academicJournals

Vol. 12(29), pp. 4588-4593, 17 July, 2013
DOI: 10.5897/AJB11.1991
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Arbitrarily primed sequence-related amplified polymorphism (AP-SRAP)

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Accepted 8 May, 2012

Sequence-related amplified polymorphism (SRAP) is a new-type molecular technique that targets coding sequences in the genome and results in a moderate number of co-dominant markers. Based on the SRAP program, the random primer combinations of SRAP, amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) were used as new primers in marker analysis. We defined this technique as arbitrarily primed sequence-related amplified polymorphism (AP-SRAP). Of 256 tested AP-SRAP primers, 37.6% primers produced polymorphic patterns from the DNA of one or more species, which showed that AP-SRAP is an effective method to screen markers. Additionally, 80 SRAP primers were used to screen markers in seven plant species (Chinese cabbage, Chinese kale, eggplant, pepper, cucumber, rose and lily), which indicated obvious polymorphism. The primers of AP-SRAP combine simply and reliably. It can overcome the limitation of the number of standard SRAP primers, make greater use of the supply of alternative primers, and potentially reduce laboratory costs. We expect that AP-SRAP may be of wide application in identity testing, population studies, linkage analysis and genome mapping.

Key words: Arbitrarily primed amplification, DNA markers, plants.

INTRODUCTION

Polymerase chain reaction (PCR) is a commonly used method for DNA amplification. Using this approach, highly reproducible fingerprints can be obtained from the genome DNA of a wide variety of organisms (Taylor and Logan, 1995). The ease of obtaining and screening DNA markers, which are shown to be genetically linked to a trait of interest can be used for gene cloning, medical diagnostics, marker-assisted breeding programs and for trait introgression in plant breeding programs (Agarwal et al., 2008). Multiloci fingerprinting methods based on PCR have been extensively used to study the relationships between varieties an cultivars of many different plant species.

Amplifying genomic DNA in arbitrarily primed sequence-related amplified polymorphism (AP-SRAP) is a new way of discovering DNA markers, which is fast and simple. Based on similar principles, amplifying genomic DNA in single primer amplification reactions (SPARs)

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Abbreviations: PCR, Polymerase chain reaction; **SPARs,** single primer amplification reactions; **RAPD,** random amplification polymorphism DNA; **DAF,** DNA amplification fingerprinting; **RAMPs,** random amplified microsatellite polymorphisms; **AP-PCR,** arbitrarily primed PCR; **SRAP,** sequence-related amplified polymorphism; **ORFs,** open reading frames; **AP-SRAP,** arbitrarily primed SRAP; **CTAB,** cetyltrimethylammonium bromide; **SSR,** simple sequence repeat; **AFLP,** amplified fragment length polymorphism; **MAS,** marker-assisted selection; **SNP,** single nucleotide polymorphism.

(Gupta et al., 1994), random amplification polymorphism DNA (RAPD) (Williams et al., 1990), DNA amplification fingerprinting (DAF) (Gustavo et al., 1991), random amplified microsatellite polymorphisms (RAMPs) (Wu et al., 1994), and arbitrarily primed PCR (AP-PCR) (Welsh et al., 1991, 1992) were developed. These methods were easily and rapidly carried out because it uses one primer and small amounts of DNA. Additionally, the RAPD method has been most widely used for developing genetic markers and the construction of genetic linkage maps (Reiter et al., 1992).

Sequence-related amplified polymorphism (SRAP) was developed by Li and Quiros (2001) for the purpose of amplifying open reading frames (ORFs), which 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, 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, and the following 35 cycles are run at 50°C. The SRAP marker system is a simple and efficient method that can be adapted for a variety of purposes in different species, including map construction, gene tagging, genomic and cDNA fingerprinting, and map-based cloning (Sun et al., 2007; Li et al., 2003; Gao et al., 2007). The SRAP system is more reproducible than that of RAPD and is less complicated than AFLP (Li and Quiros, 2001). In the standard SRAP, because the design of primers follows set principles, the primers used in SRAP are restricted and limit the usage of the method in many studies. Accordingly, we have developed the alternative method of arbitrarily primed SRAP (AP-SRAP) that is very rapid and simple and generates a fingerprint of PCR products. The AP-SRAP does not require a particular set of primers. Arbitrary primers are combined freely and the SRAP procedure is used to amplify the polymorphism between plants. These experiments indicate the value of a systematic designing of a series of universal primers that can be tested on species of interest.

The purpose of the present study was to evaluate the potential amplification of AP-SRAP amplifying DNA markers in a wide variety of plants. Here, we used the DNA of *Brassica rapa* (Chinese cabbage), *Brassica oleracea* (Chinese kale), *Solanum. melongena* L. (eggplant), *Capsicum annuum* L. (chili pepper), *Cucumis sativus* L. (cucumber), *Rosa* spp. (rose) and *Lilium brownii* var. *viridulum Baker* (lily) to demonstrate that such combinations of random primers can produce polymorphic band patterns from complex eukaryotic genomes. The technique of AP-SRAP is expected to produce rapid and easy DNA markers useful in breeding programs and other genetic experiments.

MATERIALS AND METHODS

The SRAP marker system was developed primarily for *Brassica* species, such as Chinese cabbage and Chinese kale, but here we also tested it on other species, namely cucumber, eggplant, pepper, rose and lily. Three different cultivars from each species were used in the study. For Chinese kale, we used three individuals from a BC_4 population, which was derived from a cross between male-sterile broccoli and a Chinese kale line as the recurrent parent.

DNA extraction

Genomic DNAs from freeze-dried leaf tissues were isolated by a modified cetyltrimethylammonium bromide (CTAB) method (Haymes, 1996). DNA concentration and purity were measured by a Beckman spectrophotometer (NanoDrop ND-1000) at an absorption wavelength ratio of 260 versus 280 nm.

Primers

Oligonucleotide primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co (Shanghai, China) using the standard procedure. In this experiment, normal SRAP primers (Li and Quiros, 2001; Wang et al., 2005; Budak et al., 2004), AFLP primers (Vos et al., 1995) and simple sequence repeat (SSR) primers derived from *B. rapa* (Suwabe et al., 2002; Choi et al., 2007) were used in AP-SRAP.

AP-SRAP conditions

The PCR procedure is the same as the standard SRAP program (Li and Quiros, 2001). DNA amplification fragments were separated on a non-denaturing 8% acrylamide gel and visualized using silver staining (Zhu et al., 2002).

Data analysis

The polymorphic bands were scored independently as being either present or absent in each genotype. Presence or absence of each SRAP fragment was coded as “1” or “0”, where “1” indicated the presence of a specific allele and “0” indicated its absence, and a letter “u” denoted it was missing. Only strong, reproducibly and clearly distinguished bands were used in the analysis. Polymorphism information content of primers was the content of the polymorphic bands in the total bands.

RESULTS

Using 80 SRAP primer combinations, we amplified DNA from seven different species, namely Chinese cabbage, Chinese kale, eggplant, pepper, cucumber, rose and lily (Table 1). A typically polymorphic pattern had several common bands and a few polymorphic bands (Figure 1). Samples from three different tissues were distinguishable for all seven species using those primers. The results show a high probability of amplifying polymorphic patterns from the primer’s compound variants of a

Table 1. Statistics of amplification of 80 sequence-related amplified polymorphism (SRAP) primers in seven plant species.

Parameter	Cucumber	Chinese cabbage	Chinese kale	Eggplant	Pepper	Rose	Lily
Total number of bands	734	697	731	530	650	651	450
Number of polymorphic bands	89	96	64	17	20	184	63
Polymorphic ratio (%)	12.1	13.8	8.8	3.2	3.1	28	14

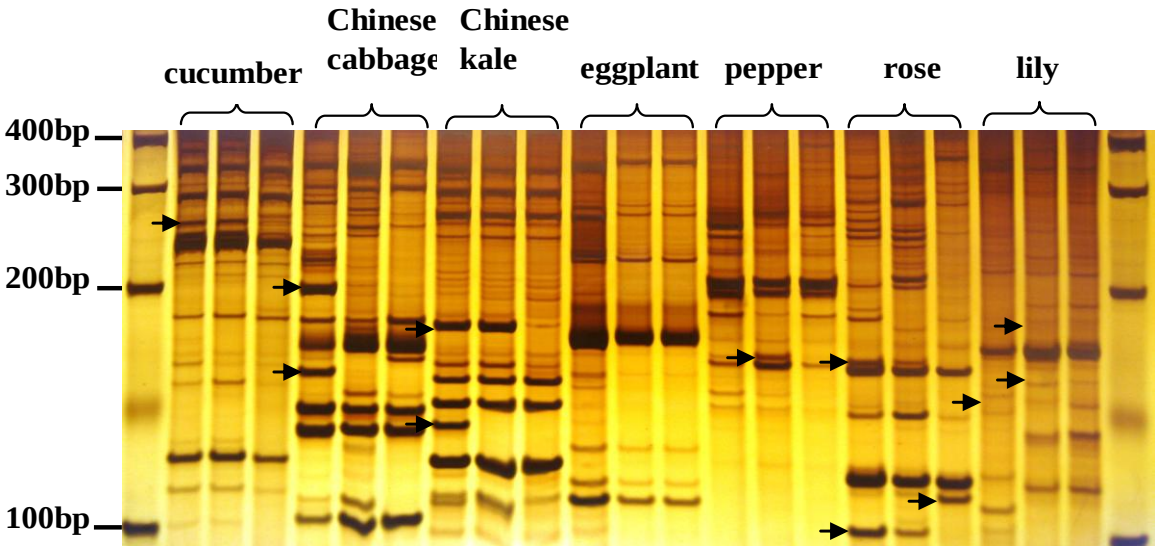

Figure 1. Polyacrylamide gel electrophoresis (PAGE) gel showing DNA patterns amplified by standard sequence-related amplified polymorphism (SRAP) primers from the DNA of seven plant species. DNA samples are numbered 1 to 21 for three different individuals taken from each of the seven species studied. M, 100 bp ladder used as a DNA size standard. Arrows show the polymorphism bands.

successful primer. A total of 4443 fragments ranging from 100 to 400 bp were recorded, 12% of which were polymorphic between different species. Depending on the particular primers and template combination, the number of amplified products had about 10 bands. These fragments could be adequately resolved and visualized by polyacrylamide gel electrophoresis and by silver staining of nucleic acids.

SRAP-based primers amplify polymorphic patterns

In addition to the primer combination of the standard SRAP method, five different primer combinations were used in the method that we defined as AP-SRAP (SRAP-SSR, SRAP-AFLP, SRAP-SNP, SSR-SSR, SSR-AFLP and AFLP-AFLP) in order to compare the various AP-SRAP DNA amplifications with that from the standard SRAP method (Figure 2). The AP-SRAP method detected changes in the DNA sequence at arbitrary sites in the genome that are defined by the primer. This resulted in multiple unspecified priming sites on each DNA strand. The results show that the AP-SRAP method had same capability as the normal SRAP method to detect polymorphic bands (Table 2). For the three selected samples from the Chinese kale BC_4 population, which included two fertile plants and one sterile plant, good DNA amplifications were obtained by using the five primer combinations of the AP-SRAP method. The results show that the use of compound SRAP-based primers was both useful and informative.

In the AP-SRAP method, primer sites are randomly distributed along the target genome and highly variable regions. For the 256 primer combinations that were screened, 37.6% of the primers produced polymorphic bands (Table 3). A total of 1889 bands were detected, among which 130 (6.88%) were polymorphic within the group of primer combinations. These bands ranged in size from 100 to 400 bp and were shared by the three individuals. The results show a high probability of amplifying polymorphic patterns from six types of combinations of compound primers.

Annealing temperature affects the pattern of AP-SRAP

AP-SRAP was performed at four annealing temperatures

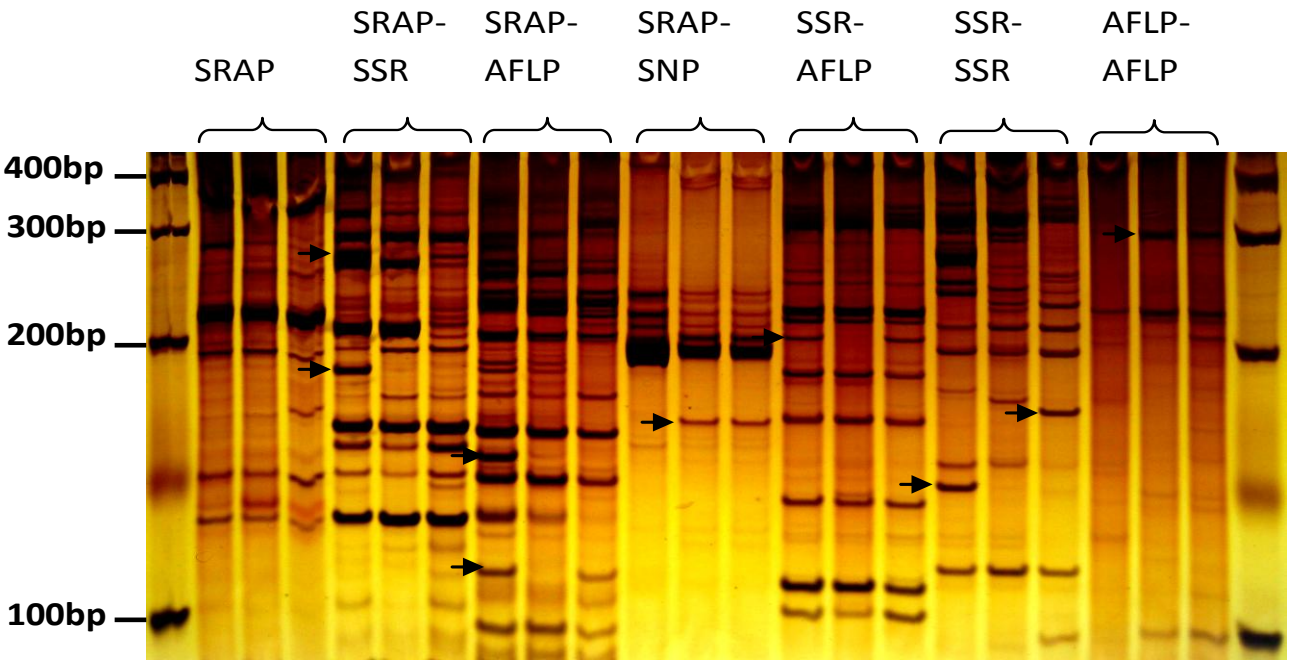

Figure 2. Examples of Chinese kale DNA patterns amplified by various compound primers denoted at the top of the photograph. The three samples were taken from the BC_4 population of Chinese kale. SRAP, SRAP-SSR, SRAP-AFLP, SRAP-SNP, SSR-SSR, SRAP-SNP and AFLP-AFLP showed the combinations of primers. M, 100 bp ladder as a DNA size standard. Arrows show the polymorphism bands.

Table 2. Description of combination of primers in arbitrarily primed - sequence-related amplified polymorphism (AP-SRAP).

Primer combination name	Primer sequence	Primer character
SRAP	BMe11F: TGAGTCCAAACCGGAAC	SRAP
	BEm8R: GACTGCGTACGAATTCAC	SRAP
SRAP-SSR	CuMe9F: TGAGTCCAAACCGGTAG	SRAP
	ENA20R: TCTGAACTACCAAAGCCAAC	SSR
SRAP-AFLP	CoMe7F:TGAGTCCAAACCGGTTG	SRAP
	M31:GATGAGTCCTGAGTAAAAA	AFLP
SRAP-SNP	CoEm7R: GACTGCGTACGAATTATG	SRAP
	SNP: CATTACGATAATTCCCAACCAAA	SNP
SSR-AFLP	nlassr016F:TTCATCCACTTTGTTCAATACAAGA	SSR
	M35: GATGAGTCCTGAGTAAACA	AFLP
SSR-SSR	Ni2E12F: TTATCTGCTTGTCTTGGGGC	SSR
	ENA14F:CTTACGGTGGAAATGCTG	SSR
AFLP-AFLP	E37: GACTGCGTACCAATTCACG	AFLP
	M83: GATGAGTCCTGAGTAATCA	AFLP

namely 30, 35, 40 and 45°C. With decrease in annealing temperature, a less intense pattern was obtained by the same annealing temperature, which could be due to non-specific annealing of the primer to the target sequences

Table 3. Statistics of the polymorphic bands detected by the AP-SRAP method for the BC_4 population of Chinese kale.

Parameter	SRAP	SRAP-SSR	SSR-SSR	SRAP-AFLP	SRAP-SNP	AFLP-AFLP	SSR-AFLP	Total
Total number of bands	304	284	267	348	230	202	254	1889
Number of polymorphic bands	24	15	23	25	14	8	21	130
Polymorphic ratio (%)	7.89	5.28	8.61	7.18	6.09	3.96	8.27	6.88

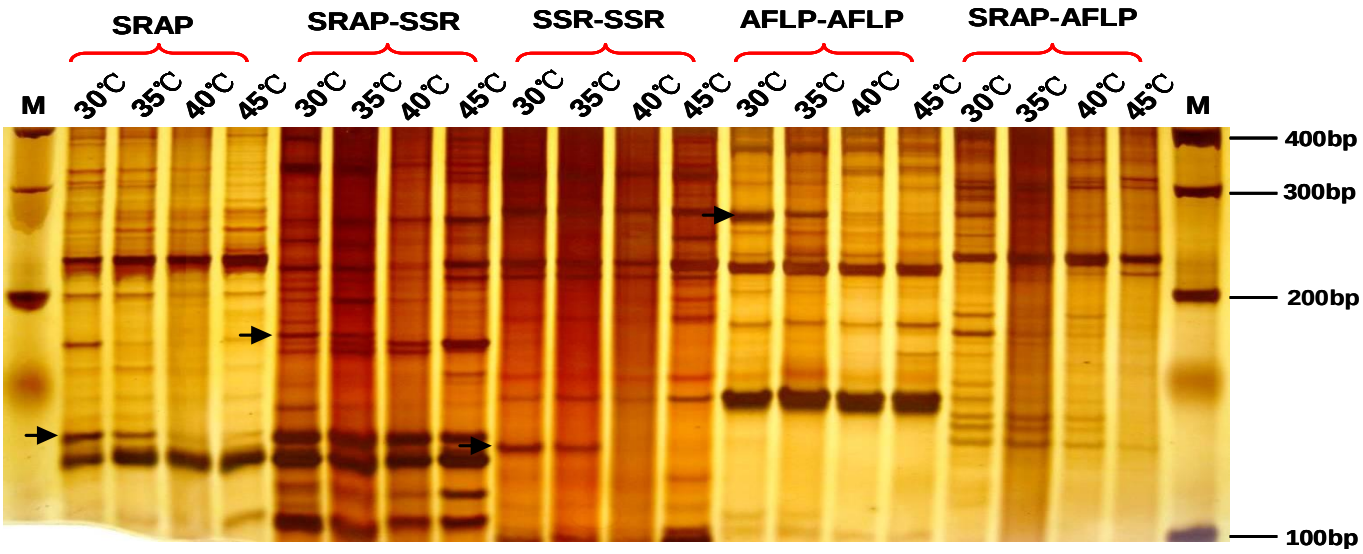

Figure 3. Analysis of Chinese kale DNA patterns on a PAGE gel amplified by variants of temperature in SRAP program. The three Chinese kale samples come from BC_4 population. In the first cycle of SRAP program, the primer annealing temperatures in the AP-SRAP are shown on top of the figure. M, 100-bp ladder as a DNA size standard; right side shows sizes of the standard DNA fragments. Arrows show the polymorphism bands.

(Figure 3). The amplified bands at 30 and 35°C which although polymorphic, were not easily discernible. The results show the most informative patterns at 30 and 35°C, but the lower annealing temperature can decrease the specificity of amplification. Separate experiments with the temperature variants proved that 35°C is the optimal AP-SRAP annealing temperature for the four temperature gradients.

DISCUSSION

We used the SRAP protocol to amplify DNA from seven plant species including cucumber, Chinese cabbage, Chinese kale, eggplant, pepper, rose and lily. According to our results, good DNA amplification and high levels of polymorphism from all of these species were obtained. Li and Quiros (2001) reported that they obtained good amplification for other plants, which included potato, rice, lettuce, rapeseed (*Brassica napus*), garlic, apple, citrus and celery. In addition, SRAP technology has been used to construct a linkage map of cotton (Lin et al., 2003) and to analyze the evolution of buffalo grass (Budak et al., 2004). Moreover, we found that there were fewer polymorphic bands for eggplant and pepper than for the other plants. Maybe they are more closely related. Polymorphism was evident for pepper, rose and lily DNA samples.

SRAP-based primers amplification

In principle, SRAP is a technique that detects randomly amplified polymorphism. Based on this concept, we explored the possibility of using five different primer combinations of SSR, AFLP and standard SRAP primers in an AP-SRAP method, and compared their performance to those of the standard SRAP primers alone. Our results indicate that this AP-SRAP method was effective in the detection of genome polymorphism at the same level as the standard SRAP method. Using this AP-SRAP method, the primer resources of a laboratory can be utilized to a greater extent and the number of primers combinations useful for SRAP is much greater.

Furthermore, this new concept can be extended to the RAPD primers and common PCR primers in the laboratory.

Since SRAP is highly sensitive in the detection of polymorphism, it should enable direct identification of cDNA from genes that are even weakly expressed (Li et al., 2003). We also anticipated the probability of using AP-SRAP for the amplification of cDNA to generate a DNA fingerprint reflecting differences in gene expression. Although the full value of using AP-SRAP is not yet established, we believe that our results provide evidence that it should be useful for identity testing, population and pedigree analysis, genetic mapping and molecular characterization.

The effect of annealing temperature to amplification

Annealing temperatures ranging from 30 to 45°C was used. Temperature variation in the small cycle was not as crucial as with conventional PCR in which cycling temperatures must be accurate and reproducible within close limits. With annealing temperature decreasing, a less intense pattern was obtained by the same annealing temperature, which could be due to non-specific annealing of the primer to the target sequences. Appropriate annealing temperature can therefore affect the stability of polymorphism.

Conclusion

In summary, AP-SRAP is an efficient system for retrieval and analysis of genetic information in plants. It does not require prior knowledge of DNA sequences which is largely independent of the amount of DNA template starting material available. It is also fast and relatively simple. Moreover, amplification products can be separated and analyzed for SCAR markers easily. In addition, it can reduce the costs of synthesizing primers and can greatly enhance the efficiency of primer utilization. On the whole, AP-SRAP can produce high quality polymorphic bands and should have immediate applications in marker-assisted selection (MAS) and other genetic experiments.

ACKNOWLEDGEMENTS

We gratefully thank Dr D. Warrington for critical reading and editing grammar and style of the manuscript. This work was supported by the National “863” High Technology Foundation (Grant Nos.2006AA100108).

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