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Detecting RAPD Markers Associated with Black Rot Resistance in Cabbage (*Brassica oleracea* var. *capitata*)

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ABSTRACT

A RAPD marker linked to resistance (assayed as absence of development of V-shaped lesions) to black rot (*Xanthomonas campestris* pv. *campestris*) in cabbage was identified using a mapping population of 200 F_2 (January King × Golden Acre) plants with January King being the resistant parent. The analysis of genetics of black rot resistance was based on parents and the F_2 generation. Resistance in cabbage appeared to be governed by a single gene (monogenic). A total of 191 ten random primers were used to survey the parental polymorphism with regard to DNA amplification by polymerase chain reaction. The primers which showed polymorphism in parental lines were used for bulked segregant analysis. The primers which amplified reproducibly in the resistant and susceptible bulks were used for single plant analysis of 200 F_2 plants. A RAPD marker C-11_{1000} (5′-AAAGCTGCGG-3′) flanking the black rot resistance gene with a distance of 3.1 cM, was identified. This marker was close enough to the black rot resistance gene to allow a dependable marker-assisted selection (MAS) for black rot resistance. However, more closely linked markers, if identified, would improve the effectiveness of MAS. Selection of plants on the basis of markers is more straightforward than that based on phenotype.

Keywords: gene tagging, linkage map, molecular markers, *X .campestris*

INTRODUCTION

Cabbage (*Brassica oleracea* var. *capitata*) is an important cole crop and belongs to the family *Brassicacae*. It is one of the oldest cultivated vegetables used by the ancient Greeks and Romans as well as one of the most popular winter vegetables in India. Cabbage attracts diseases beginning with seed; one such disease is black rot. It is caused by *Xanthomonas campestris* pv. *campestris*. Black rot was identified on crucifer vegetable crops by Pammel in 1895 on rutabaga. The disease occurs everywhere in the world and is particularly destructive when the climate is warm and wet. The infection of foliage results in a yellow V-shaped lesion (**Fig. 1**) arising from the margin of leaf and extends in the direction of the midrib. These spots are associated with typical discoloration of the veins. Planting resistant cultivars is one of the practicable strategies for management of the disease since chemical use is not only expensive but also health hazardous. Resistance to black rot is non-hypersensitive and severity of the disease is greatly influenced by the prevailing environment during its development (Bain 1955). There is limited work on the genetics of resistance for black rot in cabbage. So far there is no published report on the molecular mapping of black rot resistance in cabbage. The use of molecular markers for the identification of black rot resistant plants in the field would greatly facilitate breeding for resistance to black rot. In this paper we report genetics of black rot resistance and identification of a random amplified polymorphic DNA (RAPD) marker linked to black rot resistance in cabbage.

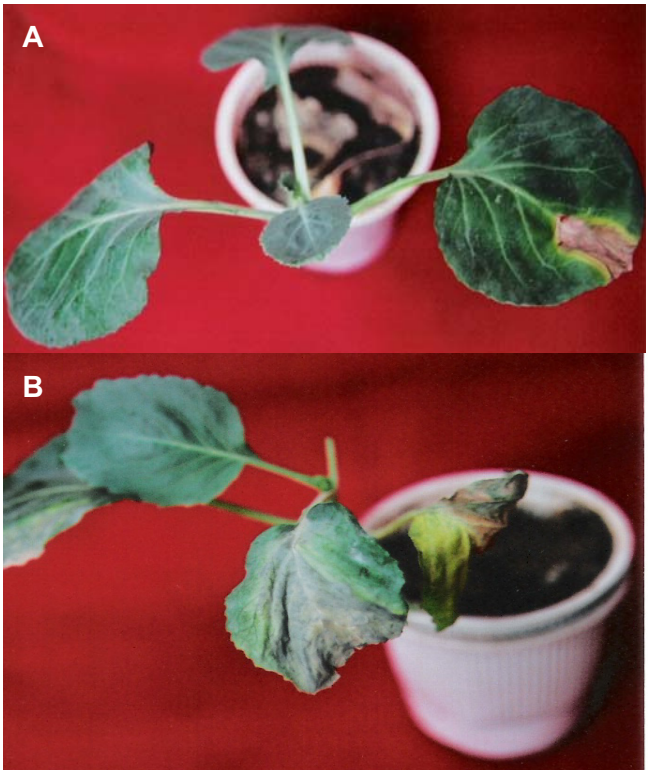

Fig. 1 (A, B) Appearance of V-shaped lesions on *Brassica oleracea* var. *capitata*.

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MATERIALS AND METHODS

Plant material

The parental lines of January King (resistant) and Golden Acre (susceptible) along with their F_2 lines were used to identify the blackrot resistance gene because these parental lines had consistently shown black rot reaction (unpublished data). The mapping population consisted of 200 F_2 (January King × Golden Acre) plants. Genetics of resistance was analyzed using the parental lines and the F_2 population.

Evaluation of black rot resistance

The infected leaves showing definite symptoms of blackrot (**Fig. 1**) were taken from 40-days-old field-grown plants of F_2 population (January King × Golden Acre) and washed under running tap water to remove soil particles. Samples (consisting of 10 pieces of infected leaf of 1 mm^3 size) were treated with sodium hypochlorite for 15-20 sec and then washed twice with distilled water. Explants were cultured into culture tubes (150 mm × 25 mm) having 1 ml of autoclaved distilled water and vortexed for 30 s-1 min and were further referred to as inocula. Slants of nutrient agar (2.5 g peptone, 1.5 g beef extract, 2.5 g NaCl, 10 g agar in 500 ml distilled water; pH 6.5-6.8) were streaked with bacterial innoculum under aseptic conditions. Cultures were incubated at 28°C in an incubator (Microsil Pvt. Ltd., India) for 3-4 days under dark conditions. Circular, yellow and mucoid colonies of bacterium were developed.

A loopful of 72-hr-old culture of bacterial cells was suspended in 100 ml of sterile water. Leaves on 40-days-old plants were inoculated with bacterial suspension (0.5 ml per leaf) by spraying through a sprayer (Modicare India Pvt. limited) in the morning hours. Inoculated plants were covered with polythene bags for 48 hrs to maintain high relative humidity (>90%). Leaves were observed daily for the appearance of symptoms.

Genetics of black rot resistance

Disease reaction, through screening of F_2 plants by pathogen inoculation, was used for studying the inheritance of the resistance gene in January King. The goodness of fit of observed F_2 ratio with expected Mendelian ratio was tested by using a χ^2 test. RAPD data were scored as presence or absence of corresponding bands from parental lines, bulked segregant DNA and single plant analysis of F_2 population. Linkage analysis was performed by using Map Maker, Ver. 3 (Lincoln *et al.* 1993). The marker distance was expressed in cM (centimorgans).

RAPD studies

DNA was extracted from healthy leaves isolated from 30-days-old seedlings of January King (resistant parent), Golden Acre (susceptible parent) and 200 F_2 plants. Genomic DNA was isolated by the Dellaporta *et al.* (1983) method with minor modifications. Bulked segregant analysis (BSA) was used to identify RAPD markers linked to black rot resistance (Michaelmore *et al.* 1991). Resistant and susceptible bulks were prepared by bulking equal quantities of DNA from 10 resistant and 10 susceptible F_2 individuals, respectively.

Genomic DNA of January King and Golden Acre were surveyed for polymorphism using 191 RAPD primers (Kit C and D from Integrated DNA Technologies Inc., San Francisco, USA; primer sets A-C from Operon Technologies Inc. Alameda, California and UBC primers 1-90 from University of British Columbia, Biotechnology Laboratory, USA). Each primer which showed polymorphism for both resistant and susceptible parents was later on tried for screening polymorphism in both parents and two bulks. After that each polymorphic primer was tested with 10 individual plants of each bulk. The primer which was polymorphic was used to screen 200 segregants of the F_2 mapping population.

For DNA amplification, a 20 µl reaction mixture contained 50 ng of template DNA, 1X PCR buffer, 1 mM of each of dNTP, 30 ng primer and 1U of *Taq* DNA polymerase. Amplification was performed for 35 cycles in a thermal cycler Cyclogene Dri-block, Techne, UK. After initial denaturation for 3 min at 95°C, each cycle consisted of 30 sec at 94°C, 1 min at 35°C and for 2 min at 72°C. Amplified products were resolved by electrophoresis in a 1.2% agarose gel with 0.5X TAE buffer containing 0.5 µg/ml ethidium bromide for 3 hr at 5 V/cm. The DNA profile was visualized on a UV transilluminator and photographed by using Alphamager Gel Documentation System.

RESULTS

Blackrot resistance was scored as symptomless on leaves after inoculating plants of the F_2 population with the pathogen. It is added that a few leaves (about 500mg) are collected from 30 days old plants for DNA isolation and for further studies before inoculating the plants. All the plants were numbered from 1 to 200. After 35 days of inoculation of plants, lack of symptoms indicated a resistant reaction and the plants showing total mortality were treated as susceptible. The observed segregation ratio, when compared to the expected ratio in order to establish a χ^2 test, showed a 3:1 ratio that fitted well with that expected for monogenically controlled traits.

For molecular tagging of the blackrot resistance gene genomic DNA of January King (resistant) and Golden Acre (susceptible) lines along with 200 F_2 used for polymorphism survey, 191 random primers were employed, out of which 165 amplified one or more bands from each parental DNA. Monomorphic bands were amplified by 150 primers and these primers were discarded and not used for further analysis. Only 15 primers produced polymorphic banding patterns between both the parents. Six primers OPA-08 (5′-GTGACGTAGG-3′), OPA-16 (5′-AGCCAGCGAA-3′), OPAK-04 (5′-AGGCTCGGTC-3′), C-11 (5′-AAAGCTGC GG-3′), C-13 (5′-AAGCCTGGTC-3′) and C-20 (5′-ACTTC GCCAC-3′) out of 15 polymorphic primers showed a polymorphic banding pattern between both parents and two bulks.

The total number of polymorphic bands identified was 40 with an average number of 6.66 polymorphic bands per primer. The six polymorphic primers used in this analysis yielded a total of 42 scorable bands comprising of 40 polymorphic bands and 2 monomorphic bands with an average of 7 bands per polymorphic primer. Out of these 6 primers, only one primer, C-11 (5′-AAAGCTGCGG-3′) produced clear polymorphic bands between resistant parent, resistant bulk, susceptible parent, susceptible bulk and ten individual plant DNA of both the bulks. A single RAPD band of 1000 bp was produced by C-11 in resistant parent, resistant bulk, and 10 resistant plants of the F_2 population, thus showing its linkage with the black rot resistance gene.

200 F_2 plants of the whole mapping population were used for single plant analysis by using primer C-11 to estimate the linkage of $C\text{-}11_{1000}$ RAPD band with resistance gene in January King. Subscript '1000' indicates that the

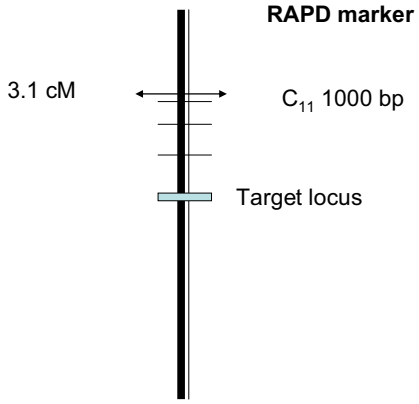

Fig. 2 Linkage map of *Xanthomonas campestris* resistance locus in *Brassica oleracea* var. *capitata* using MAPMAKER ver. 3.0, resistance marker $C\text{-}11_{1000}$ is linked at a distance of 3.1 cM from the resistance locus.

size of the band produced by C-11 is of 1000 bp. PCR amplification showed that fragment generated by C-11 was present in resistant plants at a distance of 3.1 cM from the resistant locus (**Fig. 2**) and absent in susceptible plants indicating co-segregation of the RAPD marker with the resistance gene. However, this band was absent in four plants which were phenotypically resistant but present in three phenotypically susceptible plants, indicating the presence of seven recombinants, which were detected by the marker linked to the resistance gene.

DISCUSSION

There are different DNA markers like RFLP, RAPD, AFLP and STS commonly used for molecular tagging of disease resistance genes in various crop plants (Reddy and Baraoidan 1997; Cherukuri *et al.* 2005; Muylle *et al*. 2005). In addition, these different molecular markers are also being used in marker-assisted selection, gene pyramiding and map-based cloning of *R*-genes. However, in this study, RAPD markers have been chosen to tag blackrot (*Xanthomonas campestris*) resistance gene in cabbage keeping in mind their ease and cost effectiveness.

The foremost requirement for tagging genes is a mapping population and the phenotypic screening of the same. A mapping population is the one which is raised through controlled crosses. The black rot caused by *X. campestris* is one of the most important diseases of cabbage severely affecting its production in various countries, including India. For molecular tagging of black rot resistance gene in cabbage line January King, F_2 was used as a suitable mapping population. Although many other mapping populations such as F_3, backcross, NILs, RILs and doubled haploids have been employed as quite suitable mapping populations in various crop plants for molecular tagging of different genes (Walter *et al.* 1994; Kuginuki *et al.* 1997; Cairo *et al.* 2002; Mohler *et al.* 2005; Ohara *et al.* 2005), however in the present study F_2 developed by crossing a resistant (January King) and susceptible line (Golden Acre) was used as appropriate mapping population.

An attempt was made to analyze the genetics of resistance gene to black rot. 200 plants of F_2 population were uniformly inoculated at the seedling stage with a single isolate of *X. campestris* collected from the Western Himalayan region. The number of F_2 plants was appropriate for segregation studies as it is already used by a number of researchers (Moriguchi *et al.* 1999; Govindaraj *et al.* 2005; Saxena 2006; Kashyap 2008). After screening, a 3:1 ratio was observed suggesting that resistance to black rot is controlled by a single dominant gene (Guo *et al.* 1991; Taylor *et al.* 2002; Vincente *et al.* 2002). The same genetics of resistance has been identified in some other related *Brassica* species (Guo *et al.* 1991; Westman *et al.* 1999; Tonguc *et al.* 2003). When resistance was tested as a Mendelian character, the segregation ratio of the F_2 population fitted well with that expected for a trait controlled as a single locus by a dominant gene as is clear from observation after screening with the bacterial inoculum. Since the number of genes governing the resistance was unknown, the data was analyzed by two methods. A phenotype-cut off (to distinguish resistant from susceptible individuals) was established within the F_2 population by inoculating with the pathogen. After 35 days, total lack of symptoms indicated a resistant reaction and development of V-shaped lesions followed by leaf abscission in plants as the susceptible reaction. And the observed segregation ratio, when compared to the expected ratio in order to establish the χ^2 test, showed a 3:1 ratio.

The second method is to locate the gene by a RAPD marker linked to the resistance gene. Analysis with MAPMAKER Ver. 3.0 software showed that the single RAPD marker was found at a distance of 3.1 cM from the resistance gene.

Molecular mapping of disease resistance and other important traits in cole crops started only recently. Tonguc *et al.* (2003) reported eight RAPD markers associated with completely disease-free plants following inoculations. Vijayalakshmi *et al.* (2005) reported two RAPD markers flanking the rust resistance gene (*Ruf*) in pea with a distance of 10.8 and 24.5 cM, respectively.

In our study, 191 random primers were tested. Only 15 primers produced polymorphism between parents. Bulked segregant analysis (Michelmore *et al.* 1991) was attempted to further narrow down the number of polymorphic primers. Out of 15 polymorphic primers, only six could clearly distinguish the resistant and susceptible bulks. These six primers generated a total of 42 scorable bands out of which 40 (95.23%) were polymorphic. Many studies have exemplified the fact that RAPD analysis in combination with BSA of the F_2 population provides an efficient approach to tag the target gene (Michelmore *et al.* 1991; Chida *et al.* 2000; Yang *et al.* 2004). In an earlier study, Tanhuanpaa (2004) had used 409 RAPD primers for tagging white rust resistance in *Brassica rapa* ssp. *olifera*, but none of them produced suitable markers. RAPD markers in combination with BSA of at least three F_2 populations (P_1, P_2 and P_3) have been used by Tonguc *et al.* (2003) to study segregation distortion of *Brassica carinata*-derived black rot resistance in *Brassica oleracea* var. *capitata* and they observed a significant deviation from the 3:1 ratio expected for a single dominant gene.

REFERENCES

Bain DC (1955) Resistance of cabbage to blackrot. *Phytopathology* **45**, 35-42

Cairo CA, Stein J, Delgado L, Bortolotti S, Guelman SA, Ortiz JPA, Morandi EN (2002) Tagging the juvenile locus in soybean (*Glycine max* L. Merr.) with molecular markers. *Euphytica* **124**, 387-395

Cherukuri DP, Gupta SK, Charpe A, Kaul S, Prabhu KP, Singh RB, Haq QMR (2005) Molecular mapping of *Aegilops speltoides* derived leaf rust resistance gene Lr 28 in wheat. *Euphytica* **143**, 19-26

Chida Y, Okazaki K, Karasawa A, Akashi K, Nakazawa NY, Hase S, Takahashi H, Ehara Y (2000) Isolation of molecular markers linked to the *Cry* locus conferring resistance to *Cucumber mosaic cucumovirus* infection in cowpea. *Journal of General Plant Pathology* **66**, 242-250

Dellaporta SL, Wood J, Hicks JR (1983) A plant DNA minipreperation version II. *Plant Molecular Biology Reporter* **1**, 19-21

Govindaraj P, Arumugachamy S, Maheswaran M (2005) Bulked segregant analysis to detect main effect QTL associated with grain quality parameters in Basmati 370/ASD 16 cross in rice (*Oryza sativa* L.) using SSR markers. *Euphytica* **144**, 61-68

Guo H, Dickson MH, Hunter JF (1991) *Brassica napus* sources of resistance to black rot in crucifers and inheritance of resistance. *HortScience* **127**, 1545-1547

Kashyap M (2008) Studies on gene tagging for resistance to *Xanthomonas campestris* in cauliflower (*Brassica oleracea* var. *botrytis*) using SSR markers. MSc thesis, University of Horticulture and Forestry, Solan, Himachal Pradesh, India, 47 pp

Kuginuki Y, Ajisaka H, Yui M, Yoshikawa H, Hida K, Hirai M (1997) RAPD markers linked to a clubroot resistance locus in *Brassica rapa* L. *Euphytica* **98**, 149-154

Lincoln SE, Daly MJ, Lander ES (1993) Constructing genetic linkage maps with MAPMaker EXP V3.0: A tutorial and reference manual A whitehead institute for biomedical research technical report. Available online: http://linkage.rockefeller.edu/soft/

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Genetics* **88**, 9828-9832

Mohler V, Zeller FJ, Wenzel G, Hsam SLK (2005) Chromosomal location of genes for resistance to powdery mildew in common wheat (*Triticum aestivum* L. en Thell.) a gene *MlZecI* from the *Triticum dicoccoides* derived wheat line *Zecoi-1*. *Euphytica* **142**, 161-167

Moriguchi K, Kimizuka TC, Ishii K, Nomura K (1999) A genetic map based on RAPD, RFLP, isozyme, morphological markers and QTL analysis for clubroot resistance in *Brassica oleracea*. *Breeding Science* **49**, 257-265

Muylle H, Baert J, Van Bockstaele E, Moerkerke B, Goetghebeur E, Roldan RI (2005) Identification of molecular markers linked with crown rust (*Puccinia coronata* f.sp. *lolii*) resistance in perennial ryegrass (*Lolium perenne*) using AFLP markers and a bulked segregant approach. *Euphytica* **143**, 135-144

Ohara T, Song YS, Tsukazaki H, Wako T, Nunome T, Kojima A (2005) Genetic mapping of AFLP markers in Japanese bunching onion (*Allium fistulosum*). *Euphytica* **144**, 255-263

Pammel LH (1895) Bacteriosis of rutabaga (*Bacillus campestris* n. sp.). *Bulletin of the Iowa State College of Agriculture Experimental Station* **27**, 130-

134

Reddy JN, Baraoidan MR (1997) Application of marker assisted selection in rice for bacterial blight resistance gene *Xa21*. *Current Science* **73**, 7-12

Saxena B (2006) Studies on identification of stalk rot (*Sclerotinia sclerotiorum*) resistance gene in cauliflower (*Brassica oleracea* var. *botrytis)* using RAPD markers. MSc thesis, University of Horticulture and Forestry, Solan, Himachal Pradesh, India, 60 pp

Tanhuanpaa P (2004) Identification and mapping of resistance gene analogs and a white rust resistance locus in *Brassica rapa* ssp. *olifera*. *Theoretical and Applied Genetics* **108**, 1039-1046

Taylor JD, Conway J, Roberts SJ, Astley D, Vincente JG (2002) Sources and origin of resistance to *Xanthomonas campestris* pv. *campestris* in *Brassica* genomes. *Phytopathology* **92**, 105-111

Tonguc M, Earle ED, Griffiths PD (2003) Segregation distortion of *Brassica carinata* derived black rot resistance in *Brassica oleracea. Euphytica* **134**, 269-276

Vijayalakshmi S, Yadav K, Kushwaha C, Sarode SB, Srivastava CP, Chand R, Singh BD (2005) Idntification of RAPD markers linked to the rust (*Uromyces fabae*) resistance gene in pea (*Pisum sativum*). *Euphytica* **144**, 265-274

Vincente JG, Taylor JD, Sharpe AG, Parkin IAP, Lydiate DJ, King GJ (2002) Inheritance of race specific resistance to *Xanthomonas campestris* pv. *campestris* in *Brassica* genomes. *Phytopathology* **92**, 1134-1141

Walter KJ, Hosfield GL, Kelly JD (1994) Identification of RAPD markers associated with canning quality in navy beans. *HortScience* **29**, 497-504

Westman AL, Kresovich S, Dickson MH (1999) Regional variation in *Brassica nigra* and other weedy crucifers for disease reaction to *Alternaria brassicola* and *Xanthomonas campestris* pv. *campestris. Euphytica* **106**, 253-259

Yang D-F, Zhang C-L, Zhang D-S, Jin D-M, Weng M-L, Chen S-J, Nguyen H, Wang B (2004) Genetic analysis and molecular mapping of maize (*Zea mays* L.) stalk rot resistance gene *RfgI*. *Theoretical and Applied Genetics* **108**, 706-711