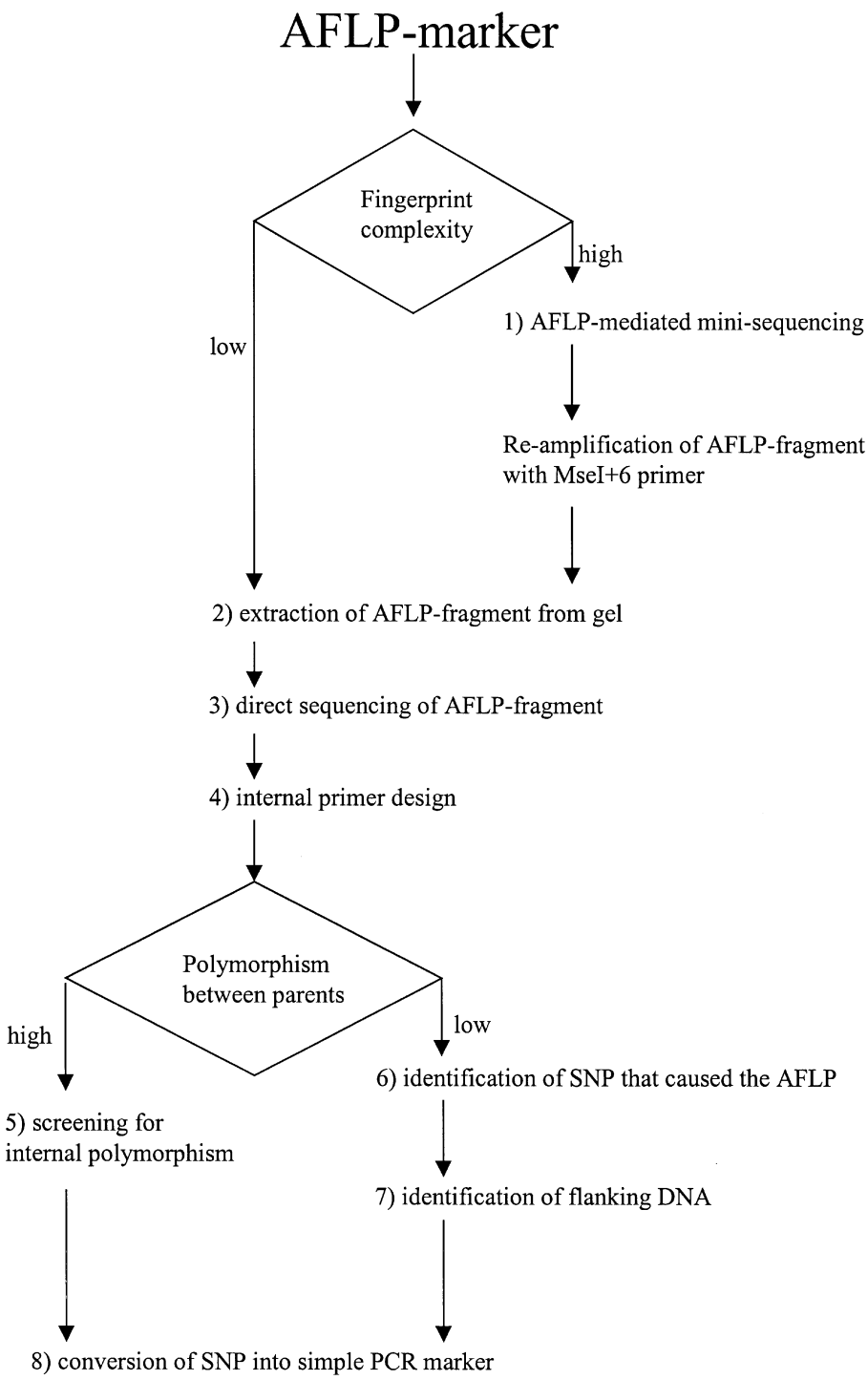


Figure 1. Overview of the steps of the protocol to convert any AFLP marker into a single locus PCR-based marker assay (our flowchart).

nucleotides. This confirms that DNA from excised bands, re-amplified from diluted +3/+3 template with a *Mse*I+6 primer, is highly pure. Therefore, this procedure avoids the need to clone AFLP fragments and to sequence multiple clones. Isolation of fragments from ^{33}P- and IRDye™ 700-labelled AFLP fingerprints provided sequences that were essentially identical (<1% differences, data not shown). The sequences of all fragments have been filed in the GenBank database under accession numbers AY244747–AY244759.

Design of internal locus-specific primers

To amplify a DNA fragment internal of the AFLP marker, primer pairs were designed on the basis of the sequence data obtained from the excised AFLP fragments (Table 4). To make the PCR as stringent as possible, only primers with annealing temperatures higher than 54°C were designed. In addition, this will allow the re-use of the primers for the Genome Walker procedure if needed. Preferably, the fragment

Table 3. Ten selected AFLP-marker conversions with their extra selective nucleotide for the *Mse*I primer, the restriction enzymes that provided a CAPS with the internal fragment and the site in which a SNP was present that caused the AFLP

Fragment and fragment length (bp)	Fourth, fifth and sixth selective nucleotide for the *Mse*I primer	CAPS enzyme of internal fragment	Place of SNP that caused the AFLP and the exact SNP for the three elongated fragments
E-ACT/M-CAG-287	GTC	[a]	*Eco*RI restriction site GAA(G/T)TC
E-AGA/M-CAG-188	AAG	*Mnl*I	*Mse*I restriction site
E-AGA/M-CCT-131	AAA	[a]	*Eco*RI restriction site GAATT(C/T)
E-ATC/M-CAC-251	AAA	*Apo*I/*Mnl*I	*Mse*I selective nucleotide
E-ATG/M-CTT-239	TTT	*Hinf*I	*Mse*I restriction site
P-AC/M-ATA-320	AAT	*Sau*96I/*Nla*IV	*Pst*I restriction site
P-AG/M-ATG-359	AAG	*Nla*III	*Pst*I selective nucleotide
P-AT/M-AGC-326	CAA	*Nla*III/*Mnl*I	*Pst*I selective nucleotide
P-TG/M-AGA-198	CCA	[a]	*Mse*I selective nucleotide (A/T)GA
P-TG/M-AGT-321	GTA	*Hpy*CH4IV	*Pst*I restriction site

[a]No restriction enzyme from Table 2 provided a CAPS marker.

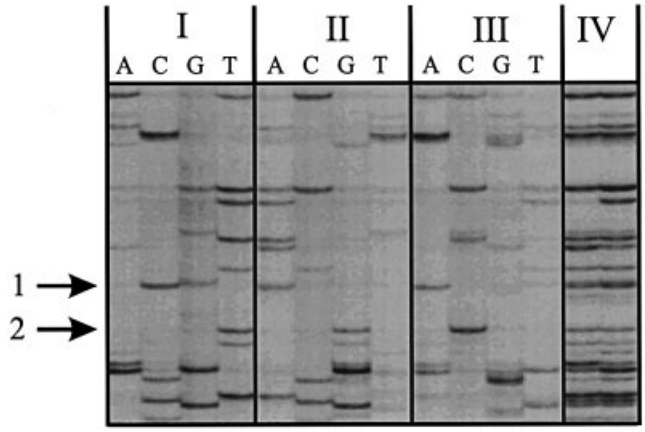


Figure 2. Products obtained after PCR using the 12 degenerated *Mse*I primers (listed in Table 3) on 100 times diluted *Eco*RI+3/*Mse*I+3 template loaded on polyacrylamide gel. The first four lanes allow the determination of the fourth selective nucleotide (I), the second four lanes allow the determination of the fifth selective nucleotide (II) and the third four lanes allow the determination of the sixth selective nucleotide (III). As a control the *Eco*RI+3/*Mse*I+3 PCR product from both parents was loaded on gel (IV), e.g. fourth, fifth and sixth selective nucleotides for fragment 1 are C-A-A and for fragment 2 are T-G-C.

between the primers was kept as large as possible to increase the probability of a SNP within the fragment, but the annealing temperature was considered of greater importance.

For all 10 markers a single amplification product was obtained using the internal primers using genomic DNA as template. The mobilities of the amplification products in 3% agarose gel corresponded to the expected sizes. This demonstrates the feasibility of obtaining SCARs (21) that can be used to screen genomic libraries. In addition, a SCAR can be used or converted into genetic marker corresponding to the AFLP locus.

For two markers the internal fragment was only amplified in genomic DNA from genotypes that also displayed the original AFLP marker, providing a dominant allele-specific PCR marker or SCAR marker. For the remaining eight markers the SCAR primers resulted in only one amplification product, although a difference between the homologous chromosomes was recognised by the AFLP marker. Subsequent steps are

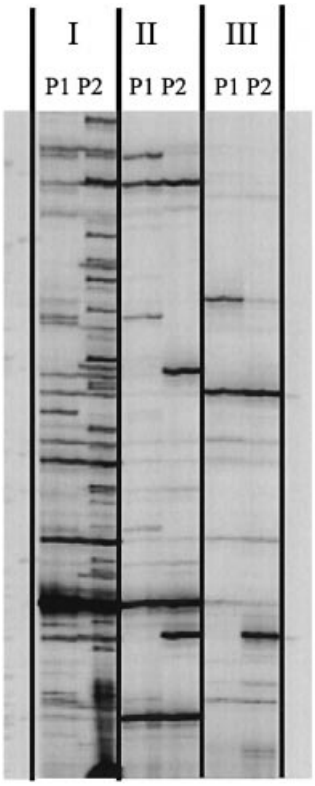


Figure 3. Reduction of the AFLP fingerprint complexity by application of the '*Mse*I+6-primer' to allow the isolation of an AFLP band by excision from the gel without the co-isolation of contaminating DNA fragments. In this image the *Eco*RI+3/*Mse*I+3 amplification products of both parental genotypes (I) are compared with their respective *Eco*RI+3/*Mse*I+6 amplification products (II and III).

required to convert these eight SCARs into another marker type before they can be used as genetic markers with the same locus and allele specificity as their corresponding AFLP markers.

Screening for additional internal polymorphic sites

New primers were designed and tested for the two markers where SCAR primers only amplified specific alleles. These

Table 4. Primers designed upon the internal sequence of the AFLP fragment to amplify the internal fragment out of genomic DNA

Fragment	Forward primer (5′–3′)[a]	Reverse primer (5′–3′)[b]
E-ACT/M-CAG-287	GAAAAATGTAATCGGTGGGAG	ACTCTCAACTTATGGATTTTC
		GGGTGGAAATATAATAATAGTAG[c]
E-AGA/M-CAG-188	ATTGTCTCTCTCTTGTCAC	CCAATAATGTAGTAGAACCA
E-AGA/M-CCT-131	ATTTGAATTGGAAGAGTTTTAC[d]	TAAAGCCAATTTTCCCCCATC[d]
	AGCAATATTCAGATTGTCTG	CCCCCATCTATATTTTTCAATC
		CTCCCACGTTACCTAACATAC[c]
E-ATC/M-CAC-251	ATGCAGTGATATATTCTATTTG	ATTTTGGCACTTCTATCTTTGC
E-ATG/M-CTT-239	GGGCCTCGGGTGTCCGT	GTCACACATCTCTCCCCTTA
P-AC/M-ATA-320	ACTCCTCAGATTCTGATGATG	GGTGTGTAACATTGTCAGC
P-AG/M-ATG-359	GAATCTAGACTTGGAACTCATG	TGAGTGAGTCATATAGGCAG
P-AT/M-AGC-326	CCAGTTTACCAGAGGCCATTC	CACACTGTACTCTCTGTCC
P-TG/M-AGA-198	GGTTGCCGGTGCTGATG	CTCCTAAACAGCGCACGATTC
	AGTTTGGTTTGACTAATGCTCCT[c]	
P-TG/M-AGT-321	GGATGAGGTATTGGAGCTTTGC[d]	GATAGTTCCTATGATATTATAGTA[d]
	GGAGCTTTGCAGAGATAGGAAG	TATTTTACTTTGTTGTAGGTTACGAAAG

[a]This primer amplifies towards the *Mse*I site.
[b]This primer amplifies towards the *Eco*RI or *Pst*I site.
[c]These primers were designed externally of the AFLP marker upon the sequence gained after usage of the Genome Walker Kit.
[d]These primer combinations gave a dominant SCAR.

new primers are also listed in Table 4. Consequently, 10 fragments could be tested for their suitability to be converted into multi-allelic CAPS markers, rather than into SCARs.

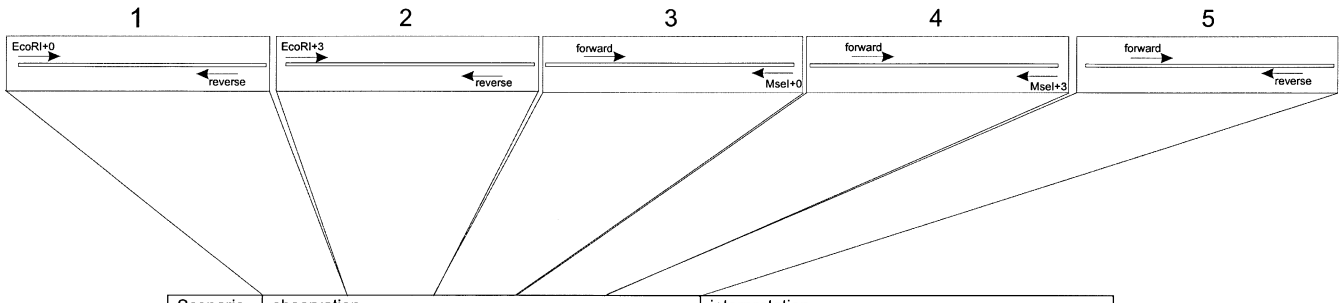
To detect internal DNA sequence variation among alleles in various potato genotypes, the monomorphic SCAR fragments were tested for the presence of restriction enzyme recognition sites using a set of restriction enzymes. To increase the possibility of detecting a SNP in a restriction site, four or five base cutter enzymes were selected, rather than restriction enzymes having more selective nucleotides. The enzymes were selected on the basis of recognition site and low cost per unit, whereby restriction enzymes with the same recognition site were avoided. To this end a collection of 24 different four or five base cutter restriction enzymes (Table 2) was chosen. Restriction fragment length polymorphisms between the potato genotypes were detected for 7 out of 10 tested SCAR fragments. Some SCAR fragments displayed polymorphisms detected by more than one restriction enzyme. The names of the enzymes are listed in Table 3. In addition, multiple alleles could be displayed with combinations of restriction enzymes, as inferred from the CAPS banding patterns observed among various potato genotypes (data not shown). In all cases the segregation of the diagnostic CAPS marker allele in the potato offspring was identical to the segregation of the AFLP marker allele, which confirms the locus specificity of the AFLP-derived CAPS marker.

Identification of the SNP that originally caused the AFLP marker

If no internal polymorphism could be detected, it was required to determine which SNP caused the AFLP marker. The majority of the polymorphisms displayed with AFLP seem to reflect SNPs, but we are aware that other molecular events, such as indels, could play a role. Whenever SNP is used in this paper, those other cases are also implied. Exploitation of that SNP would still allow the conversion of the AFLP marker into a simple PCR marker, even without internal SNPs. To determine the SNP, we performed a set of five PCRs on AFLP template (restriction–ligation mix) derived from the parent in which the AFLP band could not be amplified (Fig. 4) using combinations of an internal primer and the selective and non-selective AFLP primers. When the selective and non-selective primers differentially amplified a product, then obviously the SNP should be within the selective nucleotides. When the combination of internal and non-selective AFLP primers still cannot amplify the expected band, then the SNP is part of the recognition site of the restriction enzyme. Knowing that the SNP is within the recognition site, the strategy to develop a polymorphic CAPS marker should exploit this enzyme to cleave the PCR product.

For 4 out of 10 markers it was demonstrated that the rare cutter restriction site (*Eco*RI or *Pst*I) contained the SNP between the parents (Table 3). In these cases the *Pst*I+0 or the *Eco*RI+0 primer combined with the locus-specific internal primer did not give a PCR fragment for the sample in which the particular AFLP marker had been missing (scenario A, Fig. 4). Two polymorphic AFLP bands were caused by a SNP within the *Mse*I restriction site (Table 3). In this case, the *Mse*I+0 primer combined with the specific primer did not give a PCR fragment for the AFLP negative sample (scenario C, Fig. 4). Primers with selective nucleotides showed that three AFLP markers were caused by a SNP between the parents in the selective nucleotides flanking the *Eco*RI or *Pst*I restriction site (scenario B, Fig. 4). The last marker is an example where the SNP between the parents was present in the selective nucleotides flanking the *Mse*I restriction site (scenario D, Fig. 4).

It should be noted that for some tests the annealing temperature appeared critical. For these markers, sub-optimal annealing temperature provided several fragments or a smear rather than the predicted single fragment after PCR. By performing the tests at several annealing temperatures using a temperature gradient PCR machine, which simultaneously can handle 12 different annealing temperatures, these problems



Scenario	observation					interpretation
	1	2	3	4	5	
A	-	-	+	+	+	SNP in *EcoR*I restriction site
B	+	-	+	+	+	SNP in *EcoR*I selective nucleotides
C	+	+	-	-	+	SNP in *Mse*I restriction site
D	+	+	+	-	+	SNP in *Mse*I selective nucleotides
control	+	+	+	+	+	all PCR reactions were succesfull

Figure 4. Overview of the PCRs required for identification of the SNP that caused the polymorphism of the AFLP marker. With these five combinations of internal primers and selective and non-selective AFLP primers, the SNP can unambigiously be determined. Primer 1 is the *Eco*RI or *Pst*I matching AFLP primer without selective nucleotides; primer 2 is like primer 1 but with three selective nucleotides; primer 3 is the *Mse*I primer without selective nucleotides; primer 4 is the *Mse*I primer with selective nucleotides. Assay 5 is the positive control for the internal primers.

were overcome (data not shown). For one fragment, varying annealing temperature alone was not sufficient to detect the SNP. For this sample a two-step PCR, which used two specific internal primers successively in combination with the same AFLP primer, was needed. Whether or not two specific primers are needed to determine the SNP is a peculiarity of the primer sequence in relation to template complexity, which cannot be predicted *a priori.* However, species that are known to contain large numbers of repetitive sequences are supposed to need a two-step PCR. Eventually in all 10 cases the SNP between the parents could be detected without ambiguity.

Conversion of the SNP that caused the AFLP into a CAPS or dCAPS marker

To exploit polymorphisms in the restriction site used for AFLP template preparation, or among the selective nucleotides, it is required to obtain sequence information of the genomic DNA flanking the AFLP amplicon. This would allow the design of a PCR primer pair encompassing the restriction site or selective nucleotides with the SNP. Polymorphisms within the restriction site will immediately provide a CAPS marker when using *Eco*RI, *Pst*I or *Mse*I. Polymorphisms within the selective nucleotides can be exploited to design a dCAPS marker assay (32). The dCAPS method is a marker technique where mismatches in a PCR primer are used to create restriction site polymorphism based on the target mutation.

Of the three AFLP markers that had not yet been converted in earlier steps of the protocol, markers E-ACT/M-CAG-287 and E-AGA/M-CCT-131 were found to be based on a polymorphism in the *Eco*RI restriction site used for AFLP-template preparation. The third marker P-TG/M-AGA-198 was found to be based on a SNP within the selective nucleotides following the *Mse*I restriction site (Table 3). Flanking DNA of these three markers was obtained using the Genome Walker Kit (Clontech). For several enzyme/adapter combinations of the Genome Walker Kit, amplification products were obtained, but only one amplification product was selected and sequenced from both parents. New PCR primers were designed upon the flanking DNA to allow the amplification of a fragment that comprises the SNP. These primers are listed in Table 4. As expected, the first two markers E-ACT/M-CAG-287 and E-AGA/M-CCT-131 provided a CAPS marker when *Eco*RI was used as restriction enzyme. The polymorphism of marker P-TG/M-AGA-198 was converted into a dCAPS by using the program of Neff *et al.* (32). This resulted into a dCAPS by using the restriction enzyme *Ase*I.

In our experience the Genome Walker Kit is a robust method of obtaining flanking DNA sequences. Alternatively, the more laborious method of inverse-PCR (I-PCR) could be exploited. For organisms with large numbers of repetitive sequences I-PCR seems to perform better (33). Advantageous of I-PCR is the simultaneous use of two specific internal primers, which enlarges the chance of amplifying a single unique fragment.

APPLICATION

At the moment AFLP is applied in many organisms for many types of study, because it is an efficient technique to study DNA polymorphisms without start-up costs or the need of sequence information. As soon as useful AFLP markers have been identified that tag a specific trait, locus or map position, it is often preferred to replace AFLP markers with a simpler, agarose-based single locus PCR marker assay.

This paper describes a general and efficient protocol for the conversion of AFLP markers into single locus PCR assays (which can be fully non-radioactive, if required). Its universal applicability was demonstrated by the conversion of 10 AFLP markers of rather small (131 bp) as well as large (359 bp) size. In principle, there is no minimal size of an AFLP marker, as long as the internal sequence of the AFLP band is sufficiently long to allow the design of a highly specific PCR primer. This is the minimal requirement to isolate the flanking DNA sequence to design a reverse PCR primer to provide a CAPS or dCAPS in combination with the SNP that caused the AFLP

marker. By following this approach there is no need to search for internal polymorphisms. The possibility to detect another internal polymorphism may be low and is highly dependent on the genetic diversity within the species. In this study, internal polymorphisms were detected in 7 out of 10 cases. This is due to the high level of DNA polymorphism typical for potato caused by its outbreeding mode of reproduction. In our laboratory, the protocol has been tested on cultivated tomato (*Lycopersicon esculentum*) and flax (*Linum ussitatisimum*). Both are self-fertilising species with a very narrow gene pool. Searching for internal polymorphisms had a lower success rate, even in fragments exceeding 400 bp (data not shown). In such species it might be recommended to start with the identification of the SNP in the restriction sites or selective nucleotides that caused the original AFLP.

When the flanking DNA contains repetitive elements, multiple PCR fragments may be obtained, despite the specificity of the primers used. Nevertheless, as long as the AFLP marker is a single locus marker (not a multitude of co-migrating amplification products), there is no obstacle to exploit the unique AFLP causing SNP to allow the conversion of the AFLP marker into a simple PCR marker.

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