RAPD markers for identification of sex in pointed gourd
(Trichosanthes dioica Roxb.)

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Received 31 March 2011; revised 23 June 2011; accepted 7 August 2011

Fifty two sexual progeny of pointed gourd (Trichosanthes dioica Roxb.), raised through embryo culture, were used for identification of RAPD markers associated with sex expression traits. Genomic DNA from male and female progeny plants and from a parthenocarpic clone (IIVRPG-105) were extracted individually and bulked by sex type. Forty-one random decamer primers were screened with the three bulks and a total of 509 amplification products were obtained, of which five were found to be associated with sex expression. The five markers were then tested with individual plant DNA samples, and two sex-associated RAPD markers were identified. A 1000 bp amplification product from the primer OPC05 was found to be present only in males and absent in both the female and parthenocarpic plants. Similarly, a 400 bp amplification product from the primer OPC14 was found to be present only in female individuals. Another marker, OPN011030, was also identified, which was present in male and female plant but was absent in parthenocarpic line. The two RAPD markers, the male-specific OPC051000 and the female-specific marker OPC14400 together can reliably differentiate male and female plants of T. dioica long before the plants attain maturity and flowering.

Keywords: Pointed gourd, RAPD, sex identification, Trichosanthes

Introduction

Sex differentiation is a phenomenon, which assures the continuous generation of new gene combinations as substrates for evolution and genetic improvement through hybridization followed by selection. In addition, sex usually forms the largest single division within a species and causes difference in physiology, behaviour and economic usefulness of different individuals of a species. Dioecy is prevalent in animals, where it is controlled by sex chromosomes. In contrast, sex determination in dioecious plants, which comprises ~6% of the reported plant species, can often be genic or environmental, and only a small fraction of such plants has evolved sex chromosomes. Dioecy exists in a number of cucurbitaceous genera, including Trichosanthes, but the mechanism of sex determination has been established in only few of them, e.g., XY system in Coccinia¹.

Pointed gourd (Trichosanthes dioica Roxb.), commonly known as Parwal or Patal is one of the most nutritive cucurbit vegetables, holding a coveted position in the Indian market during the summer and rainy seasons². It is a perennial crop and is highly accepted due to its availability for about 8 months (February-September) in a year³. T. dioica is extensively grown in Eastern parts of India and in sub-tropical and tropical regions of South Asia, especially Bangladesh³. T. dioica is morphologically distinct from the other cucurbitaceous species due to its well-established dioecism, perennial nature and vegetative means of propagation. Fruits of pointed gourd are rich in proteins and vitamin A and many reports indicate its role in circulatory system, especially in lowering blood sugar and serum triglycerides². It is typically propagated through stem cuttings mainly derived from mature plants to ensure the sex and fruit types. Each fruit contains several well-developed seeds with hard seed coat, but propagation through seeds is not feasible primarily due to poor germination and slow-growth of the seed-derived plants⁴. In T. dioica, male and female plants strictly maintain their respective sexual phenotypes, which indicate presence of a clear genetic basis of differences between them. Homology in chromosome bands between male and female individuals of T. dioica has been reported, but there is
no report on sex-specific peculiarities in chromosome morphology in the species. In case of dioecious species, sex identification at juvenile stage is of considerable importance to cultivation practices since female plants are usually valued for their fruits and few male plants are sufficient to supply pollen for good fruit set. Dioecy represents an inconvenience in pointed gourd breeding since at present there is no dependable method to distinguish male and female plants prior to flowering. Random amplified polymorphic DNA (RAPD) has been used to identify sex-associated markers in plants like Humulus, papaya, and Pistacia, such as, great tit (Parus major) and zebra finch (Taenopygia guttata). A reliable method for determining the gender of plants before flowering would facilitate economy of the various resources, including time and effort of the breeders. In addition, the availability of DNA markers linked to sex determining genes would allow identification of the gene(s) involved in this process. Therefore, the present study was undertaken to identify RAPD markers associated with male, female or parthenocarpic sex expression traits in T. dioica.

Materials and Methods

Plant Material and DNA Isolation

The plant material consisted of the 52 sexual progenies raised as per the procedure of Kumar et al and a parthenocarpic clone IIVRPG-105. The young leaf samples were collected from the sexual progeny growing in a glasshouse of Indian Institute of Vegetable Research (IIVR), Varanasi (India) and genomic DNA was isolated using Qiagen DNeasy Plant Mini Kit (Qiagen, Germany). DNA bulks were prepared by separately pooling equal quantities of DNA from 5 individual progeny of each sex type (male, female, and parthenocarpic bulks).

RAPD Analysis and PCR Amplification

A total of 41 primers were used in RAPD analysis; 20 of these primers were selected since they were previously reported to be associated with male sex expression in Humulus, Carica papaya, Asparagus officinalis, Atriplex garettii, Cannabis sativa, and Actinidia deliciosa or female sex expression Pistacia vera, Salix viminalis, Piper longum, and T. dioica. The remaining 21 primers (OPA and OPC series) were selected from the random primer kit (Operon Biotechnologies, Germany). For the initial screening of primers, bulked segregant analysis (BSA)-based approach was used with the three bulks. Once a polymorphic fragment was observed with a primer, the concerned primer was then used to amplify the DNAs isolated from individual plants of either sex. The identified markers were verified for their consistency and reproducibility using DNAs from nine random male plants from accession IIVRPG-M, and one plant each from the following nine female plant accessions: IIVRPG-1, VRPG-2, VRPG-7, IIVRPG-11, IIVRPG-16, IIVRPG-73, IIVRPG-78, IIVRPG-91 and IIVRPG-107, selected randomly from the germplasm available at IIVR, Varanasi.

Amplification reaction was carried out in a 25 µL reaction volume containing 50 ng genomic DNA, 2.5 µL PCR buffer (MBI Fermentas, Hanover, USA), 200 µM dNTPs (Bangalore Genei, Bangalore, India), 1.5 U Taq DNA polymerase (MBI Fermentas), and 0.4 µL primer using a thermal cycler (MyCycler, BioRad, USA). The first amplification cycle consisted of the following steps: at 94°C for 4 min, at Tm-5°C for 1 min and at 72°C for 1 min. This was followed by 39 cycles with 1 min at 94°C, 1 min at Tm-5°C, and 1 min and 72°C with the final extension of 10 min at 72°C. The amplified DNA fragments were resolved through electrophoresis in 1.5% agarose gel prepared in TBE buffer [54.0 g Tris-base, 27.5 g boric acid, 0.5 M EDTA (pH 8.0) in 1000 mL volume] and visualized in a gel documentation system (Alfa Imager 2200, Alfa Innotech Corporation, California, USA). The 1 Kb DNA ladder (MBI Fermentas) was used as molecular size marker. The amplification was repeated 2-3 times to ensure that the amplification obtained with the primers is reproducible and consistent.

Results and Discussion

In the present study, 41 decamer primers were tested with the DNA bulks of 3 reproductive types and a total of 509 amplification products were obtained. On an average, each primer amplified 3.95, 4.29 and 4.17 bands from the DNAs of male, female and parthenocarpic plants, respectively. In BSA, most of the primers failed to amplify reproducible RAPD markers occurring in one sex type alone. Only 5 primers detected polymorphism between the sexes (Table 1): one product of 400 bp from primer OPC14 (TGGTGTTT) was found to be female-specific, while a 350 bp product from primer OPO08 (CCTCCAGTGT) and a 1000 bp fragment due to primer OPC05 (GATGACCGCC) were found to be male-specific. One primer, OPO01
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(CTCACGTTGG) produced amplification product of 1030 bp, which was absent from the parthenocarpic bulk but present in both the male and female bulk DNA. Another primer, OPAF14 (GGTGCGCACT), generated an amplification product of 1375 bp specific to the parthenocarpic bulk; however, this band was light and thus not considered further.

Subsequently, these five primers showing polymorphism for the sex type were used to amplify DNAs from individual plants of each sex type. The primer OPC14, which generated a female-specific product from the bulk DNAs, produced a consistent and reproducible amplification pattern with individual plant DNA samples as well. It amplified a 400 bp fragment in all the six female individuals (Fig. 1); this fragment was absent from the six male and the three parthenocarpic plants. Therefore, it was concluded that the marker \(\text{OPC14}_{400}\) is associated with female sex.

Among the two primers, OPO08 and OPC05, which were found to yield male-specific products from the bulk DNAs, only OPC05 gave a reproducible male-specific amplification product from the individual plant DNAs. OPC05 amplified a 1000 bp fragment in all the six male individuals; this fragment was absent from the 7 female and the 3 parthenocarpic plants tested (Fig. 2). Thus, the RAPD marker \(\text{OPC05}_{1000}\) appears to be adequate for the detection of \(T.\ dioica\) male plants, and it can be used for an early determination of male sex in \(T.\ dioica\) well before the plants reach flowering. But the primer OPO08 failed to produce the unique male-specific band in 1 of the 6 male plants, and it generated fragment in the 3 female plants tested; therefore, due to these inconsistencies it is not a reliable marker for male phenotype. The primer, OPN01, which produced an amplification product of 1030 bp from bulk male and female DNAs but failed to do so from the parthenocarpic bulk, gave the same result when tested with individual plant DNA samples. It generated an amplification product of

| Table 1—Amplification product profile of the primers generated with the bulks and individual plant DNAs from male, female and parthenocarpic plants of \(T.\ dioica\) |
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| Primer | No. of bands amplified | Size range of fragments (bp) | Sex-associated fragment (bp) |
| | | Bulk DNAs | Individual DNAs |
| OPC05 | 2\(^a\) | 800-1000 | 1000 bp; male-specific | Present in all the 6 male samples and absent from all the 7 female and 3 parthenocarpic samples tested |
| OPC14 | 6\(^b\) | 400-2000 | 400 bp; female-specific | Present in all the 6 female samples and absent from all the 6 male and 3 parthenocarpic samples tested |
| OPN01 | 3\(^c\) | 500-1031 | 1030 bp; absent from parthenocarpic bulk | Present in all the 6 male and the 6 female samples and absent from all the 3 parthenocarpic samples |
| OPO08 | 5\(^d\) | 350-700 | 350 bp; male-specific | Absent from 1 out of 6 male samples and present in 3 out of 6 female samples |
| OPAF14 | 2\(^e\) | 200-1580 | 1375 bp; parthenocarpic specific | Very light band present in the 3 parthenocarpic samples tested |

\(\text{a}\)3 bands in male bulk, \(\text{b}\)7 products in female, \(\text{c}\)2 fragments in parthenocarpic bulk, \(\text{d}\)6 bands in male bulk & \(\text{e}\)3 fragments in parthenocarpic bulk.
for use in detection of the three sex types in T. dioica (amplified the female (OPC14) used in the earlier experiment. The two primers that were tested with the DNAs isolated from 9 male plants and 9 female accessions selected randomly were tested with the DNAs isolated from 9 male samples, but their intensity was low. Thus, due to the inconsistencies in amplification patterns, the two of the primers, viz., OPO08 and OPAF14, were ruled out for use in detection of the three sex types in T. dioica.

The selected markers, OPC14400 and OPC051000, were tested with the DNAs isolated from 9 male plants and 9 female accessions selected randomly from the germplasm and unrelated to the three clones used in the earlier experiment. The two primers that amplified the female (OPC14400) and male-specific (OPC051000) markers, consistently amplified these fragments in the female and male plant DNAs, respectively. This indicates that the markers are consistently associated with the two sexes and they could together be used to detect the male, female and parthenocarpic plants of T. dioica. The third primer, OPN01, amplified the 1030 bp fragment from all male and female DNAs.

Further, a previously reported female sex-associated RAPD marker in T. dioica, OPC07564, was also tested with the DNAs isolated from the sexual progeny. Surprisingly, this primer was not found to be consistent as it failed to amplify the female sex-associated 564 bp DNA fragment. In the present study, use of a different set of parental clones might be the reason for this marker’s inconsistency. Most of the markers used are anonymous DNA sequence variants and, thus, cannot correlate sexuality at genetic level. It has been suggested that functional genomics approach like transcript profiling may be promising to delineate a complicated phenomenon of sex expression. In the case of Asparagus officinalis, a male sex-associated SCAR marker was developed but this marker did not show any association with other Asparagus clones.

Family Cucurbitaceae has a number of sex forms, viz., dioecious, monoecious, hermaphrodite, andromonoecious, gynoecious, etc. Thus, the members of this family are interesting materials to study the genetic mechanisms underlying sex determination. Use of DNA markers to discriminate between the two sexes has been advocated if the genetic mechanism of sex determination is not known. DNA markers have been used to detect and select a genotype long before the phenotype becomes apparent. In present study, BSA-based approach was adopted in the initial screening of the primers. Use of BSA has been advocated instead of examining amplification products from individual plant DNAs for the detection of consistent polymorphic bands associated with the trait for which DNAs are bulked. An alternative method, AFLP-BSA has been used to develop a more reproducible banding pattern, but this approach is more expensive than PCR-BSA. A similar problem was also reported in the case of P. vera with the primer OPO08, as the marker identified through BSA (a female-specific marker OPO08900) was reported to be inconsistent and produced monomorphic pattern when it was tested with individual DNA samples. Use of touchdown PCR to overcome such marker inconsistency has been suggested, whereas some authors have suggested co-amplification of internal transcribed spacer (ITS) region together with sex-specific marker to prevent erroneous scoring of males as females.

The results herein indicate that sex determination in T. dioica is under a simple genetic control, possibly by a single gene or few tightly linked genes. If the sex expression was determined by heteromorphic sex chromosomes, a large number of sex-specific fragments should have appeared because sex determination by multiple, unlinked loci would recombine and, thereby, prevent the identification of a single male/female-specific marker. But a single male and female specific marker was obtained in this study. These results are similar to those from Ficus fulva, where a single male-specific AFLP marker was identified, suggesting DNA segment involved in sex determination is not very large and probably involved a single or a few tightly-linked genes. Most of the studies related to sex
determination has been conducted with the species having heteromorphic chromosome (highly specialized sex chromosomes, e.g., *Asparagus*, *Cannabis*, *Coccinia*, *Humulus*, *Silene*), which usually promotes dioecy in plants. But the studies related to sex determination mechanisms in plants without sex chromosomes suggest that such a model may prove to be unsuitable for majority of dioecious plants including *T. dioica*. Recently, transcript profiling of unopened male and female floral buds of *T. dioica* has been used in order to look for the differentially expressed unique and/or up-regulated gene fragments associated with sex expression; however, many of the fragments could not be annotated with any protein function reported till date, probably indicating the paucity of information in this field. The sex type of a plant species can be detected using a DNA-based test, but such test can only be useful if a sex-associated marker is available. The chances of any RAPD markers being linked to a gene or a genomic region of interest is mainly dependent on chromosome number, genome size, type of gene or genomic region, which determines the sex, and on the type of population used for marker analysis. It also depends to a great extent on chance because random sequences are used as PCR primers.

A marker linked to a specific trait can only be efficiently used in breeding programmes, if it is determined that the marker in question can predict the phenotype in genotypes other than the ones used for its detection. The two RAPD markers reliably identified nine unrelated male and nine female *T. dioica* plants. The reproducibility of RAPD markers has often been questioned, and currently the trend is to develop a more reliable and simple to use sequence characterized amplified region (SCAR) markers by cloning and sequencing the concerned RAPD fragment, and designing a pair of longer (17 bases or more) primers from the sequence. The SCAR markers could be advantageous over RAPD markers as their amplification is not sensitive to PCR conditions and they detect a single locus; thus, significantly improving the reproducibility and reliability of the marker system. Genomic information from the sex chromosomes of plants, such as, *Silene*, *Rumex* and papaya is rapidly improving. Tagging and cloning of sex determination genes will provide information that is crucial to decipher the sex-determination system. Cloning of sex determination genes is quite a challenging task because of genetic recombinations that make map-based cloning approaches ineffective. The generation of sex reversal mutants might be a good alternative approach towards identifying the sex determining genes.

**Acknowledgement**

A part of the present study was presented and published in the proceeding of 9th EUCARP1A meeting on ‘Genetics and Breeding of Cucurbitaceae’, held at INRA, Avignon, France, May 21-24, 2008.

**References**


