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# Development of a species-specific sequence-characterized amplified region marker for roses

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**ABSTRACT.** DNA fingerprints of four rose species, *Rosa centifolia*, *R. Gruss-an-Teplitz*, *R. bourboniana*, and *R. damascena*, were developed using RAPD-PCR. We identified a unique polymorphic band in *R. centifolia*. This 762-bp fragment was produced by the random primer GLI-2. The fragment was eluted and directly cloned in a TA cloning vector, pTZ57R/T. Digestion of the plasmid with *Eco*RI confirmed the cloning of GLI-$2_{762}$ in pTZ57R/T. A second enzyme, *Pst*I, used in combination with *Eco*RI, gave complete digestion of the plasmid, and the 762-bp fragment was confirmed on the gel. Subsequently, the polymorphic amplicon was sequenced with an AB1 373 DNA sequencer system using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit. After sequencing, specific primers (23 bp long) were designed based on the sequence of the flanking regions of the original RAPD fragment. These primers will effectively allow fingerprinting for the identification of *R. centifolia* species. In essence, we developed an SCAR marker to authenticate the identity of *R. centifolia* species and to distinguish it from its substitutes. Such techniques are required not only to complement conventional parameters in creating the passport data of commercial and medicinal

 

products of rose, but also for routine quality control in commercial and government rosaries and rose nurseries.

**Key words:** Cultivar discrimination; DNA marker; *Rosa*; SCAR; RAPD

## INTRODUCTION

Rose is one of the most popular crops in the floriculture industry. The genus *Rosa* includes 200 species and 18,000 cultivars (Gudin, 2000). It is further classified into four subgenera, Eurosa, Platyrhodon, Hesperhodos, and Hulthemia, distributed widely throughout the northern hemisphere (Rehder, 1940) The genetic resources of roses can be grouped into four categories: exotic varieties, indigenously developed varieties, native rose species, and exotic species.

The cultivation of roses for many purposes has been widespread throughout the world. There are about 240-300 recognized rose species, of which only 10-20 have contributed to the development of modern rose cultivars. Rose breeding is mainly carried out by highly competitive private companies and their applied genetic knowledge is proprietary and unpublished (de Varies and Dubois, 1996). Documentation in many rosaries and rose collections indicates numerous problems of misidentification, mislabeling and incomplete denomination of taxa.

The identification of rose cultivars or species has relied on morphological characters such as growth habit, floral morphology, color, etc. (Mohapatra and Rout, 2005). However, the morphological assessment of biodiversity in the genus *Rosa* is complicated due to large number of natural as well as man-made crosses. Since many of these traits are difficult, reliable fingerprinting methods have found a particular fit in the estimation of genetic divergence of Roses. Despite its high economic importance, little is known about rose genetics, genome structure, and function of rose genes. Reasons for this lack of information are polyploid cultivars, simple breeding strategies, high turnover rates for cultivars, and little public funding. During the last three decades, different strategies for the evaluation of genetic variability, such as comparative anatomy, morphology and physiology, have increasingly been complemented by molecular techniques (Weising et al., 2005). Various molecular markers such as RAPD (random amplified polymorphic DNA; Sadia, 2007; Kiani et al., 2008), SSR (simple sequence repeats; Esselink et al., 2003), and AFLP (amplified fragment length polymorphism; Wen and Pang, 2004) have been used for the analysis of *Rosa* species biodiversity in different regions. Furthermore, DNA markers have also been used for hybrid identification (Kaul et al., 2009) and the development of genetic linkage maps in roses (Rajapakse, 2003).

RAPD is frequently used for genetic diversity analysis of plants, owing to its simplicity, low cost and lower infrastructure requirements (Upadhya et al., 2004). However, the fragment polymorphisms used in RAPD markers are not always reproducible (Masuzaki et al., 2007; Shimada et al., 2008). This limitation can be overcome by converting RAPDs into codominant sequence-characterized amplified region (SCAR) markers. SCARs have been developed for several crops, including apple (Boudichevskaia et al., 2006), papaya (Deputy et al., 2002), cowpea (Boukar et al., 2004), brassica (Piquemal et al., 2005), and strawberry (Rugienius et al., 2006). However, the literature on the synthesis of SCAR markers in rose is extremely scanty, with only one report so far (Yan et al., 2005), utilizing AFLP markers. There is, however, no report available on the development of SCARs from RAPDs in rose. Hence, the present investigation is a pioneering attempt at synthesizing SCARs from RAPD markers in rose.

 

## MATERIAL AND METHODS

### Plant material

The four *Rosa* species, *R. Gruss-an-Teplitz*, *R. bourboniana*, *R. centifolia*, and *R. damascena*, used in the study were selected from the University Flora, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan.

### DNA extraction

Total cellular DNA of rose leaves was extracted using the modified CTAB method described by Khan et al. (2004). Fresh leaf samples (8-10) were collected from random plants of each species. The leaves were immediately stored at -40°C for molecular studies. Quantification of DNA was performed both spectrophotometrically and electrophoretically.

To produce the *Rosa* cultivar-specific SCAR primers presented in this paper, the unique band of interest from the less reliable RAPD analysis was selected, isolated, and used to design a candidate SCAR primer.

### RAPD analysis

Four series of RAPD primers (A, C, K, I) from Fermentas (Vinius, Lithuania) were used for RAPD analysis, and 13 polymorphic primers were selected to amplify the genomic DNA of rose germplasm (Table 1).

**Table 1.** RAPD primers and their sequences used in the study.

| Sr. No. | Primer | Sequence |
|---|---|---|
| 1 | GL Decamer I-02 | GGAGGAGAGG |
| 2 | GL Decamer I-03 | CAGAAGCCCA |
| 3 | GL Decamer I-06 | AAGGCGGCAG |
| 4 | GL Decamer I-07 | CAGCGACAAG |
| 5 | GL Decamer K-05 | TCTGTCGAGG |
| 6 | GL Decamer K-07 | AGCGAGCAAG |
| 7 | GL Decamer K-13 | GGTTGTACCC |
| 8 | GL Decamer A-07 | GAAACGGGTG |
| 9 | GL Decamer A-10 | GTGATCGCAG |
| 10 | GL Decamer A-16 | AGCCAGCGAA |
| 11 | GL Decamer C-02 | GTGAGGCGTC |
| 12 | GL Decamer C-03 | GGGGGTCTTT |
| 13 | GL Decamer C-15 | GACGGATCAG |

Polymerase chain reaction (PCR) was performed by adding 2.5 µL template DNA, 0.2 µL *Taq* DNA polymerase (MBI, Fermentas, Vinius, Lithuania), 2.5 µL 10X buffer, 2.5 µL gelatin, 3.0 µL $MgCl_2$, 4.0 µL dNTPs, 2.0 µL primers, and 8.3 µL $d_3H_2O$. A reaction volume of 25 µL was used; the reaction mixture was prepared while kept on ice. The PCR was performed in a thermocycler (AG No. 533300839; Germany) using the following reaction conditions: hot start at 95°C for 5 min, denaturation at 95°C for 1 min, primer annealing at 34°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR products were resolved along with a λ-DNA marker (Fermentas) on a 1.2% agarose gel. The DNA was stained

with ethidium bromide and photographed under UV transilluminator. All 32 primers used for screening produced distinct reproducible amplification profiles for the screened DNAs of *R. Gruss-an-Teplitz* (*Surkha*), *R. bourboniana*, *R. centifolia*, and *R. damascena*. Among these, DNA marker GLI-2 revealed a polymorphic band only for *R. centifolia* species and was selected. This 762-bp polymorphic band was gel-purified following manufacturer instructions (QIAquick Gel Extraction kit, Qiagen, USA).

### Cloning of GLI-2

The isolated fragment was cloned into a T vector (pTZ57R/T, MBI Fermentas) and transformed into competent *Escherichia coli* 10b cells. Plasmid DNA from the recombinants was purified followed by a restriction digest with *Eco*RI and *Pst*I to recover the insert DNA.

### DNA sequencing

The complete nucleotide sequences of GLI-2 were determined by dideoxynucleotide chain termination sequencing using the PCR-based BIG DYE kit (Perkin Elmer Cetus) and specific internal primers (Genosys). Reaction products were resolved on an ABI 370 automated sequencer. Sequence information was stored, assembled and analyzed, using version 7 of the Genetics Computer Group program library (Devereaux et al., 1984).

### Designing the SCAR marker

Once the complete nucleotide sequence of the *R. centifolia*-specific 762-bp fragment was obtained, one forward and one reverse primer were designed using the Primer3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi).

## RESULTS

### *R. centifolia*-specific RAPD marker

Of the 13 markers screened, one RAPD marker, GLI-2 (5'-GGAGGAGAGG-3'), consistently amplified a polymorphic fragment of 762 bp in *R. centifolia*, which was absent in the three other rose species (Figure 1). The RAPD fragment was easily re-amplified and confirmed. The PCR product was separated on a 1% agarose gel and the 762-bp band was identified and excised from it.

### Elution and cloning of the fragment

The fragment was eluted from the gel using a QIAGEN kit, and 2 μL of the eluted mixture was electrophoresed on a 0.8% agarose gel. Appearance of the single band of 762 bp showed the successful elution of the RAPD fragment. The eluted fragment was directly cloned into a TA cloning vector pTZ57R/T. The digestion of the plasmid with *Eco*RI confirmed the cloning of GLI-$2_{762}$ in pTZ57R/T. The second enzyme *Pst*I used in combination with *Eco*RI gave complete digestion of the plasmid, and the 762-bp fragment was confirmed on the gel (Figure 2). All 15 digested plasmids were run on the gel and all showed the complete digestion of the fragment.

 

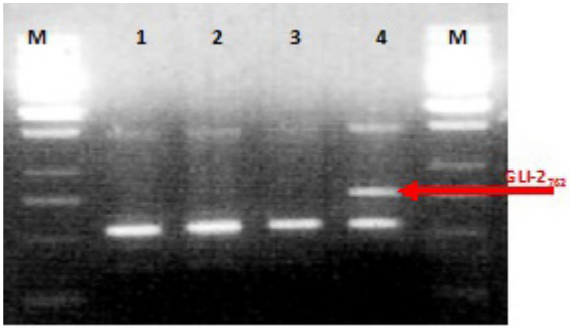


**Figure 1.** RAPD-PCR of four *Rosa* species with primer GLI-2. *Lane M* = 1-kb ladder. *Lanes 1-4* = DNA from *Rosa bourboniana*, *R. Gruss-an-Teplitz*, *R. damascena*, and *R. centifolia*, respectively.

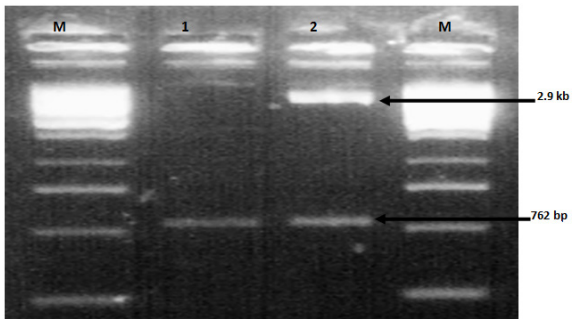


**Figure 2.** Restriction digestion with *Eco*R1 and *Pst*1. *Lane M* = 1-kb ladder. *Lane 1* = Control PCR product. *Lane 2* = Cloned fragment (762 bp).

## Sequencing of fragment GLI-$2_{762}$

The clone was then end sequenced to approximately 762 bp with an AB1 373 DNA sequencer system using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit (Figure 3). The standard PUC primers were used for the M13 binding sites, which were present in the vector. Sequencing was done in both the forward and reverse direction to make it more reliable. As the fragment size was approximately 762 bp, after sequencing 500 bp, it was subcloned into another vector, and specific primers were designed to sequence the rest of the fragment.

## Primer designing

After sequencing the DNA marker, the specific primers (forward and reverse) were designed based on the sequence of the flanking regions of the original RAPD fragment. These primers were designed so that the annealing temperature was in the range of 55°-60°C in the genomic regions and gave single locus amplification (Rugienius et al., 2006). Right primer: 5'-CCAGAAGAGGTTACGCACAA-3'; left primer: 5'-GCAACCTCTCGTCCTCATCAAGG-3'.

GCAACCTCTCGTCCTCATCAAGGTTTCAAGCATACGGAAAATTCGTTTTCTTTGC
ATTTATTACGGCTTCTTATGTATGTGAACATTTTCCTTTTTCTCCGTAACCAATCG
AATCATTTACGATTAACCTCTTCTGGGATTCTTTTTGAGCGAATACGTTCTTTCTT
ACGAATCTCACGAGTATTATAATTGGAATACTCTTATTACTCCAAAAAAATCCAT
TTTTGCAAAAAGTAATCAAAGATTATTCTTGCTCCTATATAATTTTATGAAAAAA
TAAAATATCCTGTCGAAGAAGTCTTTGCTAACGATTTTCCGGCCACCTTATGGTT
CTTCAAGGATCCTTTTATACAGTATGTTAGATATCAAGGAAAATCGATTCTGGCA
TCAAAAGATACTCCTCTTCTGATGAATAAGTGGAAATATTATCTTGTCCATTTTTG
GCAATGTCATTTTTATGTATGGTCTCAACCAGGAAGAATCCATATAAACCAATTA
TCTAAGCATTCCTTTGATTTTTTGGGTTATCTTTCAAGCATACGACCGAATATTTC
AGTGGTACGGAGTCAATTGCTAGAAAATTCGTTTTTAATGGATAATGCTATGAAG
AAGCTTGATACATTATTTCCAATTATTCCAATGATAGGATCGTTGGCTAAAGTGA
AATTTTGTAACACATCAGGGCATCCTATTAGTAAGTCCAGCTGGGCGGATTCGTC
GGATTCTGATATTATCGACCGATTTGTGCGTAATGGAGAGCAG

**Figure 3.** Sequence of the fragment GLI-$2_{762}$.

## DISCUSSION

The manipulation of genetic material using conventional breeding methods is often difficult, time-consuming and sometimes ineffective. Developments in molecular genetics offer plant breeders a rapid precise alternative approach to conventional selection schemes for improving cultivars for yield, adaptability, pest resistance, etc. DNA markers are important tools for developing genetic linkage maps (Moretzsohn et al., 2000; Kohel et al., 2001). Molecular markers have already been used to identify wild rose species and cultivars, as well as determining relationships in roses. RAPDs are the most frequently used markers in rose (Kaur et al., 2007). The RAPD reaction is affected by a number of experimental conditions (Heun et al., 1994). Nevertheless, once optimized, the technique has the potential of providing an effective and convenient method to generate molecules that could be utilized in marker-aided selection of quantitatively inherited traits (Khan et al., 2005).

For better reproducibility, RAPD markers should preferentially be converted into SCARs (Rugienius et al., 2006). So far, there is only one report of SCAR marker development in rose from AFLP markers. The present study is therefore a pioneering attempt wherein an RAPD fragment GLI-$2_{762}$ was cloned to convert into SCARs. SCAR markers have many added advantages. First, the characteristics of SCAR markers make them favorable for MAS. In addition to fast identification shared by some other PCR-based markers, SCARs can be used to genotype individuals accurately. Second, SCAR marker eliminates the use of gel electrophoresis. A direct sequencing procedure was developed by Hernández et al. (2001) to sequence RAPD products to avoid cloning, which is an expensive, time-consuming and hectic process. The development of SCARs by direct sequencing of RAPD fragment is possible if the marker is flanked by two different oligonucleotides and re-amplifies the original fragment, and the PCR product should be highly pure.

In the present study, re-amplification of nontargeted bands was the main problem for the development of SCARs by direct sequencing of RAPD products. The appearance of low-weight DNA fragments might have been due to the presence of annealing sites in the eluted DNA. Hence, it indicates primer competition with the genomic DNA to anneal at specific sites (Williams et al., 1990). Furthermore, the additional DNA fragment may also appear due

to mismatch of random primer with the annealing site, which is very likely due to the poor specificity of the short 10-mer primers (Hernández et al., 1999).

Moreover, the PCR conditions for RAPD are very sensitive to the concentration of the reagents, template concentration, primer to template ratio, etc. A template DNA concentration higher than 45 ng/mL does not give consistent amplification (Rahman et al., 2002). Similarly, the concentration of $MgCl_2$ has a profound effect on DNA amplification. Generally excess $Mg^{2+}$ will result in the accumulation of non-specific amplification products and insufficient $Mg^{2+}$ will reduce the number of amplified bands (Saiki, 1989). $Mg^{2+}$ acts as a cofactor for *Taq* DNA polymerase. Free $Mg^{2+}$ concentration is affected by dNTPs and *Taq* concentrations (Ellsworth et al., 1993; Meunier and Grimont, 1993; Schierwater and Ender, 1993). Similarly, the optimum concentration of *Taq* DNA polymerase is another important element in PCR (Meunier and Grimont, 1993; Schierwater and Ender, 1993). The recommended range of *Taq* DNA polymerase varies from 0.5 to 2.5 U per 100-µL reaction. Higher *Taq* concentration, however, produced non-specific results. Annealing temperature is another important parameter for amplification of a DNA fragment (Rahman et al., 2002). In this study, by increasing the annealing temperature while maintaining the original PCR conditions, some of the bands of fewer base pairs disappeared, but the original fragment was still not observed. Hence, we used the amplified product directly to clone into the vector.

The RAPD marker in the present study was resolved and excised from the agarose gel. Similarly, Paran and Michelmore (1993) were able to separate RAPD fragments on an agarose gel, excise the bands from the gel and re-amplify the original RAPD primer. However, poor resolution of bands on agarose gel was also reported (Paran and Michelmore, 1993). In recent reports, the problem of the poor resolution could be overcome by separating fragments on a polyacrylamide gel (Summer et al., 2009).

In essence, we developed an SCAR marker to authenticate the identity of *R. centifolia* species and to distinguish it from its substitutes. Such techniques are required not only to complement conventional parameters in creating the passport data of commercial and medicinal products of rose, but also for routine quality control in commercial and government rosaries and rose nurseries.

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