Identification of putative DNA markers for disease resistance breeding in Indian cauliflower (Brassica oleracea var. botrytis L.)

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Identification of DNA marker(s) linked to desirable gene(s) is a basic stride for speeding up of breeding procedure. To exploit this strategy, the present study was designed to identify putatively linked DNA markers to downy mildew resistance gene in Indian cauliflower (Brassica oleracea var. botrytis L.). For this, ‘BR-2’ and ‘Pusa Himjyoti’, the two genotypes having contrasting reaction traits to downy mildew were selected. Of 190 primers (115 RAPDs, 72 ISSRs and 7 SSRs) screened in the study, 171 were properly amplified and 88.3% of them were monomorphic between these two genotypes at first screening. The reproducibility analysis further reduced the polymorphic primers from 20 to 11, which included 4 RAPD primers (OPB18, 1776, OPB20, 1779, OPC14, 1106 & OPE14, 1181) and 7 ISSRs (ISSR5, 1526, ISSR8, 1125, ISSR11, 1506, ISSR12, 1050, ISSR23, 1110, ISSR30, 3575 & ISSR35, 3520). These markers showed robust reproducibility during the study. Therefore, further research in this direction has potential to identify linked markers at least for contrasting horticultural traits and disease resistance in both parents.

Keywords: Brassica oleracea, bulked segregant analysis, disease resistance breeding, DNA markers, polymorphism

Introduction

The DNA markers are highly reliable selection tool as they are stable, not influenced by environmental factors and relatively easy to score in an experienced laboratory. As compared to phenotypic assays, the DNA markers offer great advantage through increased reliability, efficiency and reducing cost in marker-assisted selection or breeding, which is known for its accelerated speed of variety development process1. Here, identification of DNA markers closely linked to desirable gene(s) is most essential step and requirement for enhancing the efficiency of ideotype breeding procedures. The gene pyramiding and marker assisted introgression also essentially depends on identification of such tool. Using linked DNA markers, the genes affecting trait(s) of interest in forward populations can be easily detected by employing statistical associations between markers and trait of interest2. Thus, it helps in rapid and confirmed identification of desirable genes/alleles in germplasm or subsequent backcross populations. Presently, the marker assisted breeding has emerged as one of the most acceptable strategies in crop improvement programmes3. In this direction, the easiest way to find linked-markers is to select key genotypes those have contrasting observations for most of the desirable traits in the target crop. This facilitates rapid identification of polymorphic markers between genotypes, then putative markers for the traits and establishes the linkage with other traits of interest.

The cauliflower (Brassica oleracea var. botrytis L.) is an important leading cole vegetable of North India, which is now gaining popularity in southern and western regions of India due to the development of tropical varieties3. However, heavy incidence of diseases and pests like black rot, downy mildew, sclerotinia and alteneria blight, and diamond back moth deteriorate its product quality and crop productivity4. Other horticultural traits in cauliflower are plant height, leaf orientation, maturity season, curd colour and compactness, which determine its productivity and curd quality. Cauliflower has fair extent of genetic diversity3-6 but five maturity groups for flowering period and harvesting restrict the choice of parent selection from adjacent maturity groups4. The parental genotypes included in the present study have opposite reaction to diseases7-9. These two genotypes also differ for important horticultural traits, such as, maturity periods, plant height, leaf
orientation, flower colour, stem length and curd compactness. Therefore, two genotypes were selected as key genotypes to identify putative markers for disease resistance, which can be exploited for other traits of cauliflower. Further, the identified DNA markers can serve as potential tool for identification of cultivars and potential parents for use in breeding programmes\(^{10}\).

Although molecular markers linked to economically important traits were identified in \textit{B. oleracea}\(^{10-14}\) but information was scarce for DNA markers linked to the resistance genes in Indian cauliflower\(^{15-16}\). The identified DNA markers in broccoli\(^{11,14}\), cabbage\(^{10}\) and European cauliflower\(^{12-13}\) have limited use in Indian cauliflower due to poor transferability of DNA markers\(^{15}\). Thus, so far, only conventional procedures are being employed for development of varieties in Indian cauliflower, which are time consuming, environment dependent and stage specific methods\(^{3}\). Therefore, present investigation was designed to generate basic information on polymorphic DNA markers between two diverse genotypes of Indian cauliflower for their possible further use in marker-assisted breeding in brassica vegetables.

**Materials and Methods**

**Plant Material**

‘Pusa Himjyoti’ and ‘BR-2’ genotypes of Indian cauliflower (\textit{B. oleracea} var. \textit{botrytis}) maintained in their pure form by the Division of Vegetable Science, Indian Agricultural Research Institute, New Delhi were taken for the study. Both genotypes had contrasting observations for diseases like downy mildew (\textit{Hyaloperonospora parasitica})\(^ {7,9}\) and black rot (\textit{Xanthomonas campestris})\(^ {8}\), and for horticultural traits like plant height, stem length, leaf orientation, curd compactness, curd colour, flower colour and maturity season\(^ {9,14}\). Thus, both have potential to serve as model in identification of putative markers for such desirable traits in cauliflower.

**DNA Extraction and DNA Bulking**

Genomic DNA from young leaves of ‘BR-2’ and ‘Pusa Himjyoti’ and\( F_2\) plants was isolated through CTAB method\(^ {18}\) with minor modifications. The laboratory analysis was done at National Research Centre on Plant Biotechnology, New Delhi. In brief, 5 g leaf tissues were taken and grounded in mortar-pestle with liquid nitrogen. The powder was resuspended in 25 mL of buffer A (200 mM Tris-HCL, pH 8.0, 250 mM NaCl, 25 mM EDTA, 1% sodium dodecyl sulphate). DNA sample was purified through addition of RNase (Ribonuclease A; Sigma-Aldrich, St. Louis, Mo.) \( @ 5 \mu g/500 \mu L \) and incubated at 37°C for 1 h. The aqueous phase was extracted with repeated process of washing with chloroform:iso-amyl alcohol (24:1). Upper aqueous phase was collected after centrifugation and mixed with \( 1/10 \)th volume of 3 M sodium acetate. Pure DNA was precipitated by adding chilled absolute alcohol through centrifugation at 5,000 rpm for 8 min. The samples were dried and dissolved in T10E1 buffer and quantified by gel electrophoresis and UV-spectrophotometer.

**DNA Marker Analysis**

Sum of 190 PCR based primers (115 RAPD, 68 ISSR & 7 SSR primers) were screened for polymorphic survey between both parents. The sequence and annealing temperatures of 154 primers were taken from earlier reports on their screening in \textit{B. oleracea} genomes\(^ {11}\). The remaining 36 ISSR primers (UBC series from Biotechnology Laboratory, University of British Columbia, Vancouver, BC, Canada) were screened through touch-down gradient polymerase chain reactions\(^ {19}\) from 48°C to 40°C with 40 cycles. Sequences for SSR primers (Table 1) were obtained from HRI website: http://www.hri.ac.uk/. The polymerase chain reaction (PCR) analyses from

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer sequence (5'-3')</th>
<th>Reverse primer sequence (5'-3')</th>
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<tr>
<td>sORA43</td>
<td>GCGCGT GTGGGATCAGAA</td>
<td>CTTCCTCCAGCT CGATCG</td>
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<td>BN72A</td>
<td>GCCCACCACCTTCTTGTCTG</td>
<td>CCCCTCACCACCTCTTCTG</td>
</tr>
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<td>BN83B1</td>
<td>GCTTTCTTCAACTGATAGCTAA</td>
<td>TCAAGTGCTTGAGTCTCC</td>
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<td>BN12A</td>
<td>GCCGTCTAGGTTTGGGGA</td>
<td>GAGGAAATGAGGACGGGATATCA</td>
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<td>sORA21</td>
<td>TTCGACATAGCTTACAGG</td>
<td>TCCCTCTAGGACACTTCTT</td>
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<tr>
<td>nga248</td>
<td>TACCGAACCAAAAACACAAAAAGG</td>
<td>TCTGTATCTCCTGGATGACTTC</td>
</tr>
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<td>MB4</td>
<td>GAACTCTGTGGCTTTTATTAC</td>
<td>TGGTTTGTGTTTCTTACTG</td>
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</table>
all three types of primers were performed in a 10 µL final volume of the reaction mixture as per protocol followed by Farinho et al. The electrophoresis for both RAPD and ISSR primers was performed with 1.5% agarose gel according to Williams et al. The amplification product of SSR primers were separated by electrophoresis in 3% MetaPhor agarose (FMC Bioproducts, USA) gel containing ethidium bromide (3 µL/100 mL). The fragment size was calculated using in built mechanism of Gel Documentation System with known marker as 100 bp and 1 kb bp DNA ladder inside wells of the gel. Same PCR reactions were repeated 3-4 times for polymorphic RAPD and ISSR primers to ensure their reproducibility. The bulked segregant analysis was performed with identified polymorphic primers for downy mildew resistance trait. For this, DNA aliquots of each 10 phenotypically resistant and susceptible plants were mixed separately and bulks were prepared. The single plant analysis was also performed using putative markers to confirm homogeneity of bulk components and identifying the recombinants.

Results
Polymorphism Survey
Genomic DNAs isolated from ‘Pusa Himjyoti’ (DMS) and ‘BR-2’ (DMR) parental lines were used for polymorphism survey with 190 (RAPDs, ISSRs & SSRs) primers. Of these, 171 primers successfully amplified one or few bands from each parental DNA. Overall, 88.3 per cent of the primers amplified monomorphic bands between both the parents and could not be used in further analysis. Only 20 markers produced polymorphic banding patterns between both the parents but their number was reduced to 13 markers at first reproducibility analysis including 4 RAPD and 7 ISSR primers (Table 2). The thirteen markers generated amplicons in the range of 3 (OPC14, OPF1, ISSR23) to 12 (ISSR 35) and amplified 22 polymorphic bands (27.5 percent) with an average of 1.69 band per markers (Table 3). The maximum polymorphic bands (3 for each) were observed with OPB20, OPE14 and OPF1, while only one polymorphic band was generated by three RAPDs and four ISSRs. Size of amplicons varied from 150 to 2300 bp. However, both OPB20 and OPF1 also produced marker band in susceptible bulk, so not included in further study (Table 3).

RAPD Analysis
Polymorphism analysis of 115 RAPD primers resulted into 6 polymorphic primers, viz., OPB18, OPB20, OPC14, OPC1, OPF1 and OPE14, between both genomic DNA (Tables 2 & 3). Though OPF10 showed polymorphic band of 180 bp but it was

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’- 3’)</th>
<th>Total no. of amplicons</th>
<th>Range of amplicon size (bp)</th>
<th>No. of monomorphic amplicons</th>
<th>No. of polymorphic amplicons</th>
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<tr>
<td>RAPD-OPB18</td>
<td>CCACAGCAGT</td>
<td>8</td>
<td>150-700</td>
<td>7</td>
<td>1(12.5)*</td>
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<td>RAPD-OPB20</td>
<td>GGACCCCTTAC</td>
<td>6</td>
<td>300-650</td>
<td>3</td>
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<tr>
<td>RAPD-OPC14</td>
<td>AAGGCTCGTC</td>
<td>3</td>
<td>450-2000</td>
<td>2</td>
<td>1 (12.5)</td>
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<tr>
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<td>TGCGGCTGAG</td>
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<td>150-1000</td>
<td>5</td>
<td>3 (37.5)</td>
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<td>200-2000</td>
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<td>1 (12.5)</td>
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<tr>
<td>RAPD-OPF1</td>
<td>ACGGATCTCG</td>
<td>3</td>
<td>600-1800</td>
<td>2</td>
<td>3 (37.5)</td>
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<td>(GA)&lt;sub&gt;b&lt;/sub&gt; YG</td>
<td>4</td>
<td>500-1600</td>
<td>3</td>
<td>2 (25.0)</td>
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<td>(AC)&lt;sub&gt;b&lt;/sub&gt; YA</td>
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<td>200-2500</td>
<td>6</td>
<td>1 (12.5)</td>
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<tr>
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<td>(GT)&lt;sub&gt;b&lt;/sub&gt; YG</td>
<td>6</td>
<td>600-900</td>
<td>5</td>
<td>2 (25.0)</td>
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<tr>
<td>ISSR12</td>
<td>(AG)&lt;sub&gt;b&lt;/sub&gt; YG</td>
<td>5</td>
<td>400-1500</td>
<td>1</td>
<td>2 (25.0)</td>
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<tr>
<td>ISSR30</td>
<td>HVH (TG)&lt;sub&gt;r&lt;/sub&gt;</td>
<td>7</td>
<td>250-1700</td>
<td>6</td>
<td>1 (12.5)</td>
</tr>
<tr>
<td>ISSR6</td>
<td>(GTGA)&lt;sub&gt;4&lt;/sub&gt;</td>
<td>12</td>
<td>280-620</td>
<td>8</td>
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<td>ISSR23</td>
<td>(CT)&lt;sub&gt;b&lt;/sub&gt; RC</td>
<td>3</td>
<td>600-2300</td>
<td>2</td>
<td>1 (12.5)</td>
</tr>
</tbody>
</table>

*Values in parentheses are percentage of polymorphic bands
showing variable pattern of faintness during reproducibility analysis; therefore excluded from the study. Primer number is further reduced to only 4, viz., OPB18, OPB20, OPC14 and OPE14, through four times reproducibility analysis. Based on size in bp of the marker generated, these markers were designated as OPB18_{1276}, OPB20_{1174}, OPC14_{1184} and OPE14_{1881} (subscripts indicates the size of amplified fragment in the base pair); while two markers OPC1 and OPF01 remained polymorphic only two times but showed monomorphic bands during reproducibility test in bulked segregant analysis (Fig. 1a).

**ISSR Analysis**

ISSRs (inter simple sequence repeats) screening showed that 89.7 percent primers were amplified in both parents (Table 2). Thirteen primers produced polymorphic banding patterns between both the genotypes. However, their number was reduced to seven on 4-time reproducibility analysis. Primers with reproducible banding pattern were ISSR5, ISSR8, ISSR11, ISSR12, ISSR23, ISSR30 and ISSR35 (Table 2). The remaining six primers were remained polymorphic only 2-times so excluded from present study.

**SSR Screening**

All the 7 microsatellite markers surveyed in present study amplified the genomic DNA of both parents ‘BR-2’ and ‘Pusa Himjyoti’ but showed monomorphic banding pattern, so excluded from further study.

**Bulked Segregant Analysis**

The bulked segregant analysis with 11 reproducible polymorphic markers, viz., 4 RAPDs (OPB18_{1276}, OPB20_{1174}, OPC14_{1184} & OPE14_{1881}) and 7 ISSRs (ISSR5_{525}, ISSR8_{125}, ISSR11_{550}, ISSR12_{1050}, ISSR23_{1103}, ISSR30_{575} & ISSR35_{620}), with both parents ‘BR-2’ and ‘Pusa Himjyoti’, and their resistant and susceptible bulks resulted in identification of only 3 markers, viz., OPC14_{1186} and OPE14_{1881} and ISSR23_{1103}, as putative markers for downy mildew resistant gene in Indian cauliflower. These 3 markers maintained similar kind of banding patterns in respective bulks by amplifying the same band only in resistant (R-)bulk and not in susceptible (S-)bulk even during reproducibility analysis (Figs 1a & b).

**Single Plant Analysis**

The PCR analysis of genomic DNA of each component plants of R- and S-bulk with 3 putative DNA markers (OPC14, OPE14 & ISSR23) resulted in amplification of marker amplicon in resistant parent, composite DNA of R-bulk and its eight component plants. However, in case of susceptible parent

![Fig. 1 (a & b)—Amplification profiles of polymorphic RAPD markers (OPE14, OPC14, OPB18, OPB20, OPF1 & OPC1) (a) and ISSR markers (ISSR5, ISSR8, ISSR11, ISSR12, ISSR30, ISSR 6 & ISSR23) (b) in P1, P2, B_s and B_r. [M_1, 1 kb DNA ladder; M_2, 100 bp DNA ladder; P_1 and P_2 are parents ‘Pusa Himjyoti’ and ‘BR-2’, respectively. B_s and B_r are S- and R-bulks, respectively.]


‘Pusa Himjyoti’ and composite DNA of S-bulk, the marker bands of 3 putative markers were absent in all the component plants. The phenotypic observations of the component plants from the field were compared with banding pattern from PCR analysis with 3 putative markers. The results showed homozygous state of the allele in susceptible genotype ‘Pusa Himjyoti’, while two recombinant plants were observed in resistant bulks, which had shown phenotypic resistance reaction against the downy mildew pathogen in field screening but did not amplify the respective marker bands during PCR analysis with 3 DNA markers (Fig. 2).

**Discussion**

Identification of genetic markers linked to desirable gene(s) facilitates marker-assisted selection and pyramiding of such genes into cultivars. The development of disease resistant varieties depends on efficiency of accurate screening procedure and selection method during breeding programme. Although conventional methods of resistance breeding are effective\(^{20}\), but they are time consuming, partially efficient and cumbersome. Their effectiveness reduces particularly with handling of obligates and/or stage specific parasites. Therefore, uses of alternative but efficient methods like marker-assisted selection are becoming more acceptable among breeders. For the speedy identification of putative markers to be used in cauliflower breeding, bulked segregant analysis approach\(^{21}\) was used in conjugation with PCR based primers. This is one of the most commonly used approaches in putative DNA markers and their further use in gene mapping or identification of linked markers to the gene of interest.

‘Pusa Himjyoti’ and ‘BR-2’ were used in the present study for their contrasting reactions against diseases and also their genetic variations for horticultural traits. ‘BR-2’ is moderately resistant to black rot\(^{8}\), sclerotinia blight and leaf and curd blight\(^{9}\) and highly resistant to downy mildew\(^{2}\), while ‘Pusa Himjyoti’ is reported to have highly susceptible reaction against all these pathogens\(^{7,9}\). ‘BR-2’ has loose curd, long stem, erect leaves and whitish flowers, while ‘Pusa Himjyoti’ has compact curd, small stem and yellowish flower (S R Sharma, personnel communication). Thus, the putative markers identified through present study can be used to investigate possible linkage with these horticultural traits in cauliflower for exploitation in marker-assisted breeding for resistance as well as for horticultural traits.

The study identified 20 polymorphic primers between both parents but their number reduced to 13 on reproducibility analysis, which happened due to low reproducibility of RAPD and ISSR primers\(^{22}\). It might be due to short length of RAPD primers, which sometimes lead to unspecified banding patterns in eukaryotic genome\(^{20}\). Similarly, small sized single fragment of ISSR primers with repeated sequences also lead to their low reproducibility. Some of the primers like OPF10 produced faint polymorphic bands, which were not observed in subsequent reactions, so excluded from further PCR analyses for polymorphic markers. It is because of the fact that faint bands generated during RAPD amplification are not reproducible and may lose polymorphism\(^{22}\) and same is true with ISSR primers. Monomorphic banding pattern of most of primers (88.3 percent) was due to the fact that both the parents were from adjacent maturity groups of Indian cauliflower\(^{4}\) and also due to small number of primers\(^{15,22}\). However, 13 primers are sufficient, to some extent, to identify linked primers to a gene of interest.

The RAPD and ISSR markers have less reproducibility and they are also of dominant nature. So, new marker systems like SSR or SCAR and AFLP can be used to strengthening such type of studies. Once linked RAPD markers are identified, they can be readily isolated, cloned and sequenced, and the sequence can then be used to develop sequence characterized amplified regions (SCARs)\(^{23}\) markers. Examples where this approach has been used successfully to mark genes include a powdery mildew resistant gene in broccoli\(^{11}\). Further, the downy mildew resistance is governed by a single dominant
gene in Indian cauliflower. So, it is suggested to screen more number of reproducible, codominant and location specific primers from genomic library of cauliflower and its orthologous species. It appears necessary in context of identification of closely linked DNA markers for fine mapping and isolation of desirable gene. Therefore, findings of the present study are initial step towards exploitation of marker-assisted resistance breeding in Indian cauliflower.

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References