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

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 AA TT 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.

Keywords cDNA · DNA-based markers · Fingerprinting · Glucosinolates

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 MseI 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* x 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 genomic DNA. A 1.5-ml tube containing approximately 0.3 g 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).

**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-32P]-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′-TGAGTCCAAAAACGGATA-3′
- me2, 5′-TGAGTCCAAAAACGGAGC-3′
- me3, 5′-TGAGTCCAAAAACGGATA-3′
- me4, 5′-TGAGTCCAAAAACGGACC-3′
- me5, 5′-TGAGTCCAAAAACGGAGA-3′

**Example of reverse primers:**

- em1, 5′-GACTGCCGTACGAAATTAT-3′
- em2, 5′-GACTGCCGTACGAAATTGGC-3′
- em3, 5′-GACTGCCGTACGAAATTGC-3′
- em4, 5′-GACTGCCGTACGAAATTGA-3′
- em5, 5′-GACTGCCGTACGAAATTAC-3′
- em6, 5′-GACTGCCGTACGAAATTCA-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. EcoRI and TaqI adapters were used (instead of MseI and EcoRI to avoid possible marker clustering), and a two-step digestion was performed. First, the genomic DNA was digested by EcoRI in TaqI buffer at 37°C, then TaqI 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).