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G. Li · C. F. Quiros

Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica*

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Abstract We developed a simple marker technique called sequence-related amplified polymorphism (SRAP) aimed for the amplification of open reading frames (ORFs). It is based on two-primer amplification. The primers are 17 or 18 nucleotides long and consist of the following elements. Core sequences, which are 13 to 14 bases long, where the first 10 or 11 bases starting at the 5′ end, are sequences of no specific constitution ("filler" sequences), followed by the sequence CCGG in the forward primer and AATT in the reverse primer. The core is followed by three selective nucleotides at the 3′ end. The filler sequences of the forward and reverse primers must be different from each other and can be 10 or 11 bases long. For the first five cycles the annealing temperature is set at 35°C. The following 35 cycles are run at 50°C. The amplified DNA fragments are separated by denaturing acrylamide gels and detected by autoradiography. We tested the marker technique in a series of recombinant inbred and doubled-haploid lines of *Brassica oleracea* L. After sequencing, approximately 45% of the gel-isolated bands matched known genes in the Genbank database. Twenty percent of the SRAP markers were co-dominant, which was demonstrated by sequencing. Construction of a linkage map revealed an even distribution of the SRAP markers in nine major linkage groups, not differing in this regard to AFLP markers. We successfully tagged the glucosinolate desaturation gene *BoGLS-ALK* with these markers. SRAPs were also easily amplified in other crops such as potato, rice, lettuce, Chinese cabbage *(Brassica rapa* L.), rapeseed (*Brassica napus* L.), garlic, apple, citrus, and celery. We also amplified cDNA isolated from different tissues of Chinese cabbage, allowing the fingerprinting of these sequences.

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G. Li · C.F. Quiros (✉)
Department of Vegetable Crops, University of California, Davis, CA 95616, USA
e-mail: cfquiros@ucdavis.edu
Tel.: 530-752-1734, Fax: 530-752-9659

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Introduction

The polymerase chain reaction (PCR) is widely used in genomic DNA analysis. One of its main applications has been in the development of DNA markers for map construction, which are useful in breeding, taxonomy, evolution and gene cloning.

Several PCR marker systems are available varying in complexity, reliability and information generating capacity. These include random amplified polymorphic DNA (RAPD), simple sequence repeat polymorphism (SSR), amplified fragment length polymorphism (AFLP) and a few others (Lee 1995; Rafalski et al. 1996). Each system has its own advantages and disadvantages. For example, RAPD is a simple method to fingerprint genomic DNA, but poor consistency and low multiplexing output limit its use. SSR has the advantage of producing mostly co-dominant markers; however, the development of these is considerably expensive and time- consuming. AFLPs are now widely used for a variety of applications due to its high multiplexing ratio (Vos et al. 1995). The main disadvantage of this method is its complexity, being necessary to perform multiple steps including DNA digestion, ligation and amplification, which makes it difficult to optimize the conditions for each step. Furthermore, methylation of genomic DNA can result in pseudo-polymorphism when the restriction enzyme used is methylation-sensitive. Also the use of the *Mse*I restriction enzyme, which recognizes AATT restriction sites, often results in uneven marker distribution in the genome of some species (Haanstra et al. 1999). The ability to isolate specific bands for sequencing is another concern when selecting a marker system, especially for the development of new markers for gene tagging. In most cases, both RAPD and AFLP markers need to be cloned into vectors, which adds to the labor. In addition, for AFLP bands it is notoriously difficult to isolate the correct fragment due to band overlapping.

In this paper we propose a new marker technique called sequence-related amplified polymorphism (SRAP), which combines simplicity, reliability, moderate throughput ratio and facile sequencing of selected bands. Further, it targets coding sequences in the genome and results in a moderate number of co-dominant markers. We used SRAP and AFLP markers to construct a genetic map of *Brassica oleracea* in order to demonstrate the application of the former in genetic mapping. This includes tagging of the *GLS-ALK* gene, which regulates the desaturation of aliphatic glucosinolates in crucifers. SRAP was also used to fingerprint genomic DNA and cDNA from other crops to show its potential broad applications.

Material and methods

Plant material

The SRAP marker system was developed primarily for *Brassica* species, but we tested it also in other crops such as potato, rice, apple, citrus, cherry, plum, garlic, lettuce and celery.

The following *B. oleracea* crops were included in the study: broccoli, cauliflower, and kale. A population of recombinant inbred (RI) lines resulting from crossing collard×cauliflower was used for mapping the *GLS-ALK* gene and sequencing some of the markers. We also used a doubled-haploid (DH) population of broccoli and cauliflower to test the reproducibility of the SRAP markers. For cDNA fingerprinting, Chinese cabbage was used as a source of mRNA from isolated pollen mother cells, meiocytes and meiotic flower buds (Li and Quiros, unpublished).

DNA extraction

A modified version of the CTAB method was used to extract genomic DNA. A 1.5-ml tube containing approximately 0.3 g of fresh leaf tissue was placed into liquid nitrogen for 30 s and its contents crushed with a small plastic bar. Then, 0.5 ml of 2×CTAB buffer was added to the tubes and incubated at 65°C for 90 min. After incubation, 0.4 ml of chloroform was added and the tubes were centrifuged at 14,000 rpm for 3 min. The supernatant was transferred into a new tube and the DNA precipitated in a 0.6 vol of 2-propanol. The DNA was then washed with 70% ethanol and dissolved in TE buffer.

Protocol for the SRAP marker system

SRAP is a PCR-based marker system with two primers, a forward primer of 17 bases and a reverse primer of 18 bases. These are labelled with [γ^{33}P]-ATP for amplification of genomic DNA.

The forward primers consist of a core sequence of 14 bases. The first ten bases starting at the 5′ end are "filler" sequences of no specific constitution, followed by the sequence CCGG and then by three selective nucleotides at the 3′ end. Variation in these three selective nucleotides generates a set of primers sharing the same core sequence. The reverse primers consist of the same components as the forward primers with the following variations: the filler is followed by AATT instead of the CCGG sequence. Following the AATT sequence, three selective bases are added to the 3′ end of the primer. The only rules for construction of the forward and reverse primers are that they do not form hairpins or other secondary structures, and to have a GC content of 40–50%. Further, the filler sequences of the forward and reverse primers must be different from each other and can be 10 or 11 bases long.

Example of forward primers:

me1, 5′-TGAGTCCAAACCGGATA-3′,
me2, 5′-TGAGTCCAAACCGGAGC-3′,
me3, 5′-TGAGTCCAAACCGGAAT-3′,
me4, 5′-TGAGTCCAAACCGGACC-3′,
me5, 5′-TGAGTCCAAACCGGAAG-3′.

Example of reverse primers:

em1, 5′-GACTGCGTACGAATTAAT-3′,
em2, 5′-GACTGCGTACGAATTTGC-3′,
em3, 5′-GACTGCGTACGAATTGAC-3′,
em4, 5′-GACTGCGTACGAATTTGA-3′,
em5, 5′-GACTGCGTACGAATTAAC-3′,
em6, 5′-GACTGCGTACGAATTGCA-3′.

DNA amplification

The first five cycles are run at 94°C, 1 min, 35°C, 1 min, and 72°C, 1 min, for denaturing, annealing and extension, respectively. Then the annealing temperature is raised to 50°C for another 35 cycles. For amplification we used the cocktail used in other routine PCR-marker applications (Vos et al. 1995). The amplicons are separated by denaturing acrylamide gels and detected by autoradiography.

AFLP protocol

The AFLP procedure was performed according to Vos et al. (1995). The sequences of all adapters and primers were the same as those in the original protocol. The oligonucleotides for the adapters and primers were commercially synthesized (Integrated DNA Technologies, Inc., USA). The adapters were produced by using similar molecular concentrations of the two complementary oligonucleotides for each adapter, denaturing the DNA for 5 min at 94°C, followed by annealing by decreasing the temperature to 24°C slowly (1°C in 2 min). All restriction enzymes, T4 DNA Ligase, and T4 polynucleotide kinase were obtained from New England Biolabs Inc. *Eco*RI and *Taq*I adapters were used (instead of *Mse*I and *Eco*RI to avoid possible marker clustering), and a two-step digestion was performed. First, the genomic DNA was digested by *Eco*RI in *Taq*I buffer at 37°C, then *Taq*I was added and the tubes incubated for 3 h at 65°C. The ligation, primer, labelling, two-step PCR, and gel analysis were the same as in the original protocol.

Sequencing of SRAP marker bands

Only bands from *Brassica* species were sequenced. We developed the following protocol to isolate DNA from the SRAP gels for direct sequencing. Denaturing, thick polyacrylamide gels (size 35×43 cm, thickness, 0.8 mm) were poured using double spacers (0.4 mm each) to run the amplified DNAs. In these thick gels, 20 µl of sample can be loaded in each well, which made it easy to collect enough DNA from a single band for direct sequencing. After electrophoresis, the gel was exposed overnight to a high-sensitivity film, (Kodak BioMax). Using the exposed film as a blueprint, the gel pieces containing the polymorphic bands were cut and introduced into a dialysis tube. The dialysis tube was placed into the buffer tank of a sequencing-gel apparatus, and the DNA was electroeluted in 1×TBE buffer (Fisher FB-SEQ 3545). The application of 2,000 V, which is the same voltage used for running sequencing gels, resulted in the complete electro-elution of DNA into buffer from the gel fragment. After ethanol-precipitation and TE buffer suspension, the DNA was used for direct sequencing, which was accomplished by an ABI377 sequencer (Perkin-Elmer Company).

Marker scoring and mapping

Sixteen primer pairs for SRAP and 20 primer pairs for AFLP were used to generate the map. Each polymorphic band was scored as a single dominant marker. Data were analyzed with Mapmaker version 2.0 for Macintosh (Lander et al. 1987). A minimum LOD score of 3.0 was used for map construction.

Glucosinolate analysis

Analysis of glucosinolates was performed with the protocol based on Kraling et al. (1990), which was modified for leaf extraction (instead of seeds). For this purpose we ground approximately 2 g of fresh leaves collected from 6 week-old seedlings in liquid nitrogen. The tissue was extracted twice with 70% methanol at 80°C for 10 min. After applying the supernatant to a DEAE-Sephadex A-25 (Sigma) column, the glucosinolates were converted into desulfoglucosinolates with sulfatase (0.5% enzyme in water for 12 h at room temperature, Sigma H-I type). The desulfoglucosinolates were then eluted by adding 1.5 ml of water. The resulting mixture was separated by HPLC (Shimadzu) using a Lichrospher 100 RP-18 column (Alltech Associates, Inc., USA) and a linear solvent gradient from 1% to 19% acetonitrile in water over 20 min. The flow rate was 1.5 ml/min at 32°C. The HPLC chromatogram was compared to the desulfoglucosinolate profile of "Linetta", a rapeseed variety widely used as a standard for glucosinolate identification to compare the peaks with the corresponding glucosinolates. The presence of desulfosinigrin and desulfoglucoraphanin peaks was confirmed by using pure authentic sinigrin (Sigma) as an internal standard. Glucosinolate content was quantified with glucotropaeolin (Merck Co.) as an internal standard.

Results and discussion

Principles of the method

Number of primers and primer sizes

While developing the SRAP protocol, we initially tried to use a single primer for the PCR amplification, resulting in the production of only a few large-size bands of approximately 0.5 to 1 kb (data not shown). We also experimented with primers of different sizes. For example, we tested smaller size primers of 10 to 15 bases, such as 10-base RAPD primers or the 14 to15-base primers consisting of the core sequences of the SBAP primers. These primer combinations produced multiple bands, but the resulting amplicons were not always consistent and the profiles had poor reproducibility. We also tried larger primers of 20–22 nucleotides, but this resulted in autoradiographs displaying a strong background. We found instead that the optimal SRAP primer size was between 17 and 18 bp. Therefore, primer size and the use of a two-primer combination are essential to successfully amplify SRAP bands.

PCR conditions

The initial annealing temperature for the first five cycles was set at 35°C. The rationale behind using this temperature is based on the fact that primer annealing to the DNA template depends on the matching-level of both sequences, and amplification efficiency is determined by the effectiveness of the primer binding. The low initial annealing temperature ensures the binding of both primers to sites with a partial match in the target DNA. The annealing temperature is then increased for the subsequent 35 cycles to 50°C. This temperature change ensures that the DNA products amplified at the first five cycles were efficiently and consistently amplified in exponential fashion during the rest of the cycles. When the annealing temperature was kept at 35°C for all 40 cycles, it resulted in bands of poor reproducibility.

Primer sequence

The purpose for using the "CCGG" sequence in the core of the first set of SRAP primers was to target exons to open reading frame (ORF) regions. This rationale is based on the fact that exons are normally in GC-rich regions. For example, in completed sequences of the chromosomes 2 and 4 of *Arabidopsis thaliana* (L.) Heynh., the GC content of exons is 43.6% and 44.08%, respectively. In introns, these values drop to 32.1% and 33.08%, respectively (Lin et al. 1999; The EU *Arabidopsis* Genome 1999). Additionally, genes are nearly evenly distributed along these two chromosomes, except within the centromeric region where less gene density is evident (Copenhaver et al. 1999). Based on this observation, we randomly selected 20 BACs from the Genbank database and checked their sequences. We found that approximately 66% of the sequence CCGG motif falls into exons in these clones. Since exons account for approximately one-third of the genome contained in chromosomes 2 and 4, (Lin et al. 1999, The EU *Arabidopsis* Genome 1999) by using the CCGG primer set, we hoped to preferentially amplify sequences containing these elements. However, since exonic sequences are generally conserved among different individuals (Quiros et al. 2000), their low level of polymorphism precludes using them as sources of markers. To counteract this potential problem, we designed the second primer set with a core containing the AATT sequence near the 3′ region in order to aim at AT-rich regions. Normally these are found more frequently on promoters and introns (Lin et al. 1999; The EU *Arabidopsis* Genome 1999). Since introns, promoters and spacers are usually variable among different individuals, this intrinsic dissimilarity makes it feasible to generate polymorphic bands based on introns and exons.

Reproducibility, polymorphism and genome coverage

Using the same primer combination, we amplified DNA from different *B. oleracea* crops, including cauliflower, collard, and broccoli. A single primer combination detected more than ten polymorphic bands per individual. Although most polymorphic bands maintained similar profiles for different populations of these crops, some of

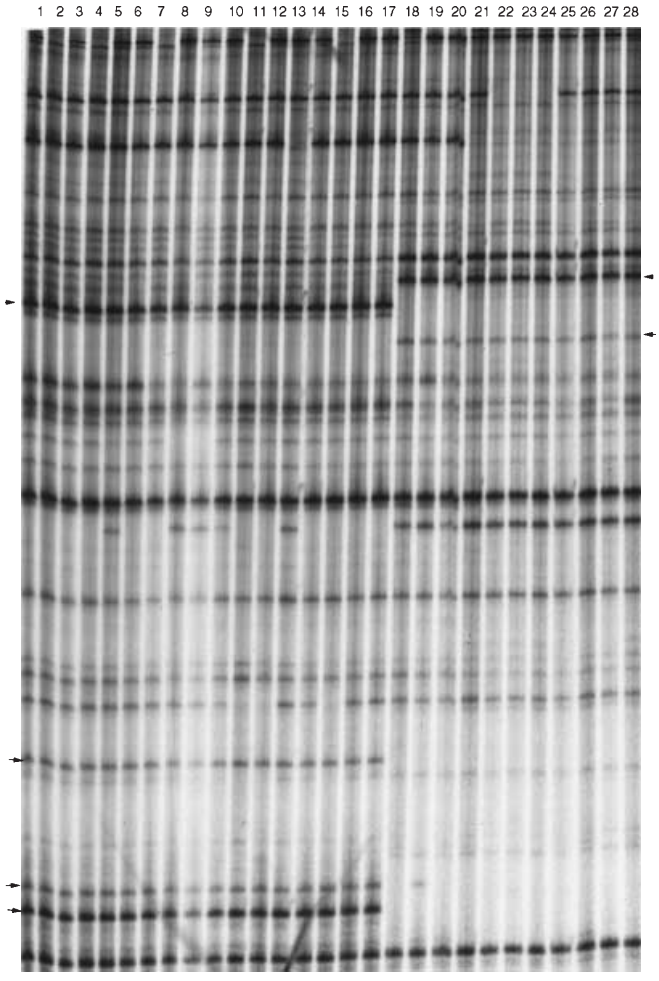

Fig. 1 SBAPs amplified by primers me1-em3 in broccoli (1 to 17) and cauliflower (18 to 28) doubled-haploid lines. *Arrows* show crop-specific markers

them were crop-specific. Different crops shared a few monomorphic bands (Fig. 1). The polymorphic bands were fully reproducible when the same DNA samples were run in independent experiments.

Map construction

To demonstrate that SRAP markers have a good coverage of the genome, which was anticipated because of targeting ORFs, we constructed a genetic map using the RI lines. The map constructed in the 86 RI lines of collard×cauliflower consisted of 130 SRAP and 120 AFLP markers, plus the *GLS-ALK* gene. All AFLP markers were dominant, whereas approximately 20% of the polymorphic SRAPs (26 markers) segregated as co-dominant markers. The AFLP as well as SRAP markers were distributed fairly evenly in nine major linkage groups covering 2,165 cM (Table 1, Fig. 2). No major differences of genome coverage were observed for the two marker techniques. Therefore, similar to AFLPs, the wide genome coverage of SRAP markers and their high reproducibility results in the construction of genetic maps with optimal marker distribution.

Table 1 Distribution of SRAP and AFLP markers in nine linkage groups (L1–L9) of *B. oleracea*

Marker	Linkage group								
	L1	L2	L3	L4	L5	L6	L7	L8	L9
SRAP	26	23	21	9	14	16	13	12	6
AFLP	23	21	21	7	16	8	7	13	4

As expected, each co-dominant marker pair (each band was scored independently, Fig. 3) was located in the map at almost the same position, at distances between 2 to 10 cM. They failed to fall exactly at the same location because, being scored separately, their genetic distance theoretically matched the level of residual heterozygosity ($1/2^5$) which exists in this RI population after five rounds of selfing. The relative high frequency of co-dominant SRAP markers is another important advantage of this technique over AFLP markers.

Tagging the BoGLS-ALK gene

The presence of specific aliphatic glucosinolates in the RI population and its parental lines allowed us to follow up the segregation and tagging of the *BoGLS-ALK* gene. Similar to their cauliflower parent, 36 of the RI lines contained only glucoiberin and glucoraphanin, thus indicating that they had the homozygous recessive genotype for the desaturation allele *GLS-ALK*$^-$ (Mithen et al. 1995). The collard parent as well as the rest of the 56 lines contained glucoiberin, glucoraphanin, sinigrin and progoitrin. Therefore these had the *GLS-ALK*$^+$ dominant allele. The observed segregation did not deviate significantly from the expected 1:1 Mendelian ratio for a monogenic trait (χ^2=0.14). *BoGLS-ALK* mapped on the L1 linkage group at 1.4 cM from the marker SRAP133. This distance, however, is overestimated by the residual heterozygosity of the mapping population, and by the scoring of the markers as dominant markers. Sequencing disclosed that SRAP133 had a size of 259 bp and, interestingly, it matched the sequence of an open reading frame for a putative gene on *Arabidopsis* BAC clone F4C21 (Table 2). This gene is located on chromosome IV, in the same position where Mithen et al. (1995) mapped a desaturation gene. Thus SRAP133 is inside this gene and the functional genes for the desaturation of glucosinolates in *B. oleracea* and in *Arabidopsis* are very similar and probably orthologs.

Direct sequencing of SRAP markers

With the ability to sequence from complex profiles, a specific marker tagging a given trait is important for marker-assisted selection. Marker-based selection permits the rapid screening of large-size segregating populations.

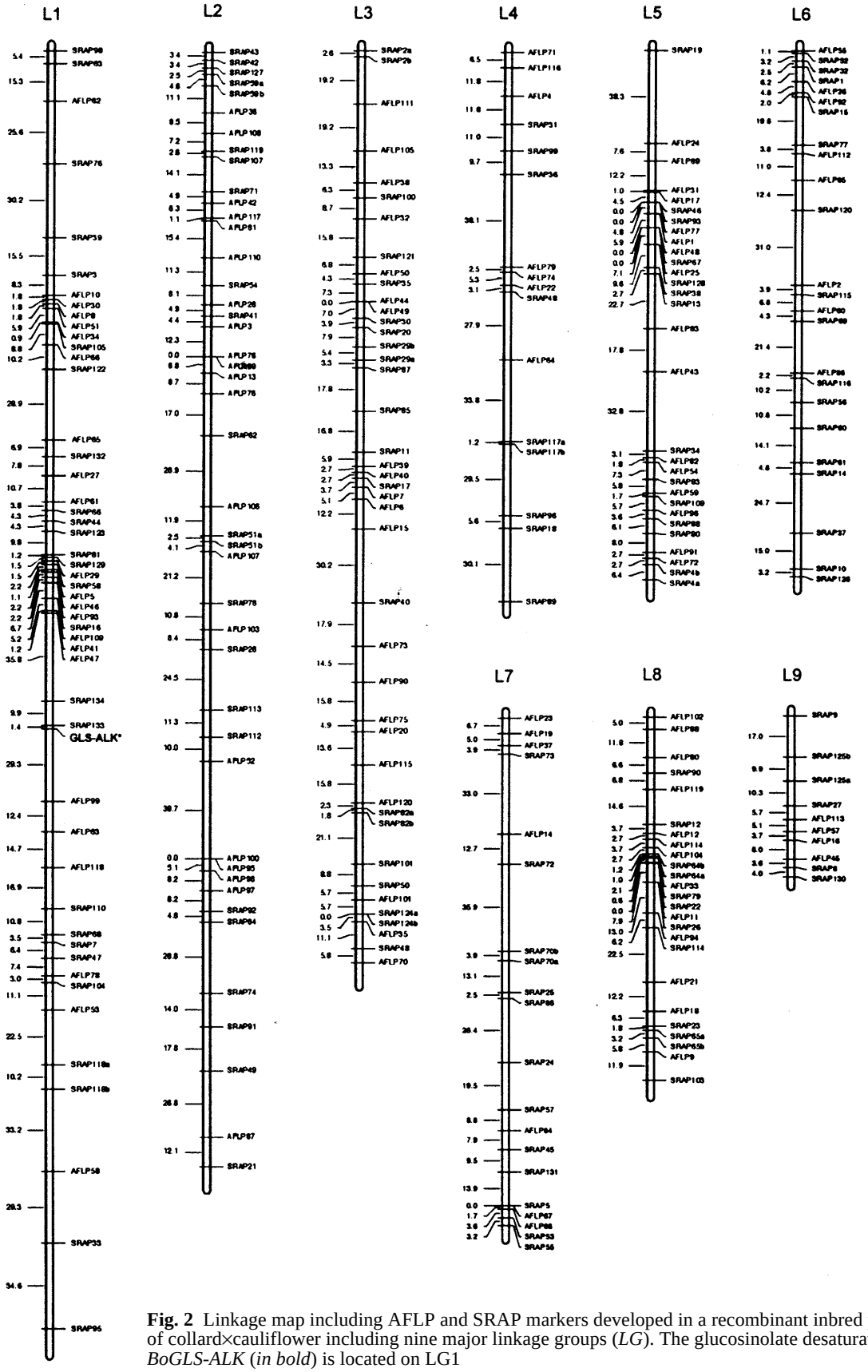

Fig. 2 Linkage map including AFLP and SRAP markers developed in a recombinant inbred population of collard×cauliflower including nine major linkage groups (*LG*). The glucosinolate desaturation gene *BoGLS-ALK* (*in bold*) is located on LG1

Table 2 Analysis of a sample of SRAP sequences isolated from acrylamide gels

Marker name [a]	Primers	Size (bp)	GC (%)	BLASTn or× score (bits) [b]	Genbank accession number
SRAP1	em1+me1	299	33.4		
SRAP2a	em1+me1	179	30.7		
SRAP2b	em1+me1	170	30.6		
SRAP3	em1+me1	137	32.8		
SRAP24	em1+me4	331	23.4		
SRAP29a	em1+me4	125	39.0		
SRAP29b	em1+me4	121	38.8		
SRAP34	em1+me5	193	51.0	28.6(Tx)	Sp/P13730/SGS3
SRAP35	em1+me5	161	26.1		
SRAP36	em1+me5	142	35.9		
SRAP37	em1+me5	390	31.5	40.2(Tx)	Gb/AAD24846.1/AC007071
SRAP41	em2+me1	170	42.9	44.5(Tx)	Gb/AAD25847.1/AC006951
SRAP42a	em2+me1	269	37.1	115(Tn)	Gb/AC101793.3/AC101793
SRAP42b	em2+me1	237	39.7	101(Tn)	Gb/AC101793.3/AC101793
SRAP45	em2+me1	158	49.4	55.0(Tx)	Pir//T01961
SRAP49	em2+me2	474	38.4	337(Tn)	Gb/AF079998.1/AF079998
SRAP50	em2+me2	454	40.5	67.9(Tn)	Gb/AC011000.3/F16P17
SRAP51a	em2+me2	227	44.8	30.5(Tx)	Pir//T10241
SRAP51b	em2+me2	217	45.4	30.5(Tx)	Pir//T10241
SRAP52	em2+me2	188	25.5		
SRAP56	em2+me3	402	41.8	113(Tn)	Gb/AC025290.3/F9P14
SRAP59a	em2+me3	293	35.8	29.3(Tn)	Ref/NP-006628
SRAP59b	em2+me3	258	35.9	29.3(Tn)	Ref/NP-006628
SRA58	em2+me3	203	37.4	218(Tn)	Dbj/AP000423.1/AP000423
SRAP133	em6+me1	276	38.4	91.7(Tn)	GbAC005275.1/AC005275

[a] Bands sharing the same number followed by the letters a or b are co-dominant bands
[b] BLAST search of Genbank database with program BLASTn or BLASTx. All nine Tn matches fall into exons of *Arabidopsis* sequences

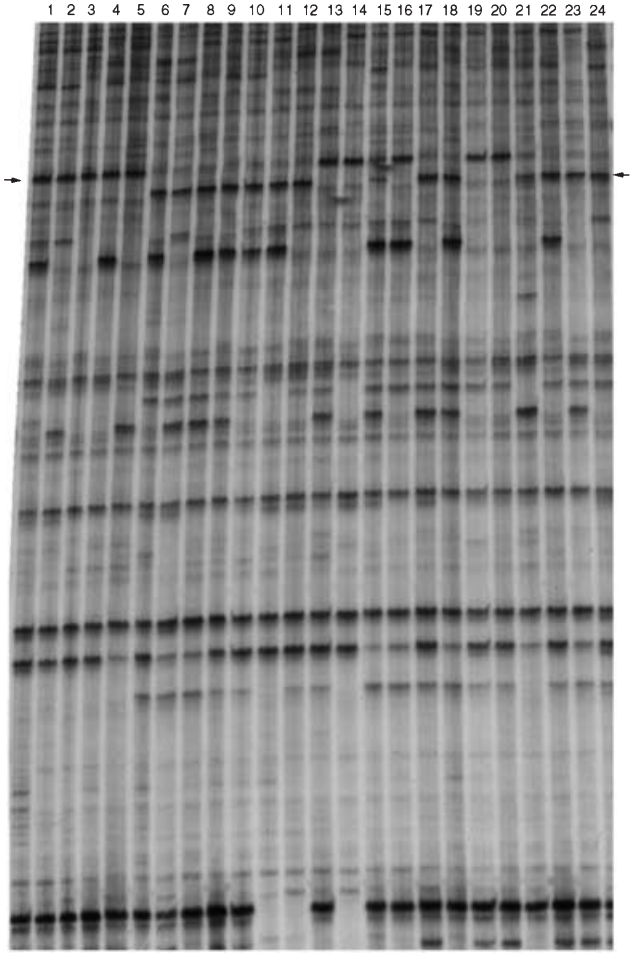

Fig. 3 SBAPs produced by primers me2-em2 in 24 recombinant inbred lines of *B. oleracea*. *Arrows* show co-dominant markers

Sequence information is useful to permit the conversion of dominant to co-dominant markers. Because most SRAP markers produce clear high-intensity bands rarely overlapping (Figs. 1, 3), it is easier than for AFLP markers to sequence polymorphisms for them by cutting directly from the gels. We isolated 29 polymorphic bands resulting from the amplification of the *Brassica* RI lines, using seven different primer combinations. Twenty five of the twenty nine sequences could be read to completion, whereas four bands could be only partially read and these were not analyzed. We found that the GC content of 16 (64%) of the sequences was over 35%, which indicates that they possibly fall into exons, assuming similar gene structures in *Brassica* and *Arabidopsis* (Lin 1999; Quiros et al. 2000). After a BLAST search, we found that 15 (60%) shared significant similarity to reported gene sequences stored in the Genbank database (Table 2). This finding confirmed that a large proportion of the bands generated by SRAPs include exons in ORFs, which are expected to be evenly distributed along all chromosomes.

Sequencing demonstrated also that SRAP polymorphism results from two events, fragment size changes due to insertions and deletions, which could lead to co-dominant markers, and nucleotide changes leading to dominant markers. Among the sequenced bands we selected five pairs that appeared to be co-dominant in the gel (Fig. 3). Sequencing of both parental bands demonstrated similar sequences differing only by small insertions or deletions. The difference in size for each pair of co-dominant markers determined by sequencing, matched the sizes estimated on the gel by migration distance. Therefore, the co-dominant markers resulted from the size change delimited by the binding sites of the two primers. The rest of the sequenced SRAP markers were

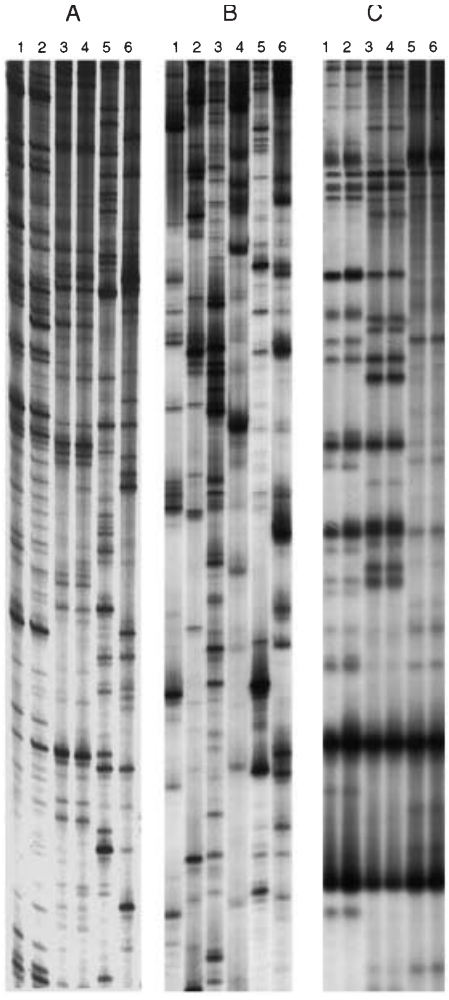

Fig. 4A–C SBAPs produced by amplification of genomic DNA from various different sources. **A** Genomic DNA amplified by primers me2-em1 for different varieties of *B. napus* (*lanes 1 and 2*), celery (*lanes 3 and 4*) and potato (*lanes 5 and 6*). **B** DNA from Fujii apple amplified by six different primer combinations all of which had primer me2 in common: em1 (*lane 1*), em2 (*lane 2*), em3 (*lane 3*), em4 (*lane 4*), em5 (*lane 5*) and em6 (*lane 6*) (autoradiograph produced by Dr. S. Struss). **C** cDNA of a *B. rapa* line derived from isolated pollen mother cells. (*Lanes 1 and 2*), from meiocytes at late meiosis (*lanes 3 and 4*) and from whole flower buds at meiotic stages (*lanes 5 and 6*)

dominant, probably resulting from nucleotide substitutions affecting primer-binding sites.

Other applications

We used the SRAP protocol to amplify DNA from other crops. These crops were potato, rice, lettuce, rapeseed (*B. napus*), garlic, apple citrus and celery. Among these crops we obtained good amplification and easily found polymorphism (Fig. 4a, b). In three of them, Chinese cabbage (Li and Quiros, unpublished), rapeseed (Riaz et al., submitted) and celery (Ruiz et al., submitted), we have found markers for a male sterility gene, a cms fertility restorer gene and a virus resistance gene, respectively.

In order to determine the feasibility of using SRAPs for cDNA fingerprinting, we have amplified cDNA isolated from different tissues of *B. rapa* generated in another study aimed to clone genes expressed specifically in pollen mother cells (Li and Quiros, unpublished). We found tissue-specific bands, which most likely correspond to genes expressed specifically in those tissues (Fig. 4c). Therefore, SRAP can be also used to fingerprint cDNAs.

In conclusion, the SRAP marker system is a simple and efficient marker system that can be adapted for a variety of purposes in different crops, including map construction, gene tagging, genomic and cDNA fingerprinting, and map-based cloning. It has several advantages over other systems: simplicity, reasonable throughput rate, discloses numerous co-dominant markers, allows easy isolation of bands for sequencing and, most importantly, it targets ORFs.

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