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SHORT COMMUNICATION

Identification of RAPD markers linked to sex determination in guggal [*Commiphora wightii* (Arnott.)] Bhandari

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Abstract Decamer RAPD primers were tested on dioeceious and hermaphrodite plants of *Commiphora wightii* to identify sex-specific molecular markers. Sixty different random decamer primers were screened out of which only three primers were found to be associated with sex expression. A ~1,280-bp fragment from the primer OPN06 was found to be present in all the female individuals. Another primer OPN 16 produced a unique ~400-bp amplification product in only hermaphrodite individuals. The third marker, OPA20 amplified a ~1,140-bp fragment from female and hermaphrodite DNAs, but failed to do so from the male plant DNAs.

Keywords *Commiphora wightii* · Dioecious · Hermaphrodite · RAPD markers · Bulk segregant analysis

Introduction

Guggul [*Commiphora wightii* (Arnot.) Bhandari] belongs to the family Burseraceae and is a well-known drug plant. Its exudates are pharmacologically active in controlling rheumatoid arthritis, obesity and peptic ulcer (Atal et al. 1975), and its oleoresin also acts as diaphoretic expectorant, diuretic and emmenagogue. The pharmacological and clinical studies on its crude drug constituents and various extracts have revealed its significance in the treatment of anti-inflammatory, antirheumatic, hypocholesterolemic, hypolipidemic, and antifertility activities (Kakrani 1981). Moreover, a hypolipidemic drug from guggul gum resin containing guggul sterones along with other lipids, which show the efficacy of the drug and its various formulations, has been developed and marketed and is in use in the allopathic, Ayurvedic and Unani systems to combat human ailments (Kapoor et al. 1979; Nityanand and Kapoor 1971; Taj-ud-din et al. 1994).

Africa and Asia are the centers of origin of the genus *Commiphora* (Kumar and Shankar 1982), and *C. wightii* is distributed in the arid tracts of the Rajasthan and Gujarat states of India and the Sind and Baluchistan provinces of Pakistan (Atal et al. 1975). The species is a much-branched spinous shrub or a small tree which faces extinction due to faulty extraction methods employed by traditional resin collectors. As a result, there has been a significant decline in the number of guggul plants available for extraction of drug mainly due to the death of the mature plants. The entire demand of oleo–gum–resin in pharmaceutical industry has been met from tapping of wild growing plants for centuries. The research so far carried out on guggul was aimed at increasing the exudates from the plant which resulted in the death of the plant. The population of this species has been drastically reduced due to over-harvesting from natural habitat which led to it being listed in the Red Data Book (IUCN) as an over-exploited species in India (Billore 1989). Plants raised through seeds showed relatively robust growth as compared to plants raised from cuttings (Dalal and Patel 1995). Since dioecy and bisexual plants have been reported in *C. wightii* (Kumar and Shankar 1982; Rao et al. 1984), it is not possible to discriminate between them until flowering, which takes about 1–2 years. However, there are also no phenotypic

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characters available to discriminate the male, female, and hermaphrodite plants, and the inability to identify gender at early ages can create problems in advanced-generation breeding programmes, particularly when all superior parental selections or all progeny are unknowingly composed of one gender. Understanding the molecular basis of sex expression has immense importance both in basic and applied research. The evolution of sex in plants has been variously hypothesized as the plants display a great variety of sexual phenotypes (Tanurdzic and Banks 2004).

Since there is no method available to distinguish male, female, and hermaphrodite plants in the absence of flowering in *C. wightii*, molecular markers could be utilized to diagnose sex-linked DNA markers. RAPD markers have shown their reliability for determining sex in *Pistacia vera* (Hormaza et al. 1994; Kafkas et al. 2001), *Atriplex garrettii* (Claudete et al. 1998), *Trichosanthes diocia* (Singh et al. 2002), *Salix viminalis* (Alstrom-Rapaport et al. 1998), *Borassus flabellifer* (George et al. 2007), *Simmondsia chinensis* (Agrawal et al. 2007), *Carica papaya*, and *Cycas circinalis* (Gangopadhaya et al. 2007). In this communication, for the first time, we report the identification of RAPD markers associated with sex determination among male, female, and hermaphrodite in *C. wightii*.

Materials and methods

Plant material

Plants from each of five accessions of both male and female and three of hermaphrodite grown in the field gene bank of Directorate of Medicinal and Aromatic Plants Research (DMAPR), Boriavi, Anand, Gujarat, India, were used for determining sex-associated markers in DNA by RAPD analysis; these plants were collected from diverse locations of Gujarat. Immature leaf materials, collected from a random sample of adult pistilate, staminate, and hermaphrodite plants after complete observation of flower types, were used for DNA extraction.

Genomic DNA isolation

Total genomic DNA was isolated from 3 g of leaf tissues from 13 accessions (5 male, 5 female, 3 hermaphrodite) with the modified CTAB method (Samantaray et al. 2009). DNA was treated with RNaseA (Quiagen, USA) for eradication of RNA followed by two washings with chloroform:iso-amyl-alcohol (24:1; v/v). Subsequently, quality and quantity were checked by running the dissolved DNA in 0.8% agarose gel along side uncut λ DNA (Bangalore Genei, Bangalore, India) of known concentration. The DNA was diluted to 30 ng/μl for RAPD analysis.

Bulk segregant analysis

Three bulk samples were prepared separately by pooling an equal amount of DNA from individual plants of 5 male, 5 female, and 3 hermaphrodite accessions and amplified with 60 decamer primers. A DNA marker present in the corresponding male, female, and hermaphrodite bulks and absent in the alternate sex bulk was considered as a potential sex-linked markers. Bulked segregant analysis (BSA) (Michelmore et al. 1991) was used to screen each individual of known sex independently to identify the sex specificity of the marker.

RAPD marker analysis

Sixty 10-base primers (series OPA, OPN and OPP; Operon Technologies, Alameda, USA) were used for polymerase chain reaction (PCR) for screening of known sex to ascertain their potential of clear amplification in polymorphism and also the reproducibility. Amplification reactions were performed in volumes of 25 μl containing 2.5 μl of 10× assay buffer (100 mM Tris–Cl; pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 0.2 mM of each dNTPs (dATP, dCTP, dGTP and dTTP) (MBI Ferment, Maryland, USA) 5 pg of primer, 1.0 unit of Taq DNA polymerase (Bangalore Genei) and 30 ng of template DNA. The amplification reaction was carried out in a DNA Thermal Cycler (Eppendorf, Hamburg, Germany) programmed for 44 cycles as follows: 1st cycle of 5 min at 94°C followed by 43 cycles each of 1 min at 92°C, 1 min at 37°C, 2 min at 72°C. The final step consisted of one cycle of 7 min at 72°C for complete polymerization. After completion of the PCR, 2.5 μl of 6× loading dye (MBI Ferment) was added to the amplified products and were electrophoresed in a 1.5% (m/v) agarose (Bangalore Genei) gels with 1× TAE buffer, stained with ethidium bromide and documented by a gel documentation system (Syngene, Cambridge, UK). The size of amplification products was estimated by comparing with standard DNA ladder (O'Gene Ruler 1.0 kbp DNA ladder; MBI Ferment). All the reactions were repeated three times. Eighteen primers were amplified on the basis of the clarity of the banding patterns.

Results and discussion

A total of 60 oligonucleotide primers were used to amplify the bulk DNA of male, female, and hermaphrodite individuals of which only 18 primers showed reproducible results; however, 3 primers, OPN 06, OPN 16, and OPA 20, produced distinct markers for sex differentiation. In these 3 primers, the number of amplified bands varied from 7 to 12 and the fragment size ranged between 400 and 2,400 bp in male, female and hermaphrodite bulks. In BSA, among 3

oligonucleotide primers taken for study, only 2 primers, OPN 06 and OPN16, showed sex specificity of female and hermaphrodite, respectively. However, one marker specific to both female and hermaphrodite amplified by OPA 20 showed an 1,140-bp DNA marker which is absent in male counterparts. The primer OPN 06 (5′GAGACGCACA3′) produced a unique ~1,280-bp fragment in female bulk DNA, which was absent in male and hermaphrodite (Fig. 1a). Similarly, another unique band of 400 bp specific to hermaphrodite amplified by OPN 16 primers (5′AAGCGACCTG3′) was observed which was not present in male and female DNA markers (Fig. 1b). In addition, OPA 20 primer (5′GTTGCGATCC3′) produced amplification fragment size of ~1,140 bp which is not present in male plants (Fig 1c); this could be used for identification of male plants of *C. wightii*. Bulked segregant analysis involving RAPD products has been used successfully to identify molecular markers associated with sex determination in several dioecious plant species instead of assessing amplification products from individual plant DNAs (Mulcahy et al. 1992; Hormaza et al. 1994; Parrish et al. 2004). Subsequently, Parrish et al. (2004) suggested using a method of AFLP-BSA for development of more reproducible results; however, this approach is more expensive than PCR-BSA.

Subsequently, for confirmation of this observation, the three primers showing polymorphism for the sex type were used to re-test the DNAs amplification with individual plants of each sex type of male, female, and hermaphrodite taken for study. The unique bands of ~1,280 and ~400 bp produced by bulk showed similarity for all individuals of female and hermaphrodite, respectively (Fig. 1a, b). Similarly, OPA 20 showed amplified bands in female and hermaphrodite which are not present in male individuals (Fig. 1c). Thus, the RAPD markers OPN 06_{1280} and OPN 16_{400} could be recognized as putative sex-linked markers

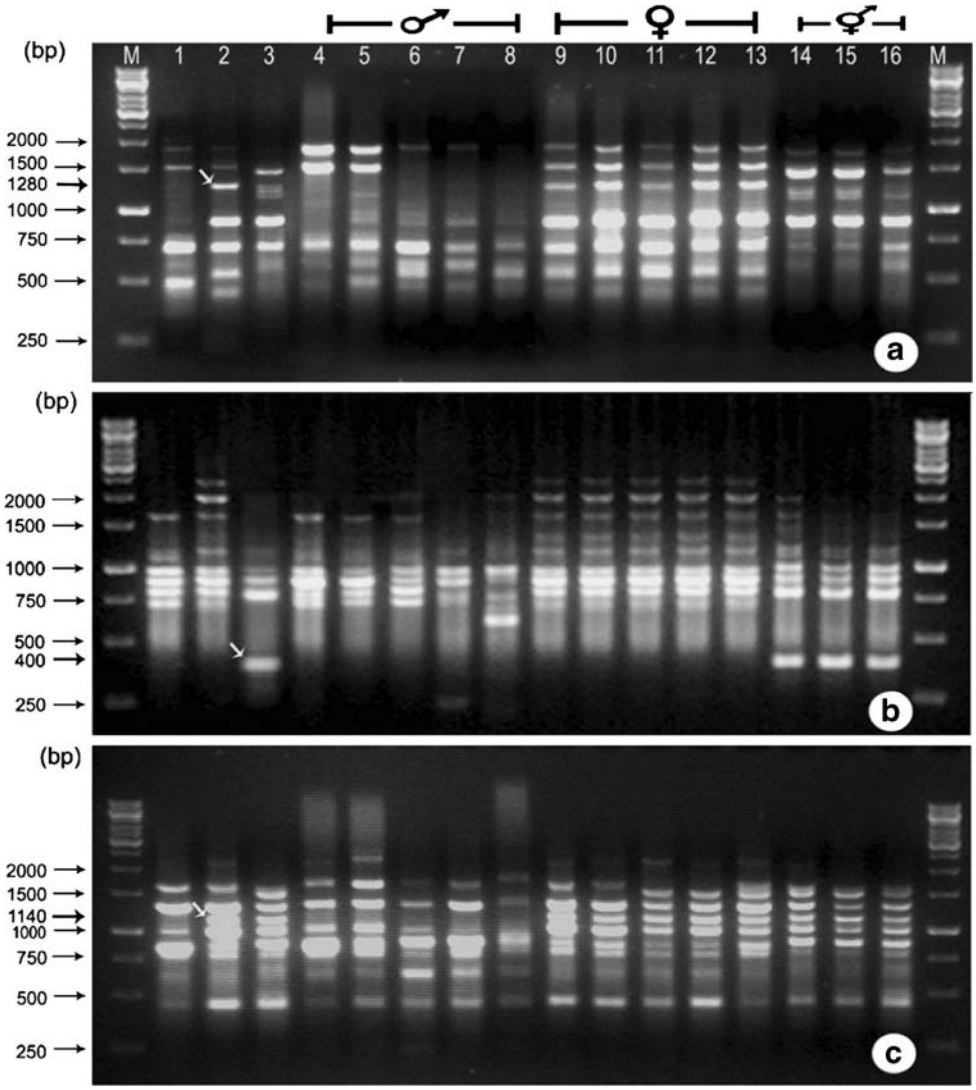

Fig. 1 RAPD banding profile of bulk and individual male, female and hermaphrodite plants of *C. wightii* using **a** OPN06; *arrow* indicates the unique band of ~1,280 bp present in female bulk. **b** OPN16; *arrow* indicates the unique band of ~400 bp present in hermaphrodite bulk. **c** OPN 20; *arrow* indicates the unique band of ~1,140 bp present in female and hermaphrodite bulk. *M* 1 Kbp ladder, *lane 1* bulk male, *lane 2* bulk female, *lane 3* bulk hermaphrodite, *lanes 4–8* male individuals, *lane 9–13* female individuals, *lane 14–16* hermaphrodite individuals

for female and hermaphrodite plants of *C. wightii*. Sex-linked markers OPA 08_{945}, UBC $345_{560,}$ and OPC 07_{567} were amplified in female individuals of *Pistachio vera*, *Salix viminalis*, and *Trichosanthes diocia*, respectively, reported by many researchers (Hormaza et al. 1994; Alstrom-Rapaport et al. 1998; Singh et al. 2002). The third primer, OPA 20, could also be used to discriminate male from female and hermaphrodite plants.

The most extreme type of sex determination system is found where highly specialized sex chromosomes are found; this type of system usually promotes dioecy in plants (Ming and Moore 2007). The evolution of dioecy directly from a hermaphrodite species is considered unlikely since the occurrence and establishment of two independent mutations, one for male and the other for female sterility, must occur simultaneously, and the mutant genes or multiple loci must be tightly linked so that the generation of hermaphrodites does not occur by recombination (Ainsworth et al. 1995). Since dioecism has arisen in different families and genera of plants (Westergaard 1958), the development of molecular strategies for early sex identification of dioecious taxa has been a priority in breeding programmes for their greater economic potentials. Moreover, studies on marker technology regarding dioecy in general would render a better understanding of the developmental as well as the evolutionary process of dimorphism. The use of DNA markers to distinguish the sexes has been employed when the genetic mechanism of sex determination is not available (Banerjee et al. 1999; Xu et al. 2004).

PCR-based DNA technology has been proved a reliable strategy for detection of sex-associated markers in dioecious and bisexual taxa. The RAPD technique is the cheapest reliable tool (Williams et al. 1990), though initially used for efficient fingerprinting of many more plants; however, it gradually paved the way for more advanced molecular techniques (Schlotterer 2004). In addition, SCAR markers originating from RAPD markers were also developed for distinguishing the sex specificity (Banerjee et al. 1999; Urasaki et al. 2002). Attempts have also been made earlier to generate the sex-linked marker in Asparagus and Fig using RFLPs and AFLPs (Bracale et al. 1991; Parrish et al. 2004).

In conclusion, our finding would be useful to detect the gender in *C. wightii* at any growth stage, thereby simplifying the breeding programmes by saving time and economic resources. Further, the female-specific $OPN06_{1280}$ and hermaphrodite-specific $OPN16_{400}$ could be used to generate a more reliable SCAR marker (Paran and Michelmore 1993) for better reproducibility as has been achieved in many plants such as *Mercurialis annua*, *Carica papaya*, and *Cannabis sativa* (Mondolino et al. 1999; Khadka et al. 2002; Urasaki et al. 2002). The availability of markers linked to sex-associated genes would allow cloning the gene(s) involved in this process. However, it is possible to differentiate male, female, and hermaphrodite plants of *C. wightii* accurately and rapidly using the RAPD markers.

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