academicJournals

Vol. 7(17), pp. 1154-1160, 3 May, 2013
DOI: 10.5897/JMPR12.615
ISSN 1996-0875 ©2013 Academic Journals
http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Real time sequence characterized amplified region (RT-SCAR) marker: Development and its application for authentication and quantification of *Catharanthus roseus* L. Don.

Anis Ahmad Chaudhary[1]*, Deepak Yadav[2], Hemant, Sayed Shakir Jamil[3] and Mohd Asif[2]

[1]Molecular Ecology Laboratory, Department of Botany, Faculty of Science, Hamdard University, New Delhi, India.
[2]Drug (Unani) Designing and Development Laboratory, Department of Ilmul Advia, Faculty of Medicine, Hamdard University, New Delhi, India.
[3]Department of Molijat, Faculty of Medicine, Jamia Hamdard, New Delhi-110062, India.

Accepted 10 July, 2012

The objective of the present study was to develop a Real Time-Sequence Characterized Amplified Region (RT-SCAR) of *Catharanthus roseus,* an effective molecular marker for authentication and quantification. Here, we made a comparative study of SCAR and RT-SCAR marker. These newer RT-SCAR methods are useful for the correct identification and quantification of C. roseus. In particular, this technique is very attractive and can be used as a novel tool for quantification of C. roseus when the SCAR results cannot be quantified and the RAPD results are not consistent. RAPD analysis of collected samples from different geographical locations in India was carried out with 25 random primers. The RAPD polymorphic band was cloned, sequenced and from the sequence information, primers pair for SCAR was developed. The same primers was used for RT-SCAR marker by using SYBR green fluorescence-based detection in Real Time PCR. Utilizing this newly specific RT-SCAR marker, we detected the presence and quantity of *C. roseus* in all the analyzed several samples, by aiding 10% its substitute and adulterants at time of DNA isolation. No difference was found in pure sample and 10% adulterant sample with the help of SCAR but in the case of RT-SCAR difference was found in the DNA sample. This analytical strategy could be used further for large scale quantification of *C. roseus*'s market samples.

Key words: *Catharanthus roseus,* RAPD, Sequence-Characterized-Amplified-Region (SCAR) Marker, Real Time Sequence-Characterized-Amplified-Region (RT-SCAR) Marker, Real Time PCR.

INTRODUCTION

Identification of medicinal plants has been traditionally carried out by morphological and agronomic traits. However, morphological and agronomic traits identification is carried with fresh materials. It is difficult to identify the dried material, which is generally available in the market. Polymerase chain reaction (PCR)-based genetic markers are widely used for molecular detection, genome mapping, map-based cloning, and analysis of genetic variation of biological sample. These marker systems take advantage of the analyses of random amplified polymorphic DNA (RAPD) (Williams et al., 1990), simple sequence repeats (SSRs) (Tautz, 1989;

*Corresponding author. E-mail: anis.chaudhary@gmail.com, anis_ahm2003@yahoo.co.in

Table 1. List of *Catharanthus roseus* (L.) G. Don.

Code	Accession	Source
A1	IC 49,595	NBPGR, New Delhi
A2	EC 415,024	NBPGR, New Delhi
A3	EC 49,580	NBPGR, New Delhi
A4	IC 210,607	NBPGR, New Delhi
A5	EC 120,837	NBPGR, New Delhi
A6	IC 49,581	NBPGR, New Delhi

Brown and Tanksley, 1996), inter simple sequence repeats-PCR (ISSR-PCR) (Zeitkeinicz et al., 1994), and amplified fragment length polymorphism (AFLP) (Vos et al., 1995). Since the first reports of RAPD markers by Williams et al. (1990) this method has been widely used. RAPDs are polymorphic DNA sequences that can be amplified using PCR, the resultant products can be separated as discrete bands when subjected to electrophoresis. Although RAPD markers are usually dominant markers, they are sensitive to minor changes in reaction conditions during PCR amplification, which can result in irreproducible results. To improve the reliability of RAPDs and to convert them to codominant markers, Paran and Michelmore (1993) developed a technique known as sequence-characterized amplified regions (SCAR). SCARs are based on sequencing the polymorphic fragment derived from RAPD primers and designing longer primers that will specifically bind to this fragment. SCAR markers are more advantageous than RAPD markers because they usually detect only a single locus and are, therefore, more specific but these all are qualitative not quantitative therefore we could not analyze how much quantity are present. Quantification of nucleic acids using the PCR has been significantly simplified by the development of the real-time PCR technique, it's becoming a common tool for detecting and quantifying expression profiles of selected genes. The advent of real-time PCR has dramatically changed the field of measuring gene expression. Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity. There are many benefits of using real-time PCR over other methods to quantify target DNA. It can produce quantitative data with an accurate dynamic range of 7 to 8 log orders of magnitude and does not require post-amplification manipulation. *Catharanthus roseus* L. Don is one of the most extensively investigated medicinal plants. It has more than 400 known alkaloids, some of which are approved as antineoplastic agents to treat leukemia, Hodgkin's disease, malignant lymphomas, neuroblastoma, rhabdomyosarcoma, Wilms' tumor, (antineoplastic medicines) however monoindole alkaloids ajmalicine and serpentine are antihypertension drugs (Zhao and Verpoorte, 2007; Fischhof et al., 1996; Hindmarch et al., 1991; Atta-Ur-Alrahman et al., 1983, 1984, 1985, 1988; Atta-ur-Rahman and Fatima, 1984; Auriola et al., 1990). Vinblastine sulphate is used commercially for the treatment of neoplasma and is recommended for generalized Hodgkin's disease and resistant choiocarcenoma. The plant has been early used in treatment of diabetes, hypertension, tuberculosis, laryngitis, sore throat, dyspepsia, malaria, and to regulate menstruation (Moreno et al., 1993; Soo-Young Lee et al., 2003). The major alkaloid is vincamine and its closely related semi-synthetic derivative widely used as a medicinal agent, known as ethyl-apovincaminate or vinpocetine, has vasodilating, blood thinning, hypoglycemic and memory-enhancing actions.

MATERIALS AND METHODS

Plant material

Germplasms of *C. roseus* were obtained from different geographical regions of India (Table 1). The seed accessions were grown in herbal garden of Hamdard University, New Delhi, India. Leaves of four-month-old plants were collected for research purpose.

DNA extraction and purification

Fresh samples were subsequently processed for DNA extraction. Genomic DNA was extracted from young leaves of *C. roseus* by using the CTAB (6-Cetyl Trimethyl Ammonium Bromide) method with some modification from Doyle and Doyle (1990). 400 mg leaf tissue was ground in liquid nitrogen with the help of motel pestle. The leaf power was transferred in 15 ml centrifuge tube and mixed with 5 ml of extraction buffer 1 (100 mM Tris-HCl pH 8.0, 1% CTAB, 1.4 M NaCl, 20 mM EDTA) and centrifuge at 5000xg for 5 min. Aqueous layer was then collected and double volume of extraction buffer 2 (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20mM EDTA, 2% CTAB) was added. The suspension was mixed gently and incubated at 60ºC for 30 min with occasional mixing. The suspension was cooled to room temperature and an equal volume of chloroform:isoamyl alcohol (24:1) was added. The mixture was centrifuged at 5000xg for 15 min. The clear upper aqueous phase was then transferred to a new tube, and 2/3 volume of ice-cooled isopropanol was added. Incubation was done at -20ºC for 30 min. The nucleic acid was collected by centrifuging at 1000xg for 10 min. The resulting pellet was washed twice with 80% ethanol and air-dried under a sterile laminar hood, and the nucleic acid was dissolved in TE (10 mM Tris buffer pH 8.0, 1 mM EDTA) at room temperature. The contaminating RNA was eliminated by treating the sample with RNase A (10 mg/ml) for 30 min at 37ºC. DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260 nm and 280 nm. The quality of the DNA was determined using agarose gel electrophoresis stained with ethidium bromide.

RAPD reaction

The RAPD reaction was performed according to the method developed by McClelland et al. (1995). The reactions were carried out in 20 µL volume in a tube using twenty five random primers, OPAA-1, OPAA-2, OPAA-3, OPAA-4, OPAA-5, OPAA-6, OPAA-7,

Table 2. Nucleotide sequences of selected primers with the number of amplified products and fragment size range (bp).

Primer code	Sequence 5' – 3'	No. of amplification product	Fragment size (bp)
OPAA -01	AGACGGCTCC	55	900-113
OPA A-02	GAGACCAGAC	45	969-189
OPA A-03	TTAGCGCCCC	42	1,068.89-109
OPAA -04	AGGACTGCTC	60	988-100
OPAA -05	GGCTTTAGCC	38	977-135
OPAA -06	TCAAGCTAAC	92	1,210-180
OPAA -07	CTACGCTCAC	39	10,114-116
OPAA -08	TCCGCAGTAG	76	938-106
OPAA -09	AGATGGGCAG	48	947-105
OPAA -10	TGGTCGGGTG	68	1,154-153
OPAA -11	ACCCGACCTG	38	995-175
OPAA -12	GGACCTCTTG	40	885-128
OPAA -13	GAGCGTCGCT	53	954-225
OPAA -14	AACGGGCCAA	47	964-328
OPAA -15	ACGGAAGCCC	70	998-115
OPAA -16	GGAACCCACA	80	1010-141
OPAA -17	GAGCCCGACT	77	998-116
OPAA -18	TGGTCCAGCC	57	895-281
OPAA -19	TGAGGCGTGT	29	997-135
OPAA -20	TTGCCTTCGG	Nil	Nil
BG-25	AAGCCTCGTC	112	975-186
BG-26	TGCGTGCTTG	87	1163-313
BG-27	GACGGATCAG	25	570-283
BG-28	CACACTCCAG	Nil	Nil
BG29	TGAGTGGGTG	13	476-383
Total		**1291**	

OPAA-8, OPAA-9, OPAA-10, OPAA-11, OPAA-12, OPAA-13, OPAA-14, OPAA-15, OPAA-16, OPAA-17, OPAA-18, OPAA-19, OPAA-20 (Operon Technologies Inc., USA), Bg26, Bg27, Bg28, Bg29 and Bg30 (Genei, India) (Table 2). Each reaction tube contained 50 ng templates DNA, 1.5 mM $MgCl_2$, 300 µM dNTPs, 2 µL of 10x*Taq* DNA polymerase buffer, 20 pM oligonucleotide primer and 0.5 units of *Taq* DNA polymerase (Genei, India). Amplification was performed in a DNA thermal cycler (Ependroff, USA), using the following conditions : 95ºC for 3 min; 40 cycles at 94ºC for 30 s, 50ºC for 1 min and 72ºC for 1 min; final extension at 72ºC for 5 min. PCR products were resolved on 1.2% agarose gel in 1xTAE buffer, agarose gel containing 0.5 µg/ml ethidium bromide visualised under UV light and photographed using gel documentation system (Image Master VDS, Pharmacia, USA).

Cloning and sequencing of 610 bp band

Polymorphic band of 610 bp was excised from gels and eluted using a Gel Extraction Kit (QIAGEN, Germany). The eluted DNA was cloned into pGEM®-T easy vector (Promega, USA) following the manufacturer's instruction. The ligated plasmid was introduced into *Escherichia coli* strain $DH_{5α}$, following the protocols for preparing competent cells and transformation using the calcium chloride method (Sambrook and Russell 2001). White colonies were picked from LB-X-gal plates and grown overnight in LB medium containing ampicilin. The plasmid DNA was isolated from the bacterial culture using plasmid isolation kit (QIAGEN, Germany). The inserted fragments were sequenced at The Center for Genomic Application, New Delhi, India with T7 primer.

SCAR analysis

Based on the sequence of the cloned fragments, we designed the pairs of gene-specific PCR primers cathF gatatgggttttgctaact and cathR taatacttcaggtcatccca reported. PCR reactions were carried out in a final volume of 25 µl, contained 10X PCR buffer [20 mM Tris (pH 8), 50 mM KCl and 2.5 mM $MgCl_2$], 15 mM $MgCl_2$, 300 µM dNTPs, 0.3 µM oligonucleotide each primers (Forward and Reveres Primer), 0.5 U of *Taq* DNA polymerase (Genei, India) and 50 ng templates DNA. DNA was replaced by sterile water in the negative control. Amplification was performed in a DNA thermal cycler (Ependroff, USA), using the following conditions : 95ºC for 5 min; 40 cycles at 95ºC for 30 s, 60ºC for 1 min and 72ºC for 1 min; final extension at 72ºC for 5 min. PCR products were resolved on 1.2% agarose gel in 1x TBE buffer, agarose gel containing 0.5 µg/ml ethidium bromide visualised under UV light and photographed using gel documentation system (Image Master VDS, Pharmacia, USA).

Real-time SCAR analysis

Real time PCR reactions were carried out with Light Cycler 480 (Roche, Germany) using the SYBR® Green. Reaction conditions were 10X PCR buffer [20 mM Tris (pH 8), 50 mM KCl and 2.5 mM

Table 3. Comparison between SCAR and RT-SCAR marker. Sample CR1 was 100% pure and sample CR2-CR6 were 1% serial diluted. Sample SR7 and CR8 were 10% diluted.

Sample no.	Sample dilution	Variation	
		SCAR	RT-SCAR
CR1	100:0	No	1.16 X 10^9
CR2	99:1	No	7.33 X 10^8
CR3	98:2	No	6.06 X 10^8
CR4	97:3	No	3.61 X 10^8
CR5	96:4	No	2.95 X 10^8
CR6	95:5	No	2.81 X 10^8
CR7	90:10	No	2.32 X 10^7
CR8	90:10	No	2.34 X 10^7

$MgCl_2$], 0.3 µM each primers (Forward and Reveres Primer) gene-specific PCR primers as same in SCAR analysis cathF gatatgggttttgctaact and cathR taatacttcaggtcatccca, 15 mM $MgCl_2$, 300 µM dNTPs, 1:1000 SYBR Green I, 0.5 U *Taq* DNA polymerase (recombinant), and 50 ng templates DNA in a 25 µl reaction volume. DNA was replaced by sterile water in the negative control. The program used for real time PCR was 5 min at 95°C, followed by 40 cycles of 30 s denaturation at 95°C, 60 s annealing at 60°C, 60 s elongation at 72°C. Fluorescence was detected at the end of the 65°C segment in the PCR step.

Melting curve analysis

After 40 amplification cycles, the purity of the PCR product was checked by the observation of the correct product by its specific melting temperature (Tm). The thermal profile for melting curve analysis consisted of a denaturation for 1 min at 95°C, lowered to 55°C for 30 s and then increased to 95°C with continuous fluorescence readings.

RESULTS

RAPD method was performed in search for DNA polymorphisms, which can be used for generating informative SCAR and RT-SCAR marker. Twenty five RAPD primers were used in this study. Only the fragments confirmed by repeated amplifications were considered useful for RT-SCAR markers. Preliminary screening of 25 random decanucleotide primers showed that 23 primers were able to prime genomic DNA of *C. roseus*, and resulted in amplified PCR products of a variable number of DNA bands (13-112 bands per primer). A total of 1291 DNA bands were obtained (Table 1). Amplification of *C. roseus* species with OPAA9, OPAA7, OPAA17 and OPAA19 produced good quality, reproducible fingerprint patterns and showed a high level of consistency of fingerprints among samples of the same species collected from different localities. Several specific RAPD fragments of high intensity and reproducibility were eluted cloned and sequenced (Figure 1). Nucleotide sequence of 610 bp RAPD amplicon, specific for all the six accessions of *C. roseus* (Figure 2), was used for designing primers for SCAR reaction and same primer use in RT-SCAR by using SYBR Green as detection system.

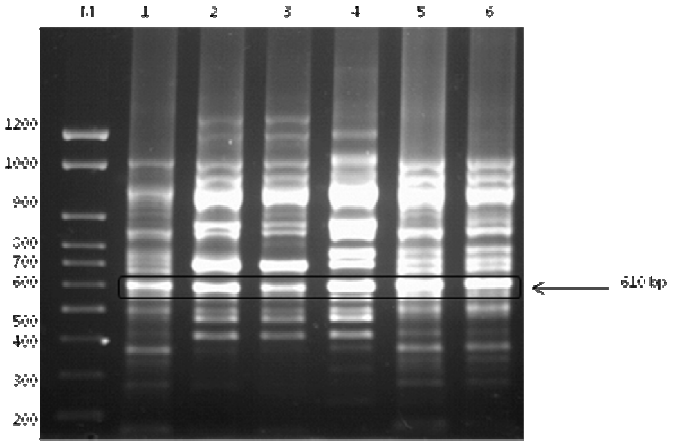

Figure 1. RAPD electrophoresis profile of 6 accessions of *C. roseus* amplified with the 10-mer primer **OPA-6.** Lanes 1–6 correspond to the 6 accessions listed in Table; Lane M, molecular marker 100 bp (inno train German). The numbers on the left of the figure indicate the DNA size markers in kilobases (kb).

A DNA marker-based method has been developed for identifying *C. roseus*, a putative 610 bp amplicon specific to *C. roseus* was identified using the RAPD technique. Further, the RAPD amplicon was converted to a sequence characterized amplified region (SCAR) and Real Time sequence characterized amplified region (RT-SCAR) marker. PCR using designed SCAR primers revealed the expected amplicon (290 bp) only in *C. roseus* (Figure 3, Table 3) and not in the other species, thus aiding 10% its substitute and adulterants at time of DNA isolation in distinguishing the authentic *C. roseus* and find out no difference in pure sample and 10% adulterant sample with the help of SCAR (Table 2) but in the case of RT-SCAR difference were found out in DNA sample (Figure 4).

DISCUSSION

In general, the characteristic profiles obtained by RAPD technique can potentially be utilized for detection, but RAPD is prone to poor reproducibility. This technique must be performed under very strictly controlled conditions. For the detection of target microorganisms, RAPD markers are normally converted into SCAR markers, because SCAR markers are more specific as they target a known sequence (Pujol et al., 2005). The SCAR technique was first applied to the identification of the downy mildew resistance genes in lettuce (Paran and Michelmore 1993), and more recently to the biological control agents *Gliocladium catenulatum* (Paavanen-Huhtala et al., 2000) and *Trichoha atroviride* (Rosa-Hermosa et al., 2001). SCAR markers have been

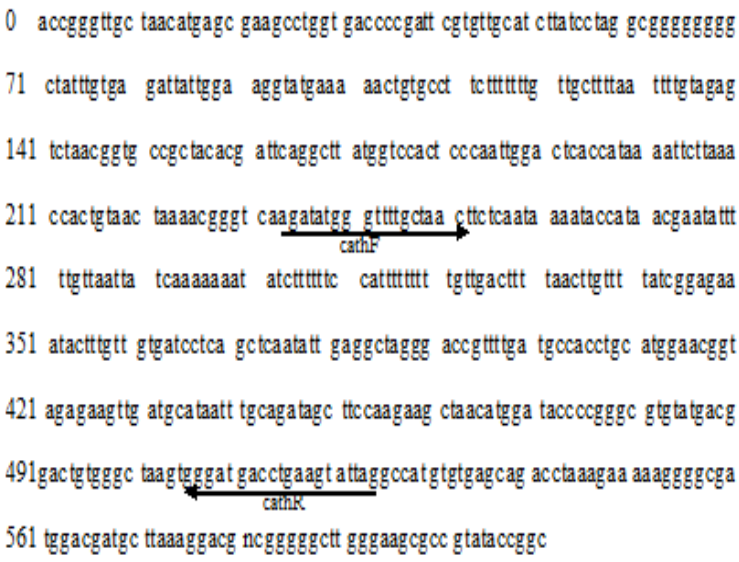

Figure 2. Nucleotide sequence of RAPD amplicon (610 bp) of *C. roseus* used for development if SCAR and RT-SCAR marker.

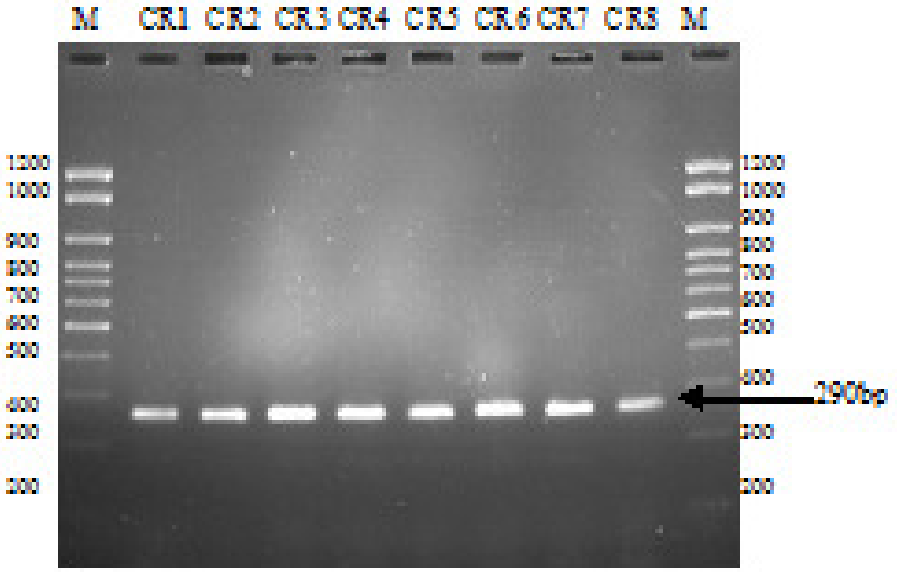

Figure 3. SCAR electrophoresis profile of 6 accessions of *C. roseus* amplified with the SCAR primer. Lanes IP1–IP2 10% impure sample and lane P1- P6 pure sample of *C. reseus* correspond to the 6 accessions, Lane M, molecular marker 100 bp (inno train German). The numbers on the left of the figure indicate the DNA size markers in (kb).

successfully developed for the detection of gut contents from the tobacco budworrn, *Helicovmpa amzigera* (Agusti et al., 1999) and the whitefly, *Trialeurodes vaporariorum* (Agusti et al., 2000). SCAR markers have also been used to differentiate between different strains of the Asian rice gall midge, *Orseolia oryzae* (Behura et al. 1999), and to distinguish the Asian from the North American gypsy moth, *Lymantria dispar* (Garner and Slavicek 1996). Similarly, SCARS have been developed for tagging genes in plants that confer resistance against insects, fungi, and nematodes (Paran and Michelmore, 1993; Williamson et al., 1994; Nair et al., 1995, 1996) but SCAR marker gave result in qualitative not in quantitative. Real-Time PCR is now a common method for measuring gene expression, it is increasingly important for users to be aware of the numerous choices available in all aspects of this technology. With a well-designed experiment per-formed with the proper controls, real-time PCR can be

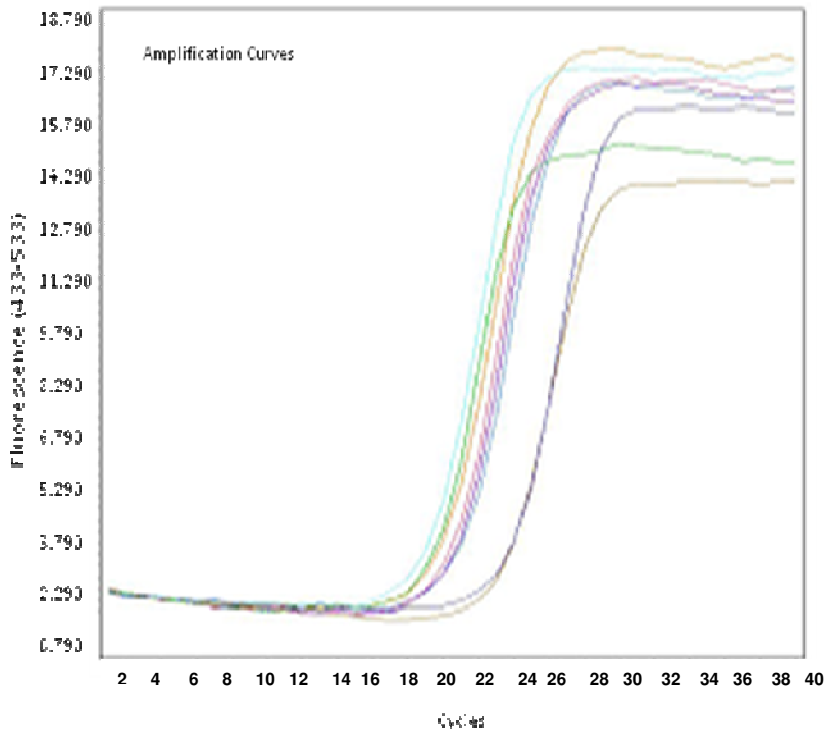

Figure 4. Amplification curves of RT-SCAR marker.

one of the most sensitive, efficient, fast, and reproducible methods of measuring gene expression. Additionally, accelerated PCR thermocycling and detection of amplified product permits the provision of a test result much sooner for real-time PCR than for conventional PCR. The combination of excellent sensitivity and specificity, low contamination risk, ease of performance and speed, has made real-time PCR technology an appealing alternative to conventional PCR method.

In this study, we find out SCAR marker was unable to distinguish in pure sample and 10% adulterant sample of *C. roseus* but in case of RT-SCAR it's very sensitive and able to distinguish in pure and 10% impure sample of *C. roseus*. A developed primer pair cathF and cathR was used to amplify the SCAR and RT-SCAR marker of *C. roseus* in this work with same pair of primers. The strategy of RT-SCAR marker had overcome the qualitative result of the SCAR marker successfully in this trial. Newly developed RT-SCAR marker was utilized to show the quantity of *C. roseus* in raw herbal medicine, but SCAR marker was unable to quantify target herbs. Authentication of medicinal raw drug identity and quantity using molecular markers holds great promise in resolving issues of controversial identity and quality control, but has not yet been capitalized by the traditional medicine sector.

ABBREVIATION

RAPD, Random amplified polymorphic DNA; **RT-SCAR,** real time sequence-characterized-amplified-region; **SCAR,** sequence-characterized-amplified-region; **SSRs,** simple sequence repeats; **ISSR-PCR**, inter simple sequence repeats-PCR; **AFLP,** amplified fragment length polymorphism; **CTAB,** 6-Cetyl trimethyl ammonium bromide.

REFERENCES

Agusti N, De Vicent MC, Gabarra R (1999). Development of sequence amplified characterized regions (SCAR) markers of Helicoverpa amrigera: a new polymerase chain reaction-based technique for predator gut analysis. Mol. Ecol. 8:1467-1474.

Agusti N, De Vicent MC, Gabarra R (2000). Developing SCAR markers to study predation on *Trialeurodes vaporariorum*. Insect Mol. Biol. 9:263-268.

Atta-ur-Rahman, Fatima J (1984). Isolation and structure of rosicine from *C. roseus*. Tetrahedron letters 25:6051-6054.

Atta-ur-Rahman IA, Chaudry (1985). Isolation and 13C-NMR studies on cathovaline on alkaloid from leaves of *C. roseus*. Planta Med. 5:447-448.

Atta-ur-Rahman, I. Ali, Bashir M (1984). Isolation of rhazinal from the leaves of *C. roseus*. J. Nat. Products 47:389.

Atta-ur-Rahman, Alam M, Ali I, Habib-ur-Rahman, Haq I (1988). Leurosinone: a new binary indol alkaloid from *C. roseus*. Perkin-Trans. 8:2175-2178.

Atta-ur-Rahman, Bashir M, Hafeez M, Perveen M, Fatima J (1983). 16-Epi-19-s-vindlinine, an indoline alkaloids from *C. rouses*. Planta Med. 47:246-247.

Auriola S, Naaranlahti T, Kostianen R, Lapinjaki SP (1990). Identification of indol alkaloids of *C. roseus* with liquid chromatography/mass spectrometry using collision-induced dissociation with the thermospray ion repeller. Biomed. Environ. Mass spectrum 19:400-404.

Behura SKI, Sahu SC, Rajamani S, Devi A, Mago R, Nair ST, Mohan M (1999). Differentiation of Asian rice gall midge, Orseolia oryzae (Wood-Mason), biotypes by sequence characterized amplified regions (SCARS). Insect Mol. Biol. 8:391-397.

Brown PI, Tanksley SD (1996). Characterization and genetic mapping of simple sequence repeats (SSR) in the tomato genome. Mol. Gen. Genet. 250:39-49.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Fischhof PK, Moslinger-Gehmayr R, Herrmann WM, Friedmann A, Russmann DL (1996). Theraupetic efficacy of Vincamine in dementia. Neuropsychobiology 34(1):29-35.

Gamer KJ, Slavicek JM (1996). Identification and characterization of a RAPD-PCR marker for distinguishing Asian and North American gypsy moths. Insect Mol. Biol. 5:81-91.

Hindmarch I, Fuchs HH, Erzigkeit H (1991). Efficacy and tolerance of vinpocetine in ambulant patients suffering from mild to moderate organic psychosyndromes. Int. Clin. Psychopharmacol. 6(1)**:**31-43.

Moreno PRH, van der Heijden R, Verpoorte R (1995). Cell and tissue cultures of *Catharanthus roseus:* A literature survey. II. Updating from 1988 to 1993 Plant Cell Tiss. Organ. Cult. 42:1–25.

Nair S, Bentur IS, Prasad Rao U, Mohan M (1995). DNA markers tightly linked to a gall midge resistance gene *(GM2)* are potentially useful for marker-aided selection in rice breeding. Theory Appl. Genet. 91:68-73.

Nair S, Kumar A, Srivastava MN, Mohan M (1996). PCR-based DNA markers tightly linked to a gall midge resistance gene Grn4t, has potential for marker-aided selection in rice. Theory Appl. Genet. 92:60-65.

Paavanen-Huhtala St Avikainen H, Yli-Mattila T (2000). Development of strain-specific primers for a strain of Glio- *cladium catmulaturn* used in biological control. Eur. J. Plant. Path. 106:187-198.

Paran I, Michelmore RW (1993). Development of reliable PCR based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genet. 85:985-933.

Pujol M, Badosa E, Cabrefiga J, Montesinos E (2005). Development of a strain-specific quantitative method for monitoring Pseudomonas fluorescens EPS62e, a novel biocontrol agent of fire blight. FEMS Microbiol. Lett. 249:343-352.

Rosa-Hermosa MI, Grondona I, Diaz-Minguez JM, Iturriaga EA, Monte E (2001). Development of a strain-specific SCAR marker for the detection of *Trichodema atroviride* 11, a biological control agent against soilborne fungal plant pathogens. Curr. Genet. 38:343-350.

Sambrook J, Russell DW (2001). Molecular Cloning: a Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory Press; New York.

Soo-Young L, Pil-Son C, Hwa-Jee C, Dong-Soo I, Dong-Woog C, Jang RL (2003). Comparison of adventitious shoot formation in petiole explant cultures of 20 cultivars of *C. roseus*. J. Plant Biotechnol. 5:59-61.

Tautz D (1989). Hypervariabiliry of simple sequences as a general source of polymorphic DNA markers. Nucleic Acids Res. 17:6463-6471.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995). AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 11:4407-4414.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.

Williamson VM, Ho JY, Wu FF, Miller N, Kaloshian I (1994). A PCR-based marker tightly linked to the nematode resistance gene, *Mi* in tomato. Theor. Appl. Genet. 87:757-763.

Zeitkeinicz E, Rafalksi A, Labuda D (1994). Genome fingerprinting by simple sequence repeats (SSR) anchored polymerase chain reaction amplification. Genomics 20:176-183.

Zhao J, Verpoorte R (2007). Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: from biochemical processing to metabolic engineering. Phytochem. Rev. 6:435-457.