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Developing RAPD-derived SCAR (Sequence Characterized Amplified Region) Marker for Flowering Time in Chili Pepper

Estri Laras Arumingtyas[1,*] Bunga Rizky Elfa Agustina[2] Joni Kusnadi[2]

[1]*Biology Department, Faculty of Mathematics and Natural Sciences, Brawijaya University*
[2]*Department of Agricultural Product Processing, Faculty of Agricultural Technology, Brawijaya University*
[*]*Corresponding author. Email: larasbio@gmail.com*

ABSTRACT
The selection step of plant breeding is a crucial stage in the process of developing new varieties which usually take a considerable long time in a conventional breeding. The development of molecular markers for selection has been carried out to reduce the time required. A Sequence Characterized Amplified Region (SCAR) molecular marker based on the Random Amplified Polymorphic DNA (RAPD) DNA profile was developed to detect fast-flowering strains in chili pepper. From the RAPD stage which was amplified using 20 RAPD primers, 1 primer (OPB6) was obtained which produced polymorphic DNA bands that could distinguish between Early-Flowering and Late-Flowering lines. A polymorphic band sequence measuring approximately 600 bp was used as the basis for the SCAR Upper F primer design: 5'-GACAACC CCACACTACA TAC-3'; R: 5'-AACT GAACGAGGAA GCGTAG-3', Lower 1 F: 5'-GGAT GGAGTGAGAG AACCTA-3'; R: 5'-GAGA AAGTTCTAGG GCAGAG-3' and Lower 2. F: 5'-GAAC CTCCAACCTC ACGTTT-3'; R: 5'-GAA GAGGTGATGA TGTCAGC-3'. The effectivity test for the SCAR primers did not provide specific and accurate result. Further refinement needs to be done to obtain more specificity.

Keywords: *Chili Pepper, Flowering, RAPD, SCAR.*

1. INTRODUCTION

Selection is an important stage in plant breeding. Conventional selection by relying on morphological characters generally takes a long time. Moreover, if the character is generative, it takes time to reach the generative phase of the plant. Therefore, many researchers have developed molecular markers to assist the selection process.

Selection using molecular markers is much developed because the application takes less time with high accuracy compared to the conventional one. One of many molecular markers developed for plant breeding is the Sequence Characterized Amplified Region (SCAR), a molecular marker which can be developed from various other markers. SCAR marker developed from RAPDs has been proven to have high effectiveness in identifying the sex of date palms [1]. SCAR markers have also been developed to identify *Pseudofabraea citricarpa* [2]. Increasing the specificity of molecular markers by combining RAPD analysis and development of SCAR primers has succeeded in detecting genetic variations in *Lonicera japonica* [3]. SCAR markers developed from ISSR markers linked to SFL allow large-scale screening of mapping progenies of *Fragaria vesca* [4]. SCAR markers were also developed for marker-assisted selection of flower shapes in chrysanthemums [5].

The development of SCAR from RAPD on chili pepper has never been done. So far, research on DNA profiles in chilies has mostly used RAPD. In India, variability of 10 commercial chili pepper varieties was detected using RAPD marker [6]. Analysis of the possibility of genetic diversity in ten chili cultivars in Bangladesh was also reported to be successful using the RAPD technique approach [7] and also for differentiating between the local chili varieties [8]. However, RAPD marker has weaknesses, including being a dominant marker and not reproducible [9].

In chili pepper and generally other plants that are expected to produce from the fruit, flowering time is a character that determines the fruit product quantity. Selection for early flowering plants is very important to get optimal product. In this study SCAR markers were developed from RAPD markers. If the RAPD mark is known as a non -reproducible mark, then the SCAR mark developed from this RAPD will be more accurate and reproducible. Several SCAR marker candidates were designed in this study based on RAPD profile.

2. MATERIAL AND METHODS

2.1. Chili pepper Lines Used

The sample used in this study was a group of chili pepper strains collected by researchers consisting of Early-Flowering (EF) and Late-Flowering (LF) lines. The genomic DNA of each line in each group were isolated. Furthermore, RAPD was carried out to obtain bands representing each line group. From these bands, the SCAR primer was designed by taking into account the requirements of a good primer design. The designed SCAR primer was then tested for its ability to detect fast-growing lines.

2.2. DNA isolation by CTAB

About 0.1-0.2 g fresh young chili leaves were ground in liquid nitrogen, then transferred into 1.5 ml tube, added with 700 µl CTAB buffer, and incubated at 65° C in a water bath for 10 minutes. After that, 1 times volume of phenol was added and centrifuged at 13000 rpm for 10 minutes at 4°C. The supernatant was taken and put into a 1.5 ml microfuge tube, followed by addition of 1/10 volume of chloroform: isoamyl alcohol (24:1). The mixture was then centrifuged again at 13000 rpm for 5 minutes at 4°C. The supernatant was carefully discharged. To precipitate the DNA, a 0.1 volume of ammonium acetate and 2.5 volume of absolute ethanol was added. The mixture was left at the temperature of 20°C overnight then centrifuged at 12000 rpm for 15 minutes at 4°C. In the next day, the pellet was rinsed with 70% ethanol at 25°C twice and dried. After the pellet was dry, then it was dissolved in 20 µl TE solution (1 mM Tris HCl pH 8; 0.1 mM EDTA) for further use.

2.3 Analysis of Genomic Variation using RAPD

There were 20 RAPD primers used (Table 1). Each RAPD primer was mixed with 2µL 10X of Taq Polymerase buffer, 1.6 µL of 200 M dNTP, 1.6 µL of 2mM MgCl2 and 1U Taq Polymerase. The conditions used consisted of 1 minute of denaturation at 93°C, 30 seconds of annealing at 37°C, 1 minute of extension at 72°C, for 40 cycles. Preheating was done for 1 minute at 93°C, and the last elongation phase was carried out for 10 minutes at 72°C. The amplification results were electrophoresed on 2% agarose gel with TBE buffer to check the DNA from the PCR results.

Table 1. RAPD Primers used in the research

No.	Primers	Sekuen (5'-3')
1.	OPA 1	CAG GCC CTT C
2.	OPA 2	TGC CGA GCT G
3.	OPA 11	CAA TCG CCG T
4.	OPB 3	CAT CCC CCT G
5.	OPB 4	GGA CTG GAG T
6.	OPB 5	CAT CCC CCT G
7.	OPB 6	TGC TCT GCC C
8.	OPB 11	GTA GAC CCG T
9.	OPB 17	GAC CGC TTG T
10.	OPD 13	GGG GTG ACG A
11.	OPL 05	ACG CAG GCA C
12.	OPF 9	CCA AGC TTC C
13.	OPF 11	TTG GTA CCC C
14.	OPF 14	TGC TGC AGG T
15.	OPF 18	TTC CCG GGT T
16.	OPU 10	ACC TCG GCA C
17.	OPD 19	CTG GGG ACT T
18.	OPU 19	GTC AGT GCG G
19.	OPW 03	GTC CGG AGT G
20.	OPW 04	CAG AAG CGG A

2.4. Determination and Establishment of SCAR Primers

From the RAPD results, polymorphic bands that differentiated each pair of NIL strains were identified. The DNA bands were then sequenced and used as the basis for the SCAR marker design. SCAR markers are designed by considering good primer requirements.

2.5. SCAR Marker Test

The SCAR primer was then used to identify varieties of chili pepper that were claimed to be early and LF using the PCR technique. Go-Taq Green Master Mix (Promega) 20 µL and 1 µL DNA sample (25 ng/µL) was added to the PCR tube which would then be homogenized and vortexed. The PCR program was set: 5 minutes at 94°C for pre-denaturation, 30 seconds at 94°C for denaturation, 30 seconds at 58°C for primers annealing, 1 minute at 72°C for elongation and 10 minutes at 72°C for final elongation. The stage of denaturation to extension will be repeated 30 times. The results obtained will be observed with 1% agarose gel electrophoresis.

3. RESULTS AND DISCUSSION

From the 20 RAPD primers, it was only 4 primers that capable in producing polymorphic bands. Among them only one primer (OPB-6) showed a quite clear-cut difference between EF and LF lines. From the amplicon of OPB-6, we found a band with a size of about 600 bp which can distinguish between groups of EF and LF lines (polymorphic band) (Figure 1). Some of the lines did not have a band-type consistency and were therefore excluded from the group. Only those lines that produced consistent bands were used for further analysis. The inconsistency of the emergence of the band which

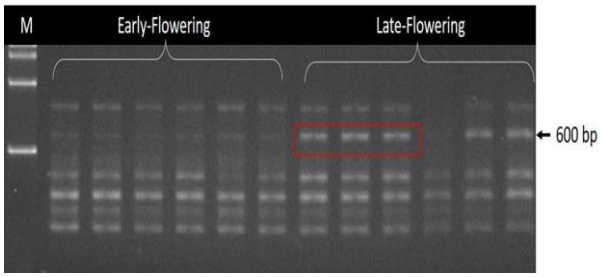

Figure 1. The RAPD Polymorphic bands (in the box) that was developed into SCAR primers

corresponds to the flowering time, maybe due to the lines

Table 2. The SCAR primers designed based on RAPD amplicon sequence

Primer Name	Sequence Length	Primer Sequence
Upper-Forward	20	5′-GACAACC CCACACTACA TAC-3′
Upper-Reverse	20	5′-AACT GAACGAGGAA GCGTAG-3′
Lower1-Forward	20	5′-GGAT GGAGTGAGAG AACCTA-3′
Lower1-Reverse	20	5′-GAGA AAGTTCTAGG GCAGAG-3′
Lower2-Forward	20	5′-GAAC CTCCAACCTC ACGTTT-3′
Lower2-Reverse	20	5′-GAA GAGGTGATGA TGTCAGC-3′

used that did not pure line yet. The line that was aroused from random mutation might still contain heterozygous alleles that cause a population to still experience segregation so that it becomes varied [10].

Bands representing EF/LF lines were sequenced and used as the basis for primer design. Three primers were designed based on this sequence (Primary upper, Lower 1 and Lower 2) (Table 1.). The primer design fulfilled the categories of good primers such as not contain primer dimer, cross dimer, running bases, repetition etc. The primers have 20 bases in length. The primers produced were tested to detect fast-flowering lines compared to slow-flowering lines.

These primers were then used for identifying LF and EF chili pepper lines. Primer Upper cannot differentiate between both lines (Figure 2). On the other hand, primer Lower 1 and Lower 2 showed a different pattern of amplification between Late and EF lines. Using primer Lower 1, two bands were developed, however for the EF line the first band (about 500 bp) seem like consisted of fewer bands than the LF, and the second band was thinner than that's of the LF line. For the primer Lower 2, the lower band (about 700 bp) of the EF showed a more dispersed smear than the LF line.

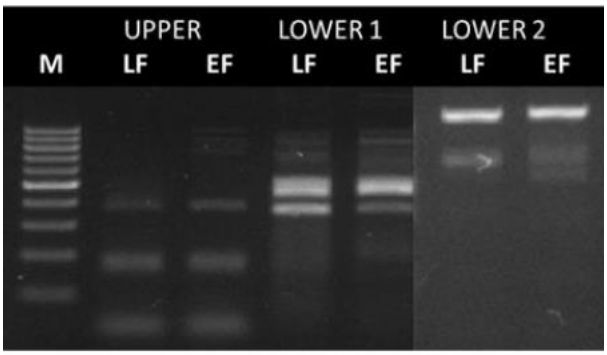

Figure 2. Bands produced by SCAR marker in Late-Flowering (LF) and Early-Flowering (EF) Chili Pepper Lines

Those patterns of bands produce by Lower 1 and Lower 2 primers might represent differences between EF and LF lines. However, the resulting band some were very thin and some others although quite thick but look like consisted of several bands. For this reason, it is necessary to improve the PCR program to obtain more clear-cut band differences between fast and slow flowering lines. Another possible work was to sequence the lower band of Lower 1 primer and used it to develop a new SCAR marker.

SCAR primers can be developed from RAPD. However, proper selection of polymorphic bands is needed in order to obtain specific and accurate primers. The primers produced in this study need further refinement to obtain this specificity.

AUTHORS' CONTRIBUTIONS

EL Arumingtyas and J Kusnadi who had ideas and designed the experiments. BRE Agustina conducted the experiments. EL Arumingtyas make a preliminary draft of the manuscript. All authors provided input and assisted in research, analysis, and manuscript writing.

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