The Journal of Animal & Plant Sciences, 24(5): 2014, Page: 1478-1486
ISSN: 1018-7081

SEQUENCE-RELATED AMPLIFIED POLYMORPHISM (SRAP) FOR STUDYING GENETIC DIVERSITY AND POPULATION STRUCTURE OF PLANTS AND OTHER LIVING ORGANISMS: A PROTOCOL

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ABSTRACT

Genetic variation is widespread in living organisms. The accurate analysis of genetic variation and studies of population structures have significant implications for understanding the genetic traits, population genetics, molecular breeding and disease control programs in plants and animals. Sequence-related amplified polymorphism (SRAP) analysis has been widely used for studying genetic diversity and population structures of living organisms in the last decade. This molecular approach preferentially targets the open reading frame (ORF) regions of the genome with universal primers to screen the mutations across the whole genome, allowing to investigate genetic variations in the genome, and to develop new specific markers for species or population differentiation and molecular breeding. This article briefly outlined the applications of SRAP technique in plants and animals, and detailed described the protocol of this technique. It will make the usage of this approach conveniently and maneuverably, and further expand its applications to a wider range of living organisms.

Key words: Sequence-Related Amplified Polymorphism (SRAP), Protocol Review, Genetic Diversity, Population Structure.

INTRODUCTION

Genetic diversity is widespread in plants, animals and micro-organisms. The accurate analysis of genetic diversity has significant implications for understanding the inheritance of genetic traits, population structure and evolution of living organisms. These features are key points in molecular breeding and disease control programs in organisms (Kumar, 1999), including species classification, germplasm identification, evolutionary study, linkage map construction, gene tagging and map-based cloning. Many genetic markers such as morphological markers (phenotypic traits) (Cheng *et al*., 2011), cytological markers (karyotype and patterns of chromosome) (Bauchan *et al*., 2003), biological markers (isozyme) (Peakall *et al*., 1995) and molecular markers have been employed to study genetic diversity. However, traditional morphological characters are based on gene expression levels that have few or lack adequate polymorphism, and are sensitive to environmental factors and not amenable for examining genetic diversity among a large set of diverse populations (Vandemark *et al*., 2006). In contrast, molecular marker technology mainly detects naturally occurring polymorphisms at DNA level with good specificity that makes it a powerful tool to assess the influence of various factors on genetic diversity and population structure. DNA markers are plentiful, independent of tissue or environmental effects and are widespread throughout the genome. They are highly polymorphic, stable, and allow organism identification in the each developmental stage (Agarwal *et al*., 2008; Xie *et al*., 2009).

Non-PCR based techniques such as restriction fragment length polymorphism (RFLP) can be useful for examining co-dominant markers, but are limited by their inability to resolve whole loci per reaction, the need of radioactive elements, the requirement for large amounts of initial template DNA as well as high expenses (Vandemark *et al*., 2006). Other PCR-coupled systems, such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), inter simple sequence repeats (ISSR), and single-strand conformation polymorphism (SSCP) have been widely used in molecular taxonomy, phylogeny, genetic mapping, gene cloning and germplasm evaluation (Gasser *et al*., 2006; Mutlu *et al*., 2008; Zhao *et al*., 2009). RAPD has been proven useful for the evaluation of genetic variation, but suffers from low stability and poor reproducibility (Welsh and McClelland, 1990). AFLP presents good rapidity, reproducibility and numerous polymorphisms but requires multiple steps, and yields pseudo-polymorphism when methylation-sensitive restriction enzymes are used (Budak *et al*., 2004). SSR produces co-dominant markers, but it needs sequencing for primer development that is expensive and time-consuming (Li and Quiros, 2001).

ISSR has the advantage of wide distribution in the genome, but could not differentiate heterozygosity for the essence of dominant marker (Budak *et al*., 2004). SSCP is suitable for high-throughput mutation screening before sequencing, but it may require the use of radio-labeled primers which has safety concerns (Gasser *et al*., 2006).

The sequence-related amplified polymorphism (SRAP) system was developed by by Li and Quiros (2001) to target overlapping coding and non-coding regions of the genome. Compared with other markers, the preferential amplification of open reading frames (ORF) by SRAP markers have the potential to strengthen the relationship between DNA polymorphisms and morphological traits that characterize different morphotypes (Ferriol *et al*., 2003). Particularly, SRAP not only amplifies the interval between genes and their non-coding flanking regions, but also tightly links to actual genes, which would generate a fingerprint of the coding sequences and permit easy isolation of these bands for sequencing (Yu *et al*., 2008). It is better than SSR as it requires comparatively little sequence information before it can be implemented in a new species with the universal primers.

SRAP has been recognized as a powerful marker system used in genetic map construction, genealogical classification, gene tagging and cloning, marker-assisted selection, germplasm resources evaluation and prediction of heterosis. It has been widely used for studying population structure, genetic diversity and genetic linkage map of plants, such as oat (Tanhuanpää *et al*., 2007), cotton (Li *et al*., 2007), *Brassica napus* (Sun *et al*., 2007), cucumber (Zhang *et al*., 2010), *Piper* spp. (Jiang and Liu, 2011), sugar beet (Nagl *et al*., 2007), *Cucurbita pepo* (Ferriol *et al*., 2003), sugarcane (Alwala *et al*., 2008), eggplant (Mutlu *et al*., 2008), castor (Zheng *et al*., 2010) and mustard (Wu *et al*., 2009), as well as grasses such as *Dendrobium* (Li *et al*., 2008), elephant grass (Xie *et al*., 2009), buffalograss (Budak *et al*., 2004), alfalfa (Castonguay *et al*., 2010), Dendrobium (Lu *et al*., 2012) and Vicia faba (Alghamdi *et al*., 2012). SRAP also has been applied to assess germplasm resources and parents selection of plants, such as tree peony (Han *et al*., 2008), citrus (Gulsen *et al*., 2010), *Hedychium* (Gao *et al*., 2008), *Dianthus* (Fu *et al*., 2008), *Carthamus* (Peng *et al*., 2008), sour orange (Polat *et al*., 2012), lotus (Deng *et al*., 2013) as well as fungi of ganoderma (Sun *et al*., 2006) and *Auricularia* (Yu *et al*., 2008). Recently, this technique has been expanded to study genetic diversity in parasites of human and animal health significance, such as *Fasciola* (Li *et al*., 2009) and *Schistosoma japanicum* (Song *et al*., 2011). The wide applicability of SRAP demonstrates that these markers are effective and reliable for investigating the degree of genetic polymorphism in different genomes, and thus could be adapted for a variety of usage on medicinal plants, endangered plant species, rare flowers and other living organisms.

SRAP is also a valuable approach to fingerprint cDNA (Li and Quiros, 2001; Que *et al*., 2012). This system has been used successfully for transcriptome map construction and comparative genome analysis of transcribed genes, which provides foundations for differential expression analysis at transcriptional level. The present study reviewed the applications of SRAP technique in genetic diversity and population structure in plant and other organisms and also detailed narrated the protocol of this technique.

MATERIALS AND METHODS

Primer design: The SRAP technique uses a combination of primers which should theoretically detect tract of DNA in open reading frames (ORFs) (Figure 1). The forward primer is usually 17 bases in length, which contains a core sequence of 14 bases (with 10 bases of non-specific constitution ‘filler’ sequences and 4 bases of CCGG sequence) at 5' end and three selective bases added to 3' end; The reverse primer is often 18 bases in length, which contains a 15 base-long core sequence (with 11 bases of ‘filler’ sequences differing from forward primer and 4 bases of AATT sequence) at 5' end and three selective bases at 3' end (Figure 2, Table 1). A variation in three selective nucleotides generated a set of primers sharing the same core sequence (Figure 2). The forward primer preferentially amplified exonic regions and the reverse primer preferentially amplified intronic regions (Li and Quiros, 2001). Therefore, the SRAP technique could detect Polymorphisms arising from variations in the length of introns, promoters, and spacers, among both genotypes and species when different forward and reverse primers are randomly combined. Examples of SRAP primers are shown in Table 1. NOTE: It is necessary to maintain good stability for the primer combination we choose. The size of the primer is significantly linked to success of SRAP amplification, and it is recommended that the length of primer is 17-18 bp. Too short primers may easily produce multiple bands and reveal bad reproducibility, while too long primers may cause high background when staining.

Polymerase chain reaction (PCR): Perform PCR in a 25 µl reaction mixture containing approximately 25 ng DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 mM of dNTPs, 4 mM of $MgCl_2$, 0.5 mM of each primer, and 1.25 unit of *Taq* polymerase. There are two kinds of SRAP amplification programs currently in use. One was established by Li and Quiros (2010) as below: the standard PCR reaction (130 min) should consist of a 5 min of initial denaturation at 94°C, then five cycles of three steps: 1 min of denaturation at 94°C, 1 min of annealing at 35°C, 1 min of extension at 72°C, followed by further 35 cycles with annealing temperature being increased to 50°C, with a final extension step of 5 min at 72°C. The other was explored by Budak *et al* (2004) with

all conditions the same as the former except that the annealing temperature was always held at 47°C.

Both methods can obtain amplicons with good reproducibility and stability. Therefore, we can choose different programs based on different materials and research purposes to achieve the best results. The primer annealing to the DNA template depends on whether it matches well with each other, and effectiveness of primer binding forms key point of PCR amplification. The initial low annealing temperature ensures the binding of both primers to the target region, and the increase of annealing temperature up to 50°C in the subsequent 35 cycles ensured the amplification efficiency and good reproducibility. The purity and quality of template DNA plays an important role in the quality of the resulting fingerprints. Standard DNA extraction methods are sufficient to yield high quality DNA from most of samples. Most commonly, 15-30 ng DNA per 1 µl reaction volume is ideal. Much higher DNA concentrations will cause smears between the bands, which is a sign of over-amplification. DNA samples should be stored at -20°C and avoid degradation. The final primer concentration(s) in the reaction can vary from 0.3 to 0.5 mM. High-quality *Taq* polymerase was recommended and the amount can vary at 1.0-1.5 unit. Although higher primer and enzyme concentrations could increase PCR efficiency and the rapidity of DNA amplification, they also produce over-amplified products shown in Figure 3. PCR thermal conditions can be varied without significant effects on the resulting band pattern. The denaturation step in PCR can be carried out at 94°C for 40 s or 98°C for 10 s. The length of the annealing step can vary from 30 s to 50 s at 50°C. The annealing temperature varies with the melting temperature of the primer; it should be between 45°C and 60°C (50°C is optimal for most of primers combinations in SRAP).

Sample preparation and loading: Add an aliquot (5 µl) of 10×loading buffer to the completed PCR reactions in tube, mix well and load 5 µl sample to the agarose gels, and load the acrylamide gels with a sample volume of 3 µl. The DNA concentration plays an important role in gel resolution, and a dilution of DNA template to 10 ng/µl is optimal for SRAP analysis. Overloaded lanes will result in poor resolution.

Agarose gel electrophoresis and pre-detection of amplicons: To pre-verify the quality/specificity of the amplicons and PCR conditions, products should be resolved firstly on 1.5-2.5% agarose-TBE gels. Prepare 100 ml of 1.5-2.5% (wt/vol) agarose containing 0.5×TBE buffer in a 200 ml bottle. This volume is required for one gel with the dimensions 0.5 cm×13 cm×15 cm. Dissolve and melt the agarose in a microwave oven. The bottle should be closed with a cap un-tightened! The agarose gel must be completely melted in the microwave and then allowed to slowly cool until its temperature drops to 70–75 °C. At that point, ethidium bromide with the final concentration of 0.25 mg per ml was allowed to be added in. NOTE: Take care not to over boil the agarose. Add ethidium bromide only after removing the agarose from the microwave oven to minimize risks from boil-over.

Load 5 µl of mixture into lanes of the 1.5–2.5% agarose-TBE gel and 2 µl of an appropriate DNA molecular size marker (e.g., DNA marker DL2000) into a lateral lane. Run the gel at 80–100 V for 30 min in 0.5×TBE buffer, and then photographed using a gel documentation system (UVItec). NOTE: The main purpose of steps 4 and 5 is to observe the size and efficiency of PCR products amplified by different primer combinations, and thus to estimate the electrophoresis time on 6% denaturing acrylamide gels in the next step.

Casting the acrylamide gels: Wash the gel matrixes (especially for glasses) clean and dry them in a ventilation area. (i) Assemble the gel matrix well according to the manufacturer's instructions. (ii) Seal the bottom and wall of the gel matrix with 1% agarose in order to ensure 6% denaturing acrylamide gels will not leak. (iii) Prepare 50 ml of 6% denaturing acrylamide gels in a 100 ml bottle (details in Table 2). This volume is required for two gels with the dimensions 0.1 cm×16 cm×20 cm. Gently mix and try not to produce bubbles. It is critical to always prepare fresh acrylamide gels in the same manner, allowing them to polymerize at room temperature for 2–3 h. If polymerization does not take place during this time, do not use the gel. Use well-combs matched with matrix. The dimensions and numbers of wells depend on the numbers of samples to be loaded. (iv) Inject liquid gel into gel matrix slowly. Insert the proper well comb and wait 1-3 h for solidification. Ensure that there is no bubble in the gel. (v) After solidification, take the matrix off carefully. Pull out comb and insert precast SRAP gels into vertical electrophoresis apparatus, filled in electrophoresis buffer. Ensure that there are no strands of semi-polymerized gel matrix stuck in the well after the removal of the comb, by flushing the well with 1×TBE buffer prior to loading. (vi) Pre-run the SRAP gel (using 1×TBE buffer) for 30 min at 90 V and 20 °C (constant). (vii) Load 2–4 µl of individual PCR amplicons (same volume for all) into wells, depending on the size of the comb used. NOTE: It is necessary to load carefully and avoid the samples drift into neighboring well. (vi) A reference marker, such as DNA marker (eg. TaKaRa DL2000) can be loaded into a lateral lane. NOTE: If the concentration of reference mark is high, please dilute it before use. The large (20cm×20cm) 2-2.5% agarose gels also could be used as an alternate reliable and sensitive system to accurately resolve multiple DNA fragments of size that wary from 100 bp up to 1.5 kb.

Gel electrophoresis: Subject the pre-run gel to electrophoresis at 90 V and 20 °C (constant) for 3-4 h,

and using a constant flow of buffer from the cooling system. NOTE: The most critical point for achieving optimal resolution is a constant temperature (< 0.1 °C fluctuation) throughout electrophoresis. We have run our gels in a temperature-controlled room (20 °C). The electrophoresis conditions need to be optimized empirically to achieve optimal band resolution for each laboratory and each application. SRAP profiles need to be demonstrated to be reproducible on different days using amplicons produced on different days.

DNA visualization: After the completion of the electrophoresis, turn off the power supply, and pour-out electrophoresis buffer. (i) Take out the glasses from electrophoresis groove plate, lift the gel from the apparatus and slice the gel from the backing sheet using knife. This step should be done carefully to avoid breaking gels. (ii) Strip the gels away from glasses and put gels into distilled water. (iii) Dip gels in the distilled water for 1-2 min and wash off electrophoresis buffer. (iv) Stain SRAP gels with 0.1% $AgNO_3$ solution for about 10 min. (v) Wash gels with distilled water for 1 min. (vi) Dip gels in developer solution (according to the details in Table 3) for 6-10 min until the bands could be seen clearly, briefly destained in distilled water for 1 min and then photograph the gel using a digital camera. Look to see if the target range of fragment sizes has been resolved and is in the desired gel position. The developing time should be controlled appropriately, because much longer time will cause smears between the bands and make background color too deep. During silver staining, strong alkali (NaOH) and weak alkali (Na_2CO_3) could all be used in silver staining. When NaOH is used, bands appear quickly but with a bit poor discrimination, whereas Na_2CO_3 permits higher sensitivity but with a shallow background and need long staining time.

Table 1. Example of SRAP primers designed by Li and Quiros (2001)

Forward primers (17 bp)	Reverse primer (18 bp)
ME1：5′-TGAGTCCAAACCGGATA-3′	EM1：5′-GACTGCGTACGAATTAAT-3′
ME2：5′-TGAGTCCAAACCGGAGC-3′	EM3：5′-GACTGCGTACGAATTGAC-3′
ME3：5′-TGAGTCCAAACCGGAAT-3′	EM4：5′-GACTGCGTACGAATTTGA-3′
ME4：5′-TGAGTCCAAACCGGACC-3′	EM5：5′-GACTGCGTACGAATTAAC-3′
ME5：5′-TGAGTCCAAACCGGAAG-3′	EM6：5′-GACTGCGTACGAATTGCA-3′

Table 2: Preparation of 6% denaturing acrylamide gels (e.g. two pieces of gels)

Componentsa	Volume (ml)
Urea pellets	6.4 g
Deionized water	30 ml
10×TBE buffer	5.4 ml
40% acrylamide (19:1)	8 ml
10% Ammonium persulfate	0.53 ml
TEMED	23.4 µl

Table 3: Preparation of developer solution (e.g. two pieces of gels).

Components	Volume (200 ml)
0.1% $AgNO_3$ (g)	0.2 g
Sodium Borate (g)	0.05 g
NaOH (g)	3 g
Formaldehyde (ml)	0.8 ml

Table 4: Troubleshooting table

Problem	Possible reasons	Solution
No amplicon	Not enough genomic DNA template	Isolate fresh genomic DNA
	Ineffective PCR reagents	Verify reagents and their concentrations
	Inefficient PCR	Optimize magnesium chloride ($MgCl_2$) concentration and cycling temperatures
	Primer mismatch	Recombinant primers
Smeary or diffuse band(s)	Primer combination not good	Modify matrix, buffer and/or electrophoresis conditions
	Incorrect or old buffer	
	Problem with acrylamide gel electrophoresis	
Bands not visible or weak	DNA amount too low	Adjust DNA amount; check on agarose gel
	Exposure/staining too short	Increase exposure/staining time
	Disabled formaldehyde	Replace formaldehyde
'Smiling effect'	Temperature higher in middle of gel compared with edges	Verify electrophoresis conditions
		Check cooling system
		Use air-condition in the room to keep temperature
Lanes not straight	Electric field not linear	Check electrodes, voltage and connections
	High voltage electrophoresis buffer not good	Replace buffer
High background	Exposure/staining too long	Decrease exposure/staining time
		Check exposure/staining conditions
	DNA amount too high	Load less DNA
Expected resolution not achieved	Lanes overloaded	Load less DNA
	Loading took too long	Load samples faster
	Migration distance insufficient	Increase electrophoresis time
Runs not reproducible	Inadequate temperature control	Check consistency of temperature control
	Electric field non-linear	Check system and electrodes
	Gel matrix inadequate	Use fresh gel
	Disabled electrophoresis buffer	Replace buffer

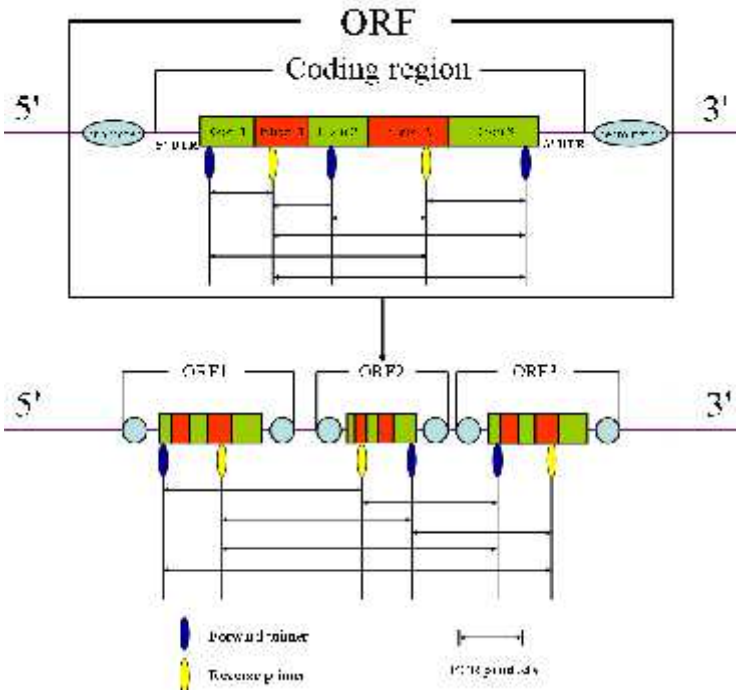

Figure. 1. Sequence-related amplified polymorphism technique. This shows the genomic features, amplification mode and the positions of the priming sites for the method described in the text. Amplification is carried out between primers matching on exons and introns. Genomic DNA (gDNA) is shown as a solid purple line, with promoter, 5′ UTR, exon, intron, terminator and 3′ UTR on it. PCR products with different length are amplified by different primer banding site as black arrows point.

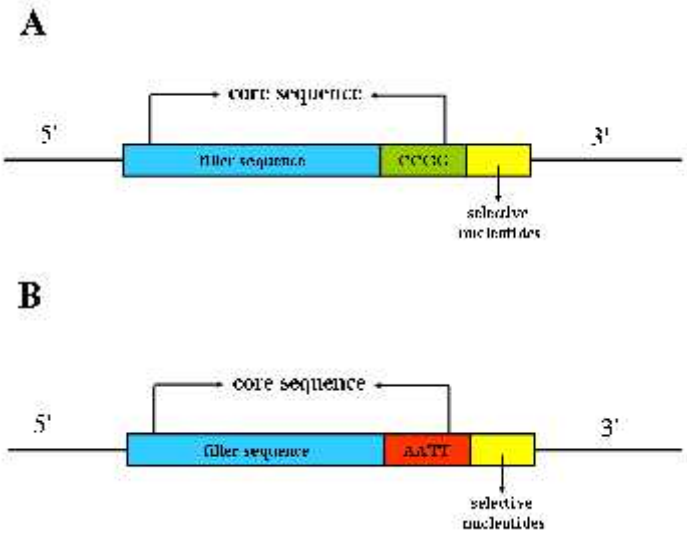

Figure. 2. The pattern of SRAP primer design. A refers to forward primer and B refers to reverse primer.

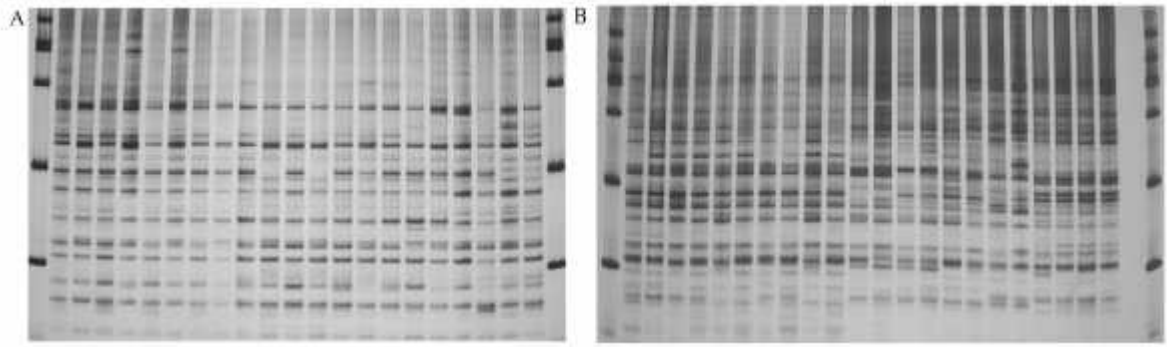

Figure. 3. SRAP gel fingerprints. This illustrates the results achieved following 6% denaturing acrylamide gels electrophoresis with correct and incorrect conditions. (A) Standard amplification, with almost all DNA samples at the same concentration. (B) PCR over-amplification, resulting from an overly high primer concentration, too many cycles, too much template or loaded sample thereof.

RESULTS AND DISCUSSION

Since SRAP method is comparatively sensitive and presents obvious differences in fingerprints under different amplification conditions, it is important to empirically optimize the PCR amplifications and the electrophoresis systems to ensure reproducible results. Proper primer combination is very important because not all primer combinations produced good polymorphism bands. Some primers generate smears under different PCR conditions and some primers just produce a single, very strong band, which are not suitable for SRAP fingerprinting. Careful casting of gels is critical to success. Small unpolymerized acrylamide gel inclusions in the gels will result in bands with spiked smears. Finally, a skilled operating process for loading samples is very important. Loading unsmooth, unflattening or lasting too long time will cause difficulty to read bands. Other critical steps and troubleshooting advices are listed in Table 4.

SRAP marker could be used to exhibit the polymorphism degree of the genome more comprehensively due to its capacity in revealing relatively more informative bands no matter on DNA or cDNA level (Li and Quiros, 2001; Que *et al*., 2012). Direct SRAP analysis on 6% denaturing acrylamide gels has been extensively used with great success for the analysis of amplicons of ~100–1000 bp (see Figure 3) and can readily obtain interesting bands by direct sequencing. Unlike other genetic marker systems, SRAP preferentially targets ORFs and estimates the variation of introns, exons, promoters and its spacers that make the patterns possessing a high degree of polymorphisms. The procedure of SRAP primer design is relatively easy and fast since there is no need of prior sequence information, and forward and reverse primers could be combined in

many ways to increase the number of polymorphisms. These primers are universal in plants and animals because they share the same characteristics of G-C rich in exons and A-T rich in introns and promoter regions, and most SRAP markers in the genome appear many copies and widely distribute.

We described here a detailed SRAP protocol for plants science study, and demonstrated the optimization of the most important parameters from primer designing to DNA visualization. This procedure can be performed with a reasonable throughput rate, and has been proved as a time efficient and cost effective marker system for displaying genetic differentiation on inter and intra-species level. Nowadays, SRAP protocol has been mostly used in crops or industrial crops, but seldom been used in medicinal plant, rare flowers, endangered plant species or other living organisms, which reveal a broad application scope for monitoring the genetic diversity as well as developing and conserving germ plasm resource. Therefore, the detailed analysis and proper optimization for SRAP procedure will further expand its applications to a wider range of living organisms.

Acknowledgements: This work is supported, in part, by the National Natural Science Foundation of China (Grant No. 31101138, 31271791), Special Funds for Basic Research (QN2012003), the Special Funds for Talents in Northwest A & F University (Grant No. 2010BSJJ033), National University Student Innovation Program in Northwest A & F University (Grant No. 201310712004) and the Provincial Natural Science Foundation of Shanxi, China (Grant No. 2011011028-1).

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