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Research Note

Identification of RAPD marker associated with gynoecious trait (*gy-1* gene) in bitter gourd (*Momordica charantia* L.)

Smaranika Mishra[1]*, Tusar K. Behera[1], Anilabh D. Munshi[1], Kishor Gaikwad[2], Trilochan Mohapatra[3]

[1]Division of Vegetable Science, Indian Agricultural Research Institute, New Delhi 110012, India
[2]National Research Centre on Plant Biotechnology, New Delhi 110012, India
[3]Central Rice Research Institute, Cuttack, Odisha 753006, India

***Corresponding author: mishrasmaranika@gmail.com**

Abstract

Bitter gourd is an important cucurbit with monoecious (both male and female flower on the same plant) sex form in general. Recently identification of some gynoecious lines has shown their advantage in hybrid breeding programme. For identification of molecular marker that can differentiate the gynoecious line from monoecious line in bitter gourd, 154 primers (67 SSR, 16 ISSR and 71 RAPD) were used to screen the parents ('Pusa Do Mausami' and 'DBGy-201'; 10 plants each) and F_2 population (86 plants) derived from 'Pusa Do Mausami' (monoecious) × 'DBGy-201' (gynoecious) using bulked segregant analysis (BSA). Phenotyping and genotyping of the 86 F_2 individuals showed a ratio of 3:1 with respect to monoecious (both male and female flower in same plant): gynoecious (only female flowers in a plant), as expected. Only one RAPD primer OPZ 13 out of 154 produced polymorphism between the monoecious and gynoecious individuals in the parent as well as in the bulks in BSA. This polymorphic marker produced 5-9 bands per lane, out of which a band size of 700 bp specific to gynoecious sex form and was absent in monoecious plants. This is the first report on targeting the gene responsible for gynoecious trait in bitter gourd.

Keywords: *Momordica charantia* L., molecular marker, gynoecious, RAPD, Bulk segregant analysis.
Abbreviations: BSA- Bulked segregant analysis; CTAB- Cetyl trimethyl ammonium bromide; MAS- Marker-assisted selection; PAGE- Polyacrylamide gel electrophoresis; QTL- Quantitative trait loci; RAPD- Random amplified polymorphic DNA.

Introduction

Bitter gourd (*Momordica charantia* L.) is quite well known in different parts of globe for its nutritive value and use in folk medicines. A compound known as 'charantin' present in bitter gourd is used for the treatment of diabetes to lower blood sugar levels. The fruit being rich in vitamin A, vitamin C, iron, phosphorus and carbohydrates, is considered as the most nutritive among cucurbits (Miniraj et al., 1993; Desai and Musmade, 1998). It increases body's resistance against pathogenic infections (Beloin et al., 2005). Ironically, this crop has not been exploited to its fullest and still confined to limited areas that too growing with local cultivars only, resulting in low production. Major reason behind this low productivity is the lack of high yielding varieties and hybrids. Hybrid in bitter gourd is generally produced by hand emasculation and pollination which is both cost and labour intensive. Furthermore, the number of seeds per fruit (cross) is also very less. Therefore, gynoecious (plant with only female flower) line can be used to produce hybrids economically in bitter gourd by sparing tedious manual emasculation and pollination. Gynoecious sex form has been reported in recent past from India (Behera et al., 2006). The subsequent generations developed using gynoecious line as one parent shows positive impact on hybrid in terms of increase in yield and earliness (Dey et al., 2010). Studies on inheritance have revealed that this trait is under the control of a recessive gene (*gy-1*) (Ram et al., 2006; Behera et al., 2009). Gene tagging refers to identification of existing DNA or introduction of new DNA that can function as a tag or label for the gene of interest. Identification of molecular marker(s) associated with the targeted trait has enormous potential for the crop improvement through molecular breeding (Collard et al., 2005). In today's context advances in molecular biology has laid down many techniques such as use of molecular marker techniques for identifying any trait of interest. These molecular or DNA markers are free from any epistatic interactions and environmental effects so that they can be used further in marker-assisted selection (MAS) to confirm the presence of gene of interest. Another advantage of this gene targeting or gene tagging is, if the marker is closely linked to the desired locus, it can be easily cloned and then introgressed in other genetic back ground either through direct gene transfer or molecular breeding. These markers will also be helpful for studying the mechanism affecting sex expression in bitter gourd. Before applying marker-assisted selection in a breeding programme, it must be ensured that the markers used are polymorphic in the populations (Huang et al., 1999), which is important for marker identification. The alternative to this step is the bulk segregant analysis (BSA) (Michelmore et al., 1991) and with this technique specific genomic regions can be studied against a random genetic background of unlinked loci by observing polymorphism generated by RAPD or other markers between two bulk DNA samples derived from a population segregating for a gene of interest. Most of the monogenic traits and major QTLs can be mapped using this technique. Previously many workers have used this BSA

technique to tag a gene of interest. The traits like gynoecy in cucumber (Lou et al., 2007), major fertility restorer gene in pepper (Zhang et al., 2000), early sex determination in *Actinidia* (Xiao et al., 1999), gall midge resistance in rice (Lima et al., 2007) and *Fusarium* wilt resistance in eggplant (Mutlu et al., 2008) have been tagged with molecular markers. In the present study, the inheritance of *gy-1* gene was evaluated by classical Mendelian procedures based on distribution of progenies based on phenotyping. BSA was used to identify marker linked to gynoecious trait based on genotyping. The degree of linkage between gynoecious trait and a RAPD marker was estimated.

Result and Discussion

Inheritance pattern of gynoecious sex form

In an earlier study by the author, seeds of the parental lines (Pusa Do Mausami and DBGy 201), B_1, B_2, F_1 and the F_2 populations were sown and observation was taken for flowering behaviour of the plants. All the F_1 plants were found monoecious, while the F_2 populations segregated for gynoecism. Out of 80 F_2 plants of Pusa Do Mausami × DBGy 201, 61 plants produced both male and female flowers, while 19 had only female flowers. The $\chi 2$ analysis indicated a good fit to a 3:1 ratio ($\chi 2 = 0.01$ and $P = 0.92$). Again in B_2 generation, 8 and 12 monoecious and gynoecious lines were obtained giving a ratio of 1:1 ($\chi 2 = 0.46$, $P = 0.50$). All monoecious plants were obtained in B_1 generation. In our study, $\chi 2$ analysis for goodness of fit indicated that gynoecism in the line DBGy 201 is under the control of a single recessive gene (*gy-1*). This study confirmed the earlier report of (Behera et al., 2009; Ram et al., 2006) which stated single recessive control of gynoecious sex form in bitter gourd. Poole and Grimball (1939) reported the similar inheritance pattern of gynoecism in melon.

Segregation of sex form in F_2 population

Phenotyping of F_2 mapping population revealed that out of 86 plants, there were 58 monoecious and 28 gynoecious plants. In order to establish a $\chi 2$ test when observed segregation ratio was compared to the expected ratio, it showed a 3:1 ratio with $\chi 2$ value of 2.620 ($P = 0.1055$) (Table 1).

Bulked Segregant Analysis (BSA) and Genotyping of F_2 population

Marker-assisted selection is considered as one of the important tool for identifying different sex form in crop plants. Furthermore, RAPD marker has been proposed as an alternative method in targeting any DNA sequences for its abundance in plant genome (Williams et al. 1990). Therefore, it is easy to tag a gene with RAPD as compared to other markers. Out of 154 markers (67 SSR, 16 ISSR and 71 RAPD), only 1 RAPD (OPZ 13; 3′ GACTAAGCCC 5′) primer showed parental polymorphism while remaining primers were found monomorphic. This primer OPZ 13 produced 5- 9 bands. From which one band of approximately 700 bp amplified in the gynoecious parent ('DBGy 201') and gynoecious bulk but absent in monoecious parent and monoecious bulk was found polymorphic. This polymorphic marker was then used to amplify the individuals of each bulk and it is observed that the band OPZ 13_{700bp} was absent in all the individuals of monoecious bulk and present in 7 individuals of gynoecious bulk except in 3 recombinants. For genotyping of the F_2 population, PCR amplification of 86 DNA samples from F_2 was carried out with OPZ 13. Banding pattern showed that a 700 bp band was present in 23 F_2 plants (be signed with G) and absent in 63 plants (be signed with M) indicating co-segregation of marker with gynoecy gene (Figure 1). Out of 86 plants, 19 were recombinant with a recombination frequency 22.09. Early identification of gynoecious sex type using marker-assisted breeding in bitter gourd has immense potential in introgression of gynoecious trait. In our investigation, BSA resulted in identification of only one RAPD primer, i.e. OPZ 13 (3′ GACTAAGCCC 5′) which could produce polymorphism between the monoecious and gynoecious bulks. In contrast SSR primers failed in doing so indicating its linkage to some traits other than gynoecy. The two bulks used in BSA have completely random genotypes for most of the genome other than the region around the gene controlling the trait of interest. The presence of polymorphism between the marker patterns of the two bulks is expected only when they are genetically linked to the underlying gene (Giovannoni et al., 1991). Hence, it is confirmed that only OPZ 13_{700bp} is linked to the *gy-1* gene. It was observed that the polymorphic primer OPZ 13 yielded 5-9 bands per lane. Significance of high number of amplified products per primer for tagging a gene has also been documented by Welsh and McClelland (1990). Out of these bands, one 700 bp amplified in gynoecious parent and gynoecious bulk and was polymorphic. A clear cut polymorphism between monoecious and gynoecious individuals were evident from the bulked segregant analysis. Thus, bulked segregant analysis with RAPD markers can provide the marker-assisted selection in F_2 population of 'Pusa Do Mausami' × 'DBGy-201' and also in other mapping populations. For genotypic characterization and mapping of the gene, amplification of DNA samples of all 86 plants of F_2 population was carried out, which yielded 19 recombinants with a recombinant frequency of 22.09% indicating the marker linked to the gene of interest at 22 cM distance. Earlier, Poulson et al. (1995) showed that BSA is the straight forward method to cope with the low levels of phenotypic misclassification, especially with bulks constructed with sufficient number of individuals. Similar to the present study, tagging of the gynoecious gene in cucumber has been reported by Lou et al. (2007) with AFLP marker through BSA, where monogenic dominant or incompletely dominant inheritance of gynoecious trait has been confirmed and their results indicated linkage between the marker and gynoecy trait at a genetic distance of 6.7 cM. The amplification product was then sequenced to generate sequence-characterized amplified region (SCAR) primers designated as SA200. Hence, in present findings, further scope is there to convert the gynoecious specific amplicons produced by OPZ 13_{700bp} into a stable SCAR marker.

Materials and Methods

Plant materials

Cross was made between a commercial monoecious variety 'Pusa Do Mausami' and 'DBGy-201', a gynoecious line in the spring-summer season of 2010. 'DBGy-201' was used as a male parent by inducing hermaphrodite flower on it through spraying of 6 mM silver thiosulfate after emergence of first female bud. The harvested F_1 seeds were grown to get the F_1 hybrid plant and then selfed to get F_2 seeds in second year. The F_2 segregating population comprising of 86 plants was planted in channel and hill methods at the research farm of Division of Vegetable Science, Indian Agricultural Research Institute, New Delhi in the spring-summer season of 2012 at a spacing of 2 m between channels and 60 cm between hills.

Table 1. Segregation of gy-1 gene and OPZ 13 marker

Data type	Observed Monoecious	Gynoecious	Expected Monoecious	Gynoecious	Ratio	$\chi 2$ value	P value
Phenotypic	58	28	64.5	21.5	3:1	2.62	0.11
Genotypic	63	23	64.5	21.5	3:1	0.14	0.71

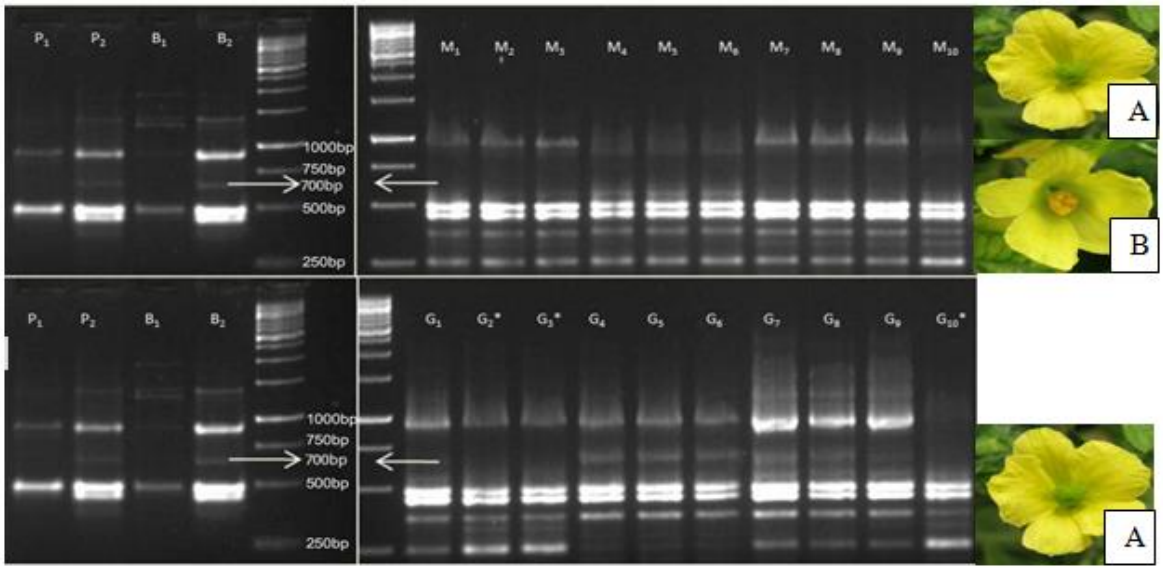

Fig 1. OPZ 13 showing polymorphism between individuals of monoecious and gynoecious bulk with a gynoecious specific band of size 700 bp, P_1 represents monoecious parent, P_2 represents gynoecious parent, B_1 represents monoecious bulk, B_2 represents gynoecious bulk, M_1, M_2…..M_{10} represents individuals of monoecious bulk, G_1,G_2….G_{10} represents individuals of gynoecious bulk and * represents the recombinants. Ladder size is 1kb.The recombinant genotypes are detected based on phenotyping and marker analysis i.e. phenotypically they are gynoecious but based on marker analysis there is absence of band (700bp). A refers to female flower and B refers to Male flower. In Monoecious plants, A & B are found together while in gynoecious plant only A is found.

Phenotyping of F_2 population for sex form

Individual plants of the F_2 population were observed for their sex form. Flowering in gynoecious plant started with appearance of female bud throughout the life cycle, whereas in monoecious plant male bud was noticed in the beginning till the end. Moreover, gynoecious lines were early in flowering and produced only female flowers throughout their life cycle, while monoecious plants produced both male and female flowers. Each F_2 plant was scored according to its sex form and entries were made as 'G' for gynoecious and 'M' for monoecious. The goodness of fit of phenotypic observation of F_2 plants with the expected Mendelian ratio (monoecious: gynoecious) was tested using chi square (χ^2) formula (Panse and Sukhatme, 1967).

DNA isolation and quantification

Good quality genomic DNA was isolated from fresh tender leaf of the parents and F_2 population by CTAB (Cetyl Trimethyl Ammonium Bromide) method given by Murray and Thomson (1980). Quantification was performed by electrophoresing DNA samples on 0.8 % agarose gel along with known quantity of uncut λ DNA. Quantification and quality analysis of the purified DNA was also carried out based on UV absorbance at 260 nm using a spectrophotometer (Nanodrop 8000, Thermo Scientific). From the ratio of absorbance at 260–280 nm, the purity of extracted DNA was ascertained. The samples were considered pure, when ratio was in a range 1.8 and 2.0. DNA working stocks were prepared by diluting a part of the original stock with appropriate amount of TE buffer to make the final concentration of DNA as 20 ng/µl. Equal quantity of DNA from 10 monoecious F_2 plants and 10 gynoecious F_2 plants were bulked (pooled) separately to form monoecious and gynoecious bulk respectively and stored at 4^0C.

Bulked Segregant Analysis (BSA)

The bulked DNA samples were subjected to BSA (Michelmore et al., 1991) along with the monoecious and gynoecious parents. Those primers differentiating the monoecious parent and monoecious bulk from gynoecious parent and gynoecious bulk was used to analyze each individuals of monoecious and gynoecious bulk. The possible inclusion of artifacts or non-specific bands in scoring as in case of RAPD was ruled out by repeated amplification.

PCR condition and electrophoresis

Sixty seven SSR primers specific to *Momordica charantia* (cited from Ph. D. thesis, Bharathi, 2010; Ph. D. Thesis, Muthu kumar, 2013 (in press) ; Guo et al., 2010; Yuan et al., 2012 and Wang et al., 2010) synthesised by SBS Genetech, Beijing, China, 16 ISSR primers (cited from Behera et al., 2008a and Ph. D. Thesis, Dalamu, 2011) synthesized from Bangalore Genei Pvt. Ltd., India and 71 arbitrary decamer oligonucleotide primers (cited from Behera et al., 2008a and available from RAPD primer kits, Operon Technologies, Almeda, California, USA) were used for PCR amplification. The PCR amplification was performed in a total volume of 25 ml reaction mixture containing 20 ng of DNA, 1X *Taq* buffer, 200 mM dNTPs (each), 25 mM of magnesium chloride, 0.5 µM Primer, 1U of *Taq* DNA polymerase

enzyme using thermal cycler (Mastercycler® pro, Eppendorf, North America) with initial DNA denaturation at 94^0C for 5 min followed by 40 PCR cycles at 94^0 C for 1 min for DNA denaturation, 37^0C for 1 min for primer annealing, 72^0C for 2 min for primer extension and final extension at 72^0C for 10 min and storage at 4^0C in case of RAPD and ISSR primers.
For SSR primers, PCR reactions were performed with initial DNA denaturation at 94^0C for 5 min followed by 35 cycles at 94^0C for 40 sec for DNA denaturation, 55^0C for 45 sec for primer annealing, 72^0C 40 sec for primer extension and final extension at 72^0C for 7 min and storage at 4^0C. All the amplified PCR products obtained from RAPD and ISSR markers were separated by electrophoresing in 1.2% agarose gel containing ethidium bromide (3 µl/ 100 ml) running in 0.5X TBE buffer (pH 8.0). The amplification products of SSR were separated by 8% PAGE. DNA fragments were visualized and photographed using a gel documentation system (Alpha Innotech, USA).

Genotyping of F_2 population

Those primers showing polymorphism in BSA were used for analyzing the whole mapping population (F_2). Individual plants producing band specific to gynoecy were noted as 'G' and absence of band considered as monoecy was noted as 'M'. Phenotyping and genotyping data were compared and when there is a difference in phenotype and genotype, it is considered as a recombinant. Number of recombinant was counted and marked with 'R'. The distance of the marker from the gene is estimated using following formula, where 1% Recombinant frequency is considered as 1 cM distance.

$$\text{Recombinant Frequency}(\%) = \frac{\text{Number of recombinant}}{\text{Size of the population}} \times 100$$

Conclusion

The frequency of tagging a gene is mainly determined by the variation present in the targeted segment irrespective of the method used (Martin et al., 1991; Michelmore et al., 1991). In general, genes transferred from wild or unrelated species are easy to tag. In this study, as the parents of the segregating population are both elite inbred lines with a similar genetic background and because the variation in the '*gy-1'* locus is a natural one, only one useful marker was identified out of total 154 primers (67 SSR, 16 ISSR and 71 RAPD) screened. This RAPD marker (OPZ 13_{700bp}) could be used in marker-assisted selection in plant breeding program as well as in identifying gene at any stage of the crop growth period to save time and cost of traditional breeding programme. This is the first report on targeting gynoecious gene (*gy-1)* in bitter gourd. The identification of more closely linked makers will lead to saturation of the map, thereby, facilitating map based cloning and introgression of gynoecy trait into commercial inbreds in bitter gourd.

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