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Full Length Research Paper

Development of a sequence characterized amplified region (SCAR) marker for identification of Thai jasmine rice (*Oryza sativa* L.) variety 'KDML105'

Juthaporn Saengprajak

Department of Biology, Faculty of Science, Mahasarakham University, Kantarawichai, Mahasarakham 44150, Thailand. E-mail: juthaporn.s@msu.ac.th. Tel: +66 4375 4245, +66 4375 4333 ext. 1122. Fax: +66 4375 4245.

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The varieties of rice are difficult to be distinguished because of their similar morphological characters, therefore rice variety identification or differentiation is very important. The authentication by using molecular marker or DNA fingerprinting is the most accurate method. In this study, random amplified polymorphic DNA (RAPD) derived sequence characterized amplified region (SCAR) markers were applied to detect aromatic rice (*Oryza sativa* L.) variety Khao Dawk Mali (KDML) 105 from its closely related varieties. Eighty 10-mer primers were screened to identify an $OPJ01_{KDML105\text{-}727}$ band which was amplified by primer OPJ-01, and this band specified to variety KDML105. SCAR marker was created by cloning and sequencing the specific band, and designing a pair of primers, $SCJ01_{KDML105\text{-}668}$ to amplify the band of 668 bp. Diagnostic PCRs were performed using the primer pair with the total DNAs of *O. sativa* var. KDML105, and its related varieties as templates, with only *O. sativa* var. KDML105 being able to be amplified. This amplification is not only rapid, but is also easy to perform. Hence, it is a feasible method for identifying *O. sativa* var. KDML105 between rice varieties.

Key words: *Oryza sativa* L., varietal identification, random amplified polymorphic DNA (RAPD) marker, sequence characterized amplified region (SCAR) marker, multiplex polymerase chain reaction (PCR).

INTRODUCTION

Rice (*Oryza sativa* L.), belonging to the family Poaceae, is one of the most commercially important food crops in world. However, Thai jasmine rice (Khao Dawk Mali 105, KDML105), a Thai commercial aromatic rice variety, is very famous and are traded or distributed at higher prices than the ordinary rice because of their high palatability, processing suitability, or special aroma, etc. Because those rice grains sell at high prices, some dishonest rice wholesalers or retailers blend low-quality cheap rice with the jasmine rice. Moreover, many varieties of rice are difficult to be distinguished because of their similar morphological characters. Under such circumstances, the technology to identify rice variety is very important for breeders, farmers, wholesalers, retailers, food industries, and consumers (Bergman et al., 2004). Therefore, it is necessary to develop a time-saving technology to clearly and precisely differentiate rice varieties.

DNA fingerprinting was developed in 1985 (Jeffreys et al., 1985) and is used for criminal investigation and trial at court. Recently, a novel cultivar identification method based on DNA polymorphism has been developed that accompanies the progress in molecular biology. DNA-based markers have the obvious advantage of sampling the genome directly, and restriction fragment length polymorphism (RFLP) analysis has been used to distinguish between species of *Oryza* (Wang and Tanksley, 1992) and particularly between *indica* and *japonica* subspecies of *O*. *sativa* (Zang et al., 1992).

Recently, a polymerase chain reaction (PCR)-based marker system has been developed by Williams et al. (1990). In this random amplified polymorphic DNA (RAPD) method, short oligonucleotides of arbitrary sequences are used to support the amplification of regions of the test plant genome and amplified DNAs are separated by gel electrophoresis. There are some reports on RAPD analysis of rice germplasms including

subspecies *indica* and *japonica* to identify suitable parents for linkage map construction and for gene tagging for drought resistance (Fukuoka et al., 1992; Yu and Nguyen, 1994).

In the case of RAPD markers, many other DNA bands than the target DNA for variety differentiation appear in the electrophoregram after PCR. Therefore, it is recommended to develop the sequence characterized amplified region (SCAR) markers based on RAPD markers (Paran and Michelmore, 1993). By the development of forward and reverse primers based on the sequence of the target DNA region, which had been proliferated using RAPD primers, it became possible to amplify only the target DNA fragment by PCR. For example, Jain et al. (2004) developed SCAR markers from the results of amplified fragment length polymorphism (AFLP) and RAPD to select the pathogen resistance gene of rice and localized those markers on the genetic map to identify the chromosomal location of the resistance gene. And, Singh et al. (2011) sequenced and designed SCAR primers from the result of RAPD to detect resistance against blast in rice variety Vallabh Basmati-21.

In the present study, the application of PCR method using RAPD markers and SCAR primers developed from RAPD markers to identify a Thai jasmine rice variety, KDML105 was investigated. Novel SCAR markers and multiplex primer were developed based on RAPD marker analyses to clearly and efficiently differentiate closely related rice varieties, such as Pathumthani 1, Ubonratchathani 80 and Homsakonnakhon, by PCR.

MATERIALS AND METHODS

The seeds of eleven varieties of rice (*O. sativa* L.) including; KDML105, Pathumthani 1, Homsakonnakhon Homsadung Homdaeng, Ubonratchathani 80 Homnangnual, RD6 Homdor, Dorhom and Homaum were obtained from the rice research institutions of Thailand namely; Khon Kaen Rice Research Center, Chumphae Rice Research Center, Sakon Nakhon Rice Research Center, Ubon Ratchatani Rice Research Center, Surin Rice Research Center and Pathumthani Rice Research Center. The seeds were germinated separately in plastic tray by labeling the names of variety. Leaves were collected after two weeks of growth for DNA extraction.

Extraction of genomic DNA

Genomic DNA was extracted from young leaf tissues following the CTAB method (Doyle and Doyle, 1987) after minor modification and adapted to small tissue quantities (Hormaza, 1999). Fresh young leaves about 0.2 g were ground in a small mortar and pestle containing liquid nitrogen. The grindate was added to 1 ml of extraction buffer [2% CTAB (hexadecyltrimethylammonium bromide), 100 mM Tris-HCl buffer, 20 mM EDTA, 1.4 M NaCl, 1% PVP-40, pH 8.0)] containing 1 µl of 2-mercaptoethanol added just before use. The extract was incubated for 1 h at 60°C with occasional swirling, mixed with an equal volume of chloroform : isoamyl alcohol (24:1, v/v) and centrifuged at 13,000 g for 10 min. The aqueous phase was transferred to a new tube and mixed with 2/3 vol of ice-cold isopropanol. The mixture was left at -20°C for 20 min and again centrifuged at 13000 rpm for 10 min. The pellet was washed with 10 mM of ammonium acetate in 76% ethanol, air dried at room temperature for 1 to 2 h, and dissolved in 40 µl of TE buffer (10 mM Tris–HCl pH 8.0, 0.1 mM EDTA). For the removal of RNA, 10 mg of RNAse A was added to the DNA solution and incubated for 10 min at 37°C. Extracted DNA samples were checked by using 1% agarose gel electrophoresis and quantified by using a spectrophotometer by measuring the absorbance at 260 and 280 nm (Sambrook et al., 1989). On the basis of the spectrophotometer readings, 50 ng/µl solutions were prepared and used for PCR amplification.

RAPD reaction

RAPD amplification was performed according to the method described in Williams et al. (1990). The final reaction volume was 25 µl and contained: 1X PCR buffer (Promega, USA), 1.5 mM $MgCl_2$ (Promega, USA), 0.32 mM dNTPs (dATP, dCTP, dGTP and dTTP), 0.96 pM of RAPD primer (Operon Technologies, Almeda, USA), 0.08 U *Taq* DNA polymerase (Promega, USA) and 8 ng of template DNA. Negative controls with distilled water instead of DNA were included in each run in order to verify the absence of contamination. PCR amplification was conducted on a programmable thermalcycler (Hybaid, USA). Template DNA was initially denatured at 95°C for 3 min, followed by 40 cycles of PCR amplification using the following parameters: 1 min denaturation at 94°C, 1 min annealing at 35°C, and 2 min primer extension at 72°C. Final 5 min incubation at 72°C was allowed for completion of primer extension.

Eighty decamer primers from the operon kits OPA, OPB, OPJ and OPW (Operon Technologies, Almeda, USA) were used for this study. Six primers (Table 1) out of 80 produced amplicons and they showed polymorphism in the varieties of rice genotypes. Amplification products were electrophoretically resolved on 1.5% agarose gels with 1X TBE (40 mM Tris-borate, 1 mM EDTA) containing 0.5 g/ml ethidium bromide, and photographed on a UV transilluminator. The size of the amplified products was determined by comparison with a 100 bp DNA ladder size standard (BioLabs, USA). Among these, primer OPJ-01 (5'-CCCGGCAATA-3') was consistently amplified as a single, intense band of 727 bp in variety 'KDML105' specimen, but absent in other varieties (Figure 1). This band named as $OPJ01_{KDML105-727}$ was selected as putative the rice variety KDML105 specific marker.

Cloning and sequencing of RAPD fragments

The RAPD fragments, $OPJ01_{KDML105-727}$ were excised from agarose gels and extracted using a QIAquick Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. The recovered DNA fragments were cloned in the pGEM-T-Easy vector system I (Promega, USA) and introduced into competent *Escherichia coli* strain JM109. Recombinants were identified as white colonies on LB plates with X-gal and IPTG. White colonies were grown in 2 ml LB medium containing 100 µg/ml ampicillin, and plasmid DNA was isolated from putative recombinants according to the method described in Serghini et al. (1989) with minor modifications.

An aliquot of purified plasmid DNA was analyzed by PCR, while another aliquot was digested by *Eco*RI restriction enzyme. The resulting products were run on 1.5% agarose gels containing 0.5 µg/ml ethidium bromides, and photographed. Again putative positive transformants were evaluated based on the size of the amplification product and the size of the plasmid insert. Plasmid DNA from several promising putative transformants were sequenced on an Applied Biosystems 377 automated sequencer, using

Table 1. Primer sequences, product length in base pairs and annealing temperature of the molecular markers used in the study.

Marker	Primer sequence (5′→3′)	Product size length (bp)	Annealing temperature (°C)	Polymorphic band of KDML105 (bp)
RAPD[a]				
OPA-08	GTGACGTAGG	340-1450	35	-
OPJ-01	CCCGGCAATA	400-1000	35	727
OPJ-16	CTGCTTAGGG	550-1250	35	-
OPJ-18	TGGTCGCAGA	680-850	35	-
OPW-01	CTCAGTGTCC	440-1250	35	-
OPW-19	CAAAGCGCTC	560-1840	35	-
SCAR[b]				
$SCJ01_{KDML105-668}$_F	AGCTTCGATAGCGGCTAGGT	668	60	668
$SCJ01_{KDML105-668}$_R	AAGTTCCCCCTCCTAACGAC			
18R rDNA				
18R rDNA_F	GACTGTGAAACTGCGAA	500	60	-
18R rDNA_R	ATACGCTATTGGAGCTGGA			

[a]RAPD, random amplified polymorphic DNA, [b]SCAR, sequence characterized amplified region, 18S rDNA, 18S ribosomal DNA.

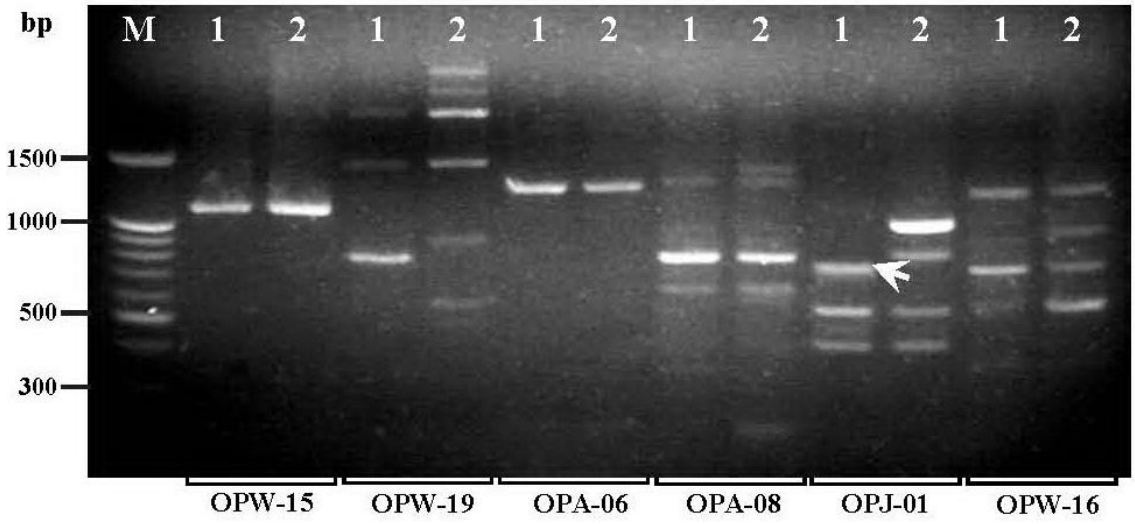

Figure 1. A sample figure of DNA polymorphism between KDML105 and Pathumthani 1 by using RAPD primers: Lane 1, KDML105; lane 2, Pathumthani 1; and lane M, DNA molecular weight marker. Arrow indicate a specific DNA fragment of KDML105.

the ABI PRISM dye terminator cycle sequencing kit at the DNA Technology Laboratory, Kasetsart University, Kampangsaen Campus, Nakorn Pathom, Thailand.

Designing and synthesis SCAR primers

Based on the sequence data of cloned RAPD fragment, specific primers were designed with the aid of using the program of GeneFisher (http//:bibiserv.techfak.uni-bielefeld/de/genefisher). Primers were synthesized with a Polygen DNA synthesizer of POLYGEN GmbH (Langen, Germany) at the DNA technology Laboratory. The specific primer was designed by adding 20 bases to the 3′ end of original random primer that produced the respective RAPD fragment to make SCAR as described by Paren and Michelmore (1993).

SCAR markers analysis and multiplex PCR

In the present study, SCAR primers for PCR were developed based on RAPD analysis to identification rice varieties by PCR. The SCAR primers designed namely; $SCJ01_{KDML105-668}$_F and $SCJ01_{KDML105-668}$_R were used to amplify DNA obtained from the eleven varieties

Table 2. Sequences of RAPD fragment, $OPJ01_{KDML105-727}$ used in the development of SCAR markers, $SCJ01_{KDML105-668}$ specific to the rice variety KDML105.

bp.	Sequences of RAPD fragment	bp
1	**CCCGGCATAA**TAGACTGAAAAACGCAATTTTATCCATTTGTAGATGGAGC	50
51	TTCGATAGCGGCTAGGTCTAGAGGGAAGTTATGAGCATTACGTTCATGCA	100
101	TAACTTCCATACCAAGGTTAGCACGGTTAATGATATCAGCCCAAGTATTA	150
151	ATTACACGACCTTGACTATCAACTACAGATTGGTTGAAATTGAACCCATT	200
201	TAAGTTGAAGGCCATAGTACTAATACCTAAAGCAGTAAACCAAATACCTA	250
251	CTACAGGCCAAGCAGCTAAGAAGAAGTGTAAAGAACGAGAGTCGTTGAAA	300
301	CTAGCATATTGAAAGATCAATCGGCGAAATTACCATGAGCCAGCTTCCAA	350
351	TATTATAAGTTTCTTCCTCTTGCCCGAATCTGTAACCCTCATTAGCAGAT	400
401	TCATTTTCTGTAGTTTCCCTGATCAAACTAGAAGTTACCAAGGACCCATG	450
451	CATAGCACTAAAAAGGGAGCCGCCGAATACACCAGCTACACCTAACATGT	500
501	GAAATGGATGCATAAGGATGTTGTGCTCAACCTGGAATACAATCATAAAG	550
551	TTGAAAGTACCCAGAGATTCCTAGAGGCATACCATCAGAAAAGCTTCCTT	600
601	GACCAATTGGGTAGATCAAGAGAACAGCAGTAGCCGCTGCCACCGGAGCC	650
651	GAATATGCAACAGCAATCCCAGGACGCATACCCAGACGGAAACTTAAGTT	700
701	CCCCCTCCTAACGACCA**TTATGCCGGG**	727

RAPD primer sequences are indicated in bold and the positions of SCAR primers are underlined.

of rice including; KDML105, Pathumthani 1, Homsakonnakhon Homsadung Homdaeng, Ubonratchathani 80 Homnangnual, RD6 Homdor, Dorhom and Homaum. The final reaction volume was 25 µl and contained: 1X PCR buffer (Promega, USA), 2.0 mM $MgCl_2$, 240 µM each of dATP, dGTP, dCTP and dTTP (Promega, USA), 0.5 µM of SCAR primers, 0.08 U of *Tag* DNA polymerase (Promega, USA), and 6 ng of genomic DNA template. Negative controls with distilled water instead of DNA were included in each run in order to verify the absence of contamination. DNA amplification was conducted on a programmable thermalcycler (Hybaid, USA).

Template DNA was initially denatured at 94°C for 2 min, followed by 42 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C, and 1 min extension at 72°C, with a final extension period of 4 min at 72°C, and a hold at 4°C until recovery. Amplification products were electrophoresed on 1.5% agarose gels containing 0.5 µg/ml ethidium bromide, and photographed on a UV transilluminator. The combinations of these SCAR primers for PCR, multiplex primer with 18S rDNA primers (18S-F, 5′-GACTGTG-AAACTGCGAA-3′ and 18S-R, 5′-ATACGCTATTGGAGCTGGA-3′) were carried out in 25 µl reaction mixtures containing the same components used for single analysis, except that 18R rDNA primers was included at a concentration of 1.0 µM. Thermal cycling condition for amplification using multiplex primers was conducted on a programmable same as described.

RESULTS

RAPD-PCR and identification of specific RAPD amplicon

RAPD analysis of eleven varieties of rice including; KDML105, Pathumthani 1, Homsakonnakhon Homsadung Homdaeng, Ubonratchathani 80 Homnangnual, RD6 Homdor, Dorhom and Homaum was carried out using 80 RAPD primers for selecting polymorphic bands between the rice genotypes. One primer, OPJ-01 (5′-CCCGGCAATA-3′) gave DNA band which was consistently amplified as a single, intense fragment of 727 bp in the rice variety ‘KDML105’ genotype, but absent in other varieties. This band named as $OPJ01_{KDML105-727}$ was identified as putative the rice variety KDML105 specific RAPD marker (Figure 1).

Cloning and sequencing RAPD fragments

The amplification product of the recombinant colonies of $OPJ01_{KDML105-727}$ which putative positive transformant were identified based on the size of the amplification product (data not shown). The positive clones were used for DNA sequencing. The entire RAPD fragment of $OPJ01_{KDML105-727}$ was sequenced using T7 and SP6 forward and reverse sequencing primers. The sequencing results showed that the cloned RAPD fragment $OPJ01_{KDML105-727}$ had the correct size of 727 bp. Based on sequence information derived from cloned RAPD band, 20-mer oligonucleotide primers pair was synthesized for further use in PCR reactions (Table 2).

Designing and synthesis of SCAR primers

The designed SCAR primer pair name as $SCJ01_{KDML105-668}$_F (5′-AGCTTCGATAGCGGCTAGGT-3′) and $SCJ01_{KDML105-668}$_R (5′-AAGTTCCCCCTCCTAACGAC-3′) were used to amplify genomic DNA from the eleven varieties of rice (including; KDML105, Pathumthani 1, Homsakonnakhon Homsadung Homdaeng, Ubonratchathani 80 Homnangnual, RD6 Homdor, Dorhom and Homaum . Thermal cycling conditions for

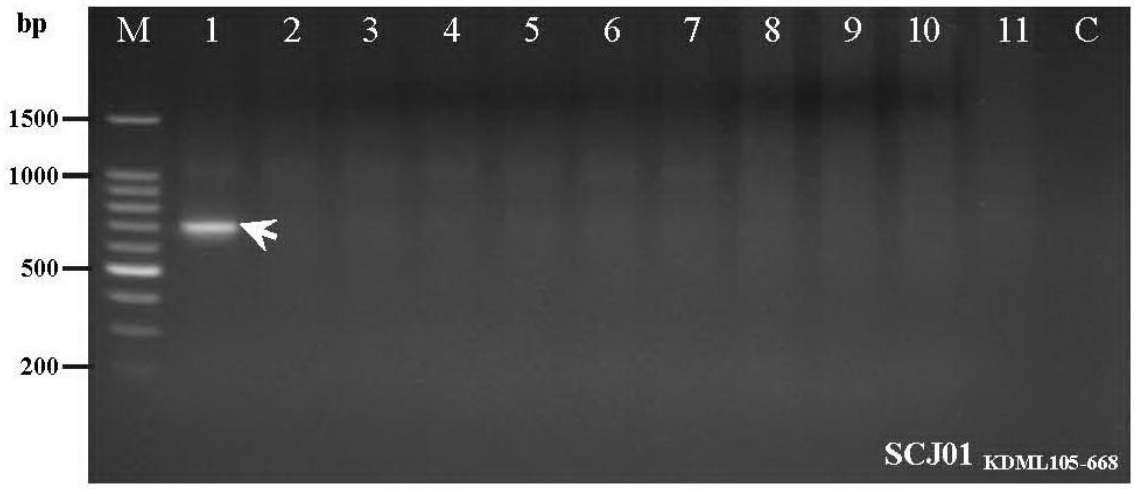

Figure 2. Results of PCR using the SCAR primers, $SCJ01_{KDML105-668}$ for identification of the variety KDML105 using various rice varieties: Lane 1, KDML105; lane 2, Pathumthani 1; lane 3, Homsakonnakhon; lane 4, Homsadung; lane 5, Homdaeng; lane 6, Ubonratchathani 80; lane 7, Homnangnual; lane 8, RD6; lane 9, Homdor; lane 10, Dorhom; lane 11, Homaum; lane C, Negative control and lane M, DNA molecular weight marker, respectively. Arrow indicate a specific DNA fragment of KDML105.

amplification using SCAR primers were optimized as described previously. A single, distinct and brightly resolved band of 668 bp was obtained in DNA isolated from only the variety 'KDML105' genotype, and no non-specific amplification was observed in the other related rice varieties (Figure 2).

SCAR markers analysis and multiplex PCR

To test the use of such an approach for rice variety identification, one polymorphic band obtained with the OPJ-01 was chosen for the development of SCAR markers, and they were cloned and sequenced. The original RAPD marker was successfully transformed into more-specific SCAR assays ($SCJ01_{KDML105-668}$_F and $SCJ01_{KDML105-668}$_R). The SCAR markers detected differences between the eleven varieties of rice (including; KDML105, Pathumthani 1, Homsakonnakhon Homsadung Homdaeng, Ubonratchathani 80 Homnangnual, RD6 Homdor, Dorhom and Homaum . $SCJ01_{KDML105-668}$_F and $SCJ01_{KDML105-668}$_R amplified a DNA fragment on 727 bp only of KDML105 variety, indicating that this marker can only be used for KDML105 variety-specific markers. All other rice varieties no non-specific amplified DNA fragments from these SCAR markers, indicating the usefulness of these markers.

The DNA amplification of eleven rice varieties using the SCARs is shown in Figures 2 and 3. These show polymorphisms suitable for variety identification and resemble the original RAPD polymorphism. The amplifications of eleven rice varieties using SCAR primers and 18R rDNA primers combinations that were determined. Among two primer pairs, DNA fragments of 668 bp amplified by the SCAR markers only in KDML105, and which did not yield in other varieties by agarose gel electrophoresis. Moreover, DNA fragments amplified by 18S rDNA primers yielded DNA bands of 500 bp were the same between these eleven varieties, indicating that these two markers were demonstrated by PCR using the mixture of the SCAR primers and 18R rDNA primers (Figure 3).

DISCUSSION

Rice is markedly diversified from the viewpoint of genetics, morphology, and properties. On the contrary, high-quality rice is closely related by inbred breeding to attain high palatability, high processing suitability, or characteristic aroma, etc. (Ohtsubo et al., 1998). The objective of this investigation was to identify suitable RAPD polymorphisms across rice varieties and to transform them into more-specific SCAR markers in an efficient and reliable manner. RAPD analysis can reveal high degree of polymorphism; does not require prior DNA sequence information of the species and is easy to perform. Therefore, various researchers have explored its application for identifying of rice cultivars and the resistance genes such as; SCAR markers developed from AFLP and RAPD markers to select the pathogen resistance gene of rice (Jain et al., 2004), Shirasawa et al. (2004) converted AFLP-markers to the SCAR markers used enables efficient sequence-specific marker for identifying closely related of rice cultivars, and SCAR primers sequenced and designed from the result of RAPD to detect resistance against blast in rice variety Vallabh Basmati-21 (Singh et al., 2011).

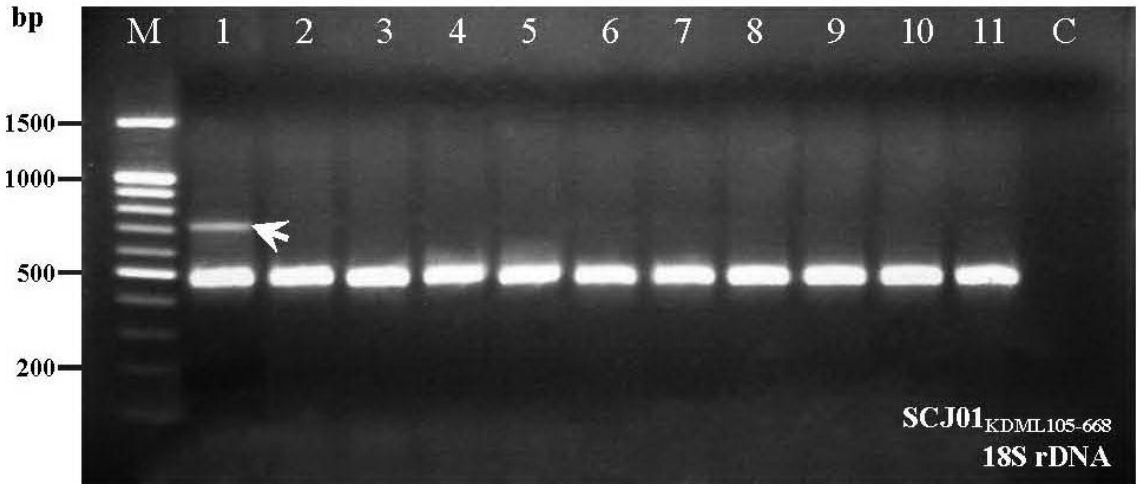

Figure 3. Screening of variety-specific SCAR primers, $SCJ01_{KDML105-668}$ and 18S rDNA primers using various Thai rice varieties: Lane 1, KDML105; lane 2, Pathumthani 1; lane 3, Homsakonnakhon; lane 4, Homsadung; lane 5, Homdaeng; lane 6, Ubonratchathani 80; lane 7, Homnangnual; lane 8, RD6; lane 9, Homdor; lane 10, Dorhom; lane 11, Homaum; lane C, Negative control; and lane M, DNA molecular weight marker, respectively. Arrow indicate a specific SCAR marker of KDML105.

In this RAPD analysis, 80 decamer primers were screened for suitable polymorphisms among the eleven rice varieties. As an example, amplification patterns obtained using the 10-mer primer, OPJ-01 is shown in Figure 1. RAPD-PCR is a widely used technique in plant genome analysis, but the practical applications of such markers are enhanced by sequencing the RAPD products and converting them into more-specific assays (Paran and Michelmore, 1993). Moreover, such conversion is more cost effective if the direct sequencing methodology (Hernández et al., 1999) is applicable. To test the use of such an approach for rice varieties identification, polymorphic band of 727 bp obtained with primer OPJ-01 in the variety KDML105 genotype, but did not yield in other varieties. This band named as $OPJ01_{KDML105-727}$ was chosen for the development of specific SCAR markers. This RAPD band was cloned and sequenced originating the SCAR markers.

In SCAR, pairs of 20 bp oligonucleotide primers specific to the sequence of polymorphic bands can be used to amplify the characterized regions from genomic DNA under stringent conditions, which makes this marker more specific and dependable as compared to RAPD marker (Paran and Michelmore, 1993). SCAR primer pairs were designed internally to the RAPD sequences, indicating that the original RAPD polymorphisms did not rely on the RAPD priming sites. Such a possibility of internal primer design increases the utility of the developed markers, allowing specific and robust multiplexing. SCAR, $SCJ01_{KDML105-668}$ amplifies a single band (Figure 2) and is thus suitable for a plus-minus screening in the electrophoregram after PCR (Figure 3).

Genomic DNA only variety KDML105 specimen was found to be fragmented, while no amplification was observed in other rice varieties. This SCAR has therefore a putative use in rice variety germplasm identification and marker-assisted selection, but its needs to be confirmed by the screening of a larger collection of varieties and cultivars. Therefore, the proposed methodology represents a simple and reliable approach for the generation of effective and specific SCAR markers potentially useful to distinguish Thai aromatic rice variety 'KDML105'. However, the development of species-specific markers has become an objective of high priority in paternity testing, genotype identification, gene mapping and breeding program.

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