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ORIGINAL ARTICLE

Sequence-tagged site-based diagnostic markers linked to a novel anthracnose resistance gene *RCt1* in chili pepper (*Capsicum annuum* L.)

Rukmini Mishra[1] · Ellojita Rout[1] · Jatindra Nath Mohanty[1] · Raj Kumar Joshi[1,2]

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Abstract

Anthracnose, caused by *Colletotrichum* spp. is the most devastating disease of chili (*Capsicum annuum*) in the tropical and subtropical regions of the world. The present study aimed at molecular mapping and development of markers linked to a new gene for anthracnose resistance in the chili cultivar 'Punjab Lal'. Phenotypic evaluation of F_1, F_2, and BC_1F_1 populations derived from a cross between 'Punjab Lal' and susceptible cultivar 'Arka Lohit' against a virulent isolate of *C. truncatum* revealed that anthracnose resistance in Punjab Lal is governed by a monogenic-dominant gene designated as *RCt1*. Forty-four (28 ISSRs and 16 AFLPs) out of 201 markers exhibited parental polymorphism and were used in bulk segregant analysis. Three ISSRs ($ISSR41_{1493}$, $ISSR58_{1485}$, and $ISSR112_{1857}$) and one AFLP marker ($E\text{-}ACA/M\text{-}CTG_{516}$) showed precise polymorphism between resistant and susceptible bulks, and were used for genotyping F_2 and BC_1 populations. The four putative fragments were converted into sequence-tagged site (STS) markers and southern blotting confirmed their association with the resistance locus. Molecular mapping revealed that the STS markers CtR-431 and CtR-594 were closely linked to the *RCt1* locus in coupling at distances of 1.8 and 2.3 cM, respectively. Furthermore, both of these markers showed the presence of resistance-linked allele in seven genotypes including the highly resistant *C. chinnese* 'PBC932' and *C. baccatum* 'PBC80' while negatively validated in 32 susceptible genotypes. Therefore, CtR431 and CtR-594 could be recommended as efficient diagnostic markers to facilitate the introgression of *RCt1* locus into susceptible chili variants towards the development of high-yielding anthracnose resistance genotypes in *C. annuum* background.

Keywords Capsicum · Anthracnose · *Colletotrichum truncatum* · Resistance locus · ISSR · AFLP · STS marker

Introduction

Capsium annuum (family Solanaceae) is the most commonly cultivated chili species and an indispensable source of vegetable and spice across the tropical and subtropical regions of the world. India is the second largest producer, consumer, and exporter of chili, accounting for 1.39 million tons and contributing 21.5% of the world's total chili production (FAOSTAT 2016). However, a significant decline in chili productivity has been realized in the recent times due to the anthracnose disease caused by the *Colletotrichum* species complex (Kim et al. 2008a; Than et al. 2008a). The pathogen causes both pre and post-harvest fruit rot and spots and blights of aerial plant parts resulting in a significant crop loss across the world including 10% in Korea (Byung 2007), 10 to 54% in India (Ramachandran and Rathnamma 2006), and 20 to 80% in Vietnam (Don et al. 2007). Although a number of *Colletotrichum* species are linked to chili anthracnose, *C. truncatum* (Schwein) (Andrus and Moore 1935) (formerly referred as *C. capsici*) is most predominant and belligerent in India (Ranathunge et al. 2012; Saxena et al. 2014). An estimated yield loss of 29.5%, amounting to US$ 491.67 million, has been reported due to chili anthracnose in India (Garg et al. 2014). Although the management of

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✉ Raj Kumar Joshi
rkjoshi@rdwu.ac.in; rajkumar.joshi@yahoo.co.in

[1] Functional Genomics Laboratory, Centre for Biotechnology, Siksha O Anusandhan University, Bhubaneswar, Odisha, India

[2] Department of Biotechnology, Rama Devi Women's University, Vidya Vihar, Bhubaneswar, Odisha 751022, India

chili anthracnose has been a burning issue for more than a decade, no effective control measures have, so far, been proposed. Currently, practiced application measures for controlling chili anthracnose with fungicides such as Azoxyxtrobin and Mancozeb is costly and only partially effective under environmental conditions that are favorable for pathogen infection (Goswami et al. 2013). Besides, the biological control of anthracnose by inoculation of antagonistic fungi and bacteria is not 100% effective due to variability in the antagonistic effect against different *Colletotrichum* species (Vasanthakumari and Shivana 2013). Thus, molecular breeding through the deployment of resistant cultivars could be the most efficacious and safer management strategy for the economically important *C. truncatum* infection problem.

The inheritance to anthracnose resistance is highly variable based on resistance source and *Colletotrichum* species involved. For instance, resistance of *C. chinense* 'PBC932' and the derived *C. annuum* progressive line 'Daepong-cho' to *C. truncatum* is controlled by a single recessive gene, while that in *C. annuum*'83-168′ is governed by monogenic-dominant gene (Lin et al. 2002; Pakdeevaraporn et al. 2005; Kim et al. 2008a, b). Similarly, *C. baccatum* 'PBC80' exhibit resistance to *C. acutatum* via a dominant gene, while the same in *C. chinense* 'PBC932' and the *C. annuum* 'AR' is controlled by a single recessive factor (Yoon and Park 2005; Kim et al. 2007). In addition, the recent evidences indicated a polygenic inheritance of anthracnose resistance. Voorrips et al. (2004) reported one major and one minor resistant QTL for *C. truncatum* and one major and three minor resistant QTLs for *C. gloeosporoides* in *C. chinense* 'PRI95030' lines. Recently, Chunying et al. (2015) reported one major QTL in chromosome 5 of *C. chinense* 'PBC932' exhibiting broad spectrum resistance against *C. acutatum*. However, none of the above-mentioned genes or QTLs have so far been linked with the *C. annuum* cultivars of Indian subcontinent and their inheritance pattern of anthracnose resistance is still unknown. The recent identification of new natural sources of resistance in the *C. annuum* background (Garg et al. 2013; Mishra 2017, 2018) makes it essential to understand the resistance mechanism in these genotypes for their suitability in marker-assisted breeding programs.

A combination of PCR-based molecular markers and high-throughput phenotyping offer the possibility of identifying disease resistance loci through molecular breeding (Ashkani et al. 2015). The molecular systems such as the amplified fragment length polymorphism (AFLP), inter-simple sequence repeats (ISSRs), short-sequence repeats (SSRs), and single-nucleotide polymorphism (SNP) have been widely used to develop markers linked to disease-resistant traits in many plant species due to their high accuracy and reproducibility (Agarwal et al. 2008). Furthermore, the subsequent cloning and conversion of the ISSRs and AFLPs into sequence-tagged sites (STS) or sequenced characterized amplified regions (SCARs) makes them more reliable, less cumbersome, and breeder friendly markers that increases their specificity in the identification of genetic loci linked to disease resistance (Kar et al. 2014). Therefore, a comprehensive characterization of *C. annuum* genotypes with suitable molecular markers will play an important role in the early identification of *C. truncatum*-resistant chili genotypes in the Indian subcontinent for their successful utilization in the marker-assisted selection program. In the present study, we have ascertained the genetics of anthracnose resistance in the *C. annuum* cultivar 'Punjab Lal' which is designated as resistant to *C. truncatum* in India (Garg et al. 2013; Mishra et al. 2018). We also employed a wide set of ISSR and AFLP markers in a bulk segregant analysis to identify putative resistant fragments linked to anthracnose resistance and their subsequent validation in chili genotypes from different genetic backgrounds.

Materials and methods

Plant material and disease evaluation

The plant materials used in this study included chili cultivars—Punjab Lal and Arka Lohit. Punjab Lal is a natural landrace developed at the Punjab Agricultural University, Ludhiana, India. It is a cayenne-type cultivar with medium-sized plants which produces two inch erect fruits that turn green to purple to red. Arka Lohit is a pure line selection variety developed at the Indian Institute of Horticulture Research (IIHR), Bangalore, India, through a mass selection from the local collection line IHR-324. It has straight dark green fruits that turn deep red on maturity which high pungency. Disease reaction from field evaluation and glass house screening has revealed that Punjab Lal is resistant, while Arka Lohit is highly susceptible to anthracnose caused by *C. truncatum* (Garg et al. 2013; Mishra 2017; Mishra et al. 2018). To analyze the inheritance of anthracnose resistance, Punjab Lal was crossed with Arka Lohit and the plants from F_1 (54 plants), F_2 (255 plants), and BC_1F_1 (99 plants) segregating populations were labeled for phenotypic identification during disease reactions. The F_2 generation chili population was also used for molecular mapping of the anthracnose resistance gene.

Capsicum truncatum virulent isolate MTCC-3414 (obtained from Microbial type Culture Collection, Institute of Microbial Technology, Chandigarh, India) was used for resistance evaluation of the parental lines along with the F_1, F_2, and BC_1 materials. Prior to inoculation, the identity of the pathogen isolate was confirmed through PCR amplification using species-specific primers as described previously (Thind and Jhooty 1990). The disease inoculation experiments were conducted in a temperature-controlled

greenhouse at the Centre for Biotechnology, Siksha O Anusandhan University, Bhubaneswar, India. The microbial isolate was cultured on potato dextrose agar (PDA) medium at 25 °C under 12 h fluorescent light/12 h dark in an incubation chamber for 7 days. A conidial suspension was made by flooding the plates with distilled water and scraping the surface to collect the conidia, and the final concentration was adjusted to 10^6 conidia/ml. Three fruits of mature red ripened stage from each plant were harvested, surface sterilized with 0.1% mercuric chloride ($HgCl_2$), and rinsed twice with distilled water. The fruit pericarp was injected with 1000 spores in 1 µl at both the proximal and distal end using a micro-injector consisting of a micro-syringe model 1705 TLL fitted with a 1 mm micro needle (1 mm length) and a PB600-1 dispenser (Hamilton company, Switzerland). The inoculated fruits were placed in acrylic boxes that were tightly sealed with plastic bags to maintain more than 90% humidity and kept in dark for 48 h. The boxes were then removed from plastic bags and incubated at 25 ± 1 °C with a 12 h dark/ light cycle in a small moist chamber with relative humidity (RH) of 95%. The disease severity was recorded 9 days after inoculation (DAI) and the disease reactions of the plants were determined according to Montri et al. (2009). The frequency of each phenotype was assessed, and a Chi-square test was used to establish the goodness of fit for a Mendelian segregation ratio in the F_2 and BC_1F_1 generations.

DNA isolation and marker analysis

Genomic DNA was extracted from the fresh leaf tissues of young seedlings following the Cetyltrimethyl ammonium bromide (CTAB) method with the desired modifications (Doyle and Doyle 1990). The DNA quality was determined on a 0.8% ethidium bromide stained agarose gel using uncut lambda DNA (Fermentas, USA) as standard. In addition, the DNA concentration was assessed on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, USA), and the DNA samples were diluted to 25 ng/µl in TE buffer for PCR amplification of molecular markers.

A total of 201 molecular markers including 154 ISSRs and 47 AFLPs were used for screening of polymorphism between Punjab Lal and Arka Lohit. Based on stable amplification in *C. annuum* genotypes, additional 86 SSR markers including 49 genomic SSRs and 37 EST-SSRs were also included in the parental polymorphic survey. These markers were retrieved from previously published data based on their stable amplification in *C. annuum* genotypes (Kumar et al. 2001; Rai et al. 2013; Krishnamurthy et al. 2015). However, none of the SSRs could result in any polymorphism between the parents and, therefore, were exempted from further analysis.

ISSR and AFLP analyses

Equal amount of DNA from ten resistant and ten susceptible F_2 plants were bulked to produce resistant and susceptible pools for bulk segregation analysis (BSA) (Michelmore et al. 1991). Based on parental screening, 28 ISSRs and 16 AFLP primer combinations were used to amplify DNA from the two parents and resistant and susceptible bulks for a co-segregating polymorphic marker study. ISSR amplification was carried out in a 25 µL reaction system consisting of 2 µL of 10× reaction buffer (16 mM $(NH)_2SO_4$, 67 mM Tris–HCl with 25 mM $MgCl_2$), 20 pM of ISSR primer, 200 µM dNTP mix, 25 ng of template DNA, and 1 unit of *Taq* DNA polymerase. The PCR reaction was executed in a Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: 94 °C for 5 min tagged on by 40 cycles at 94 °C for 1 min, 45–58 °C (for different primers) for 1 min, 72 °C for 2 min followed by a final extension at 72 °C for 10 min. The amplified products were resolved on 1.5% agarose gel in 1× TBE buffer at 60 volts for 3 h.

The AFLP analysis was performed using the standard protocol (Vos et al. 1995) with desired modifications. 100 ng of genomic DNA of each sample was digested with *EcoRI*/*MseI* restriction endonuclease solution and linked with the corresponding block adapters. Pre-amplification reaction was performed using *EcoRI* primer (5′-GACTGC GTACCAATTCA-3′) and *MseI* primer (5′-GATGAGTCC TGAGTAAC-3′) for 30 cycles at a melting temperature of 94 °C for 30 s, an annealing temperature of 56 °C for 30 s, and elongation temperature of 72 °C for 1 min. A 20-fold dilution of the pre-amplification product was used as template for selective PCR amplification involving 35 cycles including 10 touchdown cycles with annealing temperature reducing from 65 °C to 56 °C (0.7 °C per cycle) followed by 20 cycles with annealing temperature at 56 °C for 30 s. PCR products were separated on a 5% polyacylamide gel with a 50 bp DNA ladder (Fermentas, USA). The co-segregating ISSR and AFLP primers were analyzed at least twice in the entire F_2 population to confirm the putatively linked marker for anthracnose resistance.

Development and analysis of STS marker

The polymorphic-resistant specific ISSR and AFLP fragments were eluted from the gel and purified using the Wizard SV gel and PCR cleanup system (Promega, USA). The purified product was cloned into the pTZ57R/T vector (Insta clone TA cloning kit, Thermo-Fischer Scientific, Waltham, USA) and transformed into competent *Escherichia coli* strain JM109 cells. The positively cloned DNA samples were sequenced using the BigDye Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequenced fragments were subjected to similarity

check in the National Centre for Biotechnology Information (NCBI) database using the BLASTp tool. Sequence-tagged site markers were designed using the Primer3 software (Untergrasser et al. 2012) based on the sequence alignment information.

A set of 20 resistant and susceptible individuals randomly chosen from the F_2 population was used to validate the resistance specificity of the STS markers. PCR was performed on 25 µL reaction volume containing 25 ng of template DNA, 200 µM dNTP mix, 10 µM forward and reverse primers, and 0.5 units of *Taq* Polymerase in 10× PCR buffer. The PCR temperature conditions were: 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 45 s, 72 °C for 1 min, and final extension at 72 °C for 7 min. The amplified product was resolved through agarose gel electrophoresis as described earlier. Subsequently, the putatively linked STS markers were also used to analyze the entire F_2 population to confirm their linkage with anthracnose resistance locus.

Southern blot analysis

Southern blot analysis was carried out according to the protocol described by Kar et al. (2014). Three different restriction endonucleases- *EcoRI, HinDIII* and *XbaI* (Thermo Fischer Scientific, USA) were used to digest the genomic DNA from the resistant line 'Punjab Lal' and the susceptible line 'Arka Lohit'. The electrophoretically separated DNA digest was subsequently blotted onto a nylon membrane filter (Hybond-N+, Amersham Pharmacia Biotech) and baked at 80 °C for 2 h. Digoxigenin labeled probes were designed from STS amplified DNA sequence using a digoxigenin DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland). The nylon membrane blocked with DIG Easy Hyb was subsequently hybridized with DIG labeled STS probe in a hybridization chamber and incubated at 65 °C for 15 h. This was followed by repeated washing of the hybridized nylon membrane with 0.1× SSC buffer (15 mM sodium chloride, 1.5 mM sodium citrate, pH 7.0) containing 0.1% sodium dodecyl sulfate. The possible hybridization between the genomic DNA and STS probe was detected as per the manufacturer's instructions.

Data analysis and genetic mapping

Chi-square test was performed with a probability of 0.05 to determine the goodness of fit for the observed and expected segregation of anthracnose resistance gene using the anthracnose disease phenotype of each F_2 and BC_1F_1 plant derived from Punjab Lal/Arka Lohit cross. Linkage analysis was performed by importing a matrix carrying the F_2 disease phenotype and marker genotype data into the MAPMAKER software ver 3.0 (Lander et al. 1987). A logarithm of odds (LOD) score of > 3.0 was used as threshold to order the segregating markers within the linkage group. The Kosambi mapping function was applied to convert the recombination frequency into map distances in centi Morgans (cM).

Results

Genetics of anthracnose resistance in Punjab Lal

All plants in F_1, F_2, and BC_1 generations were phenotyped as resistant and susceptible based on anthracnose disease score. When inoculated with *C. truncatum* isolate MTCC-3414, Punjab Lal showed a hypersensitive reaction, whereas Arka Lohit was highly susceptible (Fig. S1A and B). The inoculated fruits in the susceptible genotypes started developing sunken necrotic tissues within 3 days after inoculation followed by concentric rings of acervuli in the following days. The disease scoring was recorded at 9 days after infection, and the plants were grouped as resistant and susceptible based on the percentage disease index (PDI) and disease score of '1' and '9', respectively. The susceptible genotypes, including 'Arka Lohit', were heavily infected with PDI ranging between 26.4–73.2%, while the resistant parent 'Punjab Lal' remained largely uninfected with a minimum PDI value of 1.1%. All F_1 genotypes, including the reciprocal F_1, resembled the resistant parent 'Punjab Lal', and the 255 plants from the F_2 population segregated as 190 resistant to 65 susceptible ($\chi^2_{3:1} = 0.033$, $df = 1$, $P = 0.857$) fitting well with the single dominant gene segregation ratio (Table 1). When F_2 plants were transplanted to the field, 197 plants survived to produce F_3 seeds. The evaluation of anthracnose response in the $F_{2:3}$ plants revealed that the ratio of homozygous resistant (RR):segregating resistant (Rr):homozygous susceptible from Punjab Lal/Arka Lohit was consistent with the expected ratio of 1:2:1 (Table 1) (Fig. S1C to S1F). Furthermore, the BC_1F_1 population consisting of 99 plants segregated as 49 resistant and 50 susceptible, thereby satisfying the expected ratio of 1:1 ($\chi^2 = 0.011$, $df = 1$ $P = 0.921$). Therefore, we concluded that anthracnose resistance in *C. annuum* cv. Punjab Lal is conferred by a single dominant gene, which was named as *Resistance to Colletotrichum truncatum 1* (*RCt1*).

Identification of molecular markers linked to *RCt1* locus

Out of 201 (154 ISSRs and 47 AFLPs) molecular markers used in parental polymorphic analysis, 44 (including 28 ISSRs and 16 AFLPs) differentiated the parental lines and were identified as polymorphic markers (Table S1 and S2). 154 ISSR markers resulted in 703 amplified bands in a size range of 200–3500 bp. The ISSR bands varied between 2 and 14 with an average of 6 bands per primer. 28 ISSR

Table 1 Segregation of resistance to *Colletotrichum truncatum* in parental, F_1, F_2, and BC_1 generations for cross of susceptible cultivar Arka Lohit with the resistant cultivar Punjab Lal

Generation	Observed phenotype		Expected ratio (R:S)	χ^2		*P*
	Resistant	Susceptible		Value	*df*	
SP (Arka Lohit)	–	35	All S	–	–	–
RP (Punjab Lal)	35	–	All R	–	–	–
F_1 (SP×RP)	32	–	All R	–	–	–
F_1 (RP×SP)	21	–	All R	–	–	–
F_2 [(SP×RP) selfing]	106	38	3:1	0.147	1	0.701
F_2 [(RP×SP) selfing]	84	27	3:1	0.027	1	0.869
F_2 Combined	190	65	3:1	0.033	1	0.857
F_3 Combined	51/100	46	1:2:1	0.299	1	0.861
BC_1(RP×SPF_1)	26	29	1:1	0.164	1	0.686
BC_1(SP×RPF_1)	23	21	1:1	0.091	1	0.763
BC_1 Combined	49	50	1:1	0.011	1	0.921

bands were found to be polymorphic resulting in 3.98% polymorphism between the parents. Likewise, 1038 fragments were amplified from 47 AFLP primer combinations with an average of 22 bands per marker. Although the majority of AFLP bands were similar in both the parents, 16 were polymorphic with respect to resistant and susceptible parents. Evaluation of 44 polymorphic markers with contrasting resistant (RB) and susceptible bulks (SB) revealed only four of them (three ISSRs and one AFLP) that were specifically distinguished in the resistant and susceptible bulks (Figs. 1, S2). The three ISSR markers, viz., $ISSR41_{1493}$, $ISSR58_{1485}$, and $ISSR112_{1857}$, were amplified specific to resistant parent 'Punjab Lal', resistant bulk and 10 resistant F_2 individuals while absent in the susceptible genotypes as well as the bulk (Fig. S2A–C). All the same, the AFLP primer E-ACA/M-CTG generated a specific amplicon of 516 bp approximately only in the resistant individual and resistant bulk (Fig. S2D). Thus, we reasoned that these four markers were co-segregating with the resistant gene.

The resistance-specific fragments corresponding to the three ISSR and one AFLP markers were cloned into pTZ57R/T vector and sequenced (Figs. S3–S6). Homology assessment of the four sequenced fragment $ISSR41_{1493}$ (Accn no. MF581010), $ISSR58_{1485}$ (MF581014), $ISSR112_{1857}$ (MF581012), and $E\text{-}ACA/M\text{-}CTG_{516}$ (MF581016) using BLASTn against the nucleotide and EST databases in the NCBI homepage revealed no significant similarity between the cloned fragments and coding sequences of any functional genes. In addition, the four sequences did not possess any distinctive open reading frames and have multiple stop codons all along the reading structures. Based on the complete sequence information of the cloned fragments, four pairs of STS markers (CtR-431, CtR-594, CtR-496, and AFLP-376) were developed (Fig. S3–S6). PCR amplification with the four STS markers resulted in distinct unambiguous bands of 431 bp, 594 bp, 496 bp, and 376 bp, respectively, in the resistant parent 'Punjab Lal' and ten resistant F_2 individuals with no bands in the susceptible parent DNA or susceptible F_2 individuals (Fig. 2). To confirm the genomic position of the cloned fragments, the genomic DNA of resistant and susceptible cultivar was allowed to hybridize with digoxigenin labeled STS probes. DNA digested with three different restriction endonucleases (*EcoRI, XbaI and HinDIII*) revealed single-resistant specific bands for all the four STS probes in the southern blots (Fig. 3). This implies that the four STS markers are characterized by single copy sequence within the genome of the resistant cultivar and could be recognized as exclusive markers for the *RCt1* dominant allele conferring resistance to chili anthracnose.

Furthermore, the entire 255 plants from the F_2 population were screened with the four STS markers and the segregation of the marker locus was assessed for goodness of fit to the expected Mendelian ratio of 3:1 to confirm their linkage with anthracnose resistance locus *RCt1*. Individual F_2 plants were scored for the presence or absence of four amplicons, i.e., CtR-431, CtR-594, CtR-496, and AFLP-376. The genotyping pattern of F_2 individuals with the molecular markers CtR-431 and CtR-594 is represented in Fig. 4. The observed phenotypes (anthracnose disease reaction) and the segregation ratio of four molecular markers fitted well with the expected ratio of 3:1 (Tables 2, S3). The information on the STS markers linked to *RCt1* locus conferring anthracnose resistance in *C. annuum* cv. Punjab Lal in provided in Table S4.

Molecular mapping and linkage assignment to *RCt1* locus

The MAPMAKER *compare* and *map* commands were used to correlate the genotypic and phenotypic data of the F_2 population to determine the most probable arrangement within the linkage group for the resistance gene *RCt1*. The analysis resulted in a linkage map of four linked markers with the resistance *RCt1* locus spanning a distance of 16.9 cM

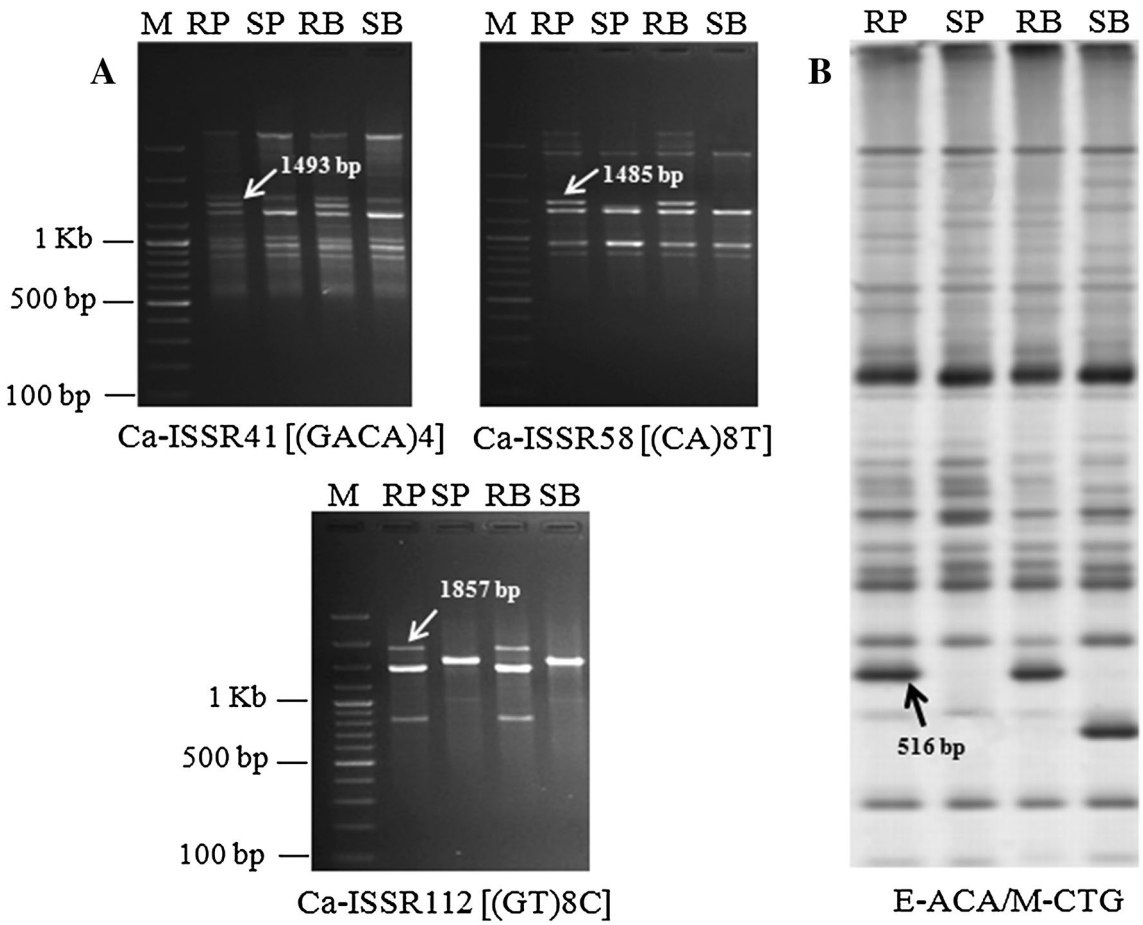

Fig. 1 Bulk segregant analysis towards identification of ISSR (**a**) and AFLP (**b**) markers linked to anthracnose resistance in *Capsicum annuum*. Amplification was performed with a total of 44 polymorphic markers (28 ISSR and 16 AFLP) with individual and bulked DNA from chili genotypes either susceptible or resistant to anthracnose. M, molecular weight marker; R, Resistant parent 'Punjab Lal'; S, Susceptible parent 'Arka Lohit'; RB, bulk DNA from ten resistant F_2 individuals; SB, bulk DNA from ten susceptible F_2 individuals. The arrow represents the polymorphic band in the resistant individual. The number represents the size of the polymorphic band

(Fig. 5). Two flanking STS markers, CtR-431 and CtR594, were estimated to be located at a genetic distance of 1.8 cM and 2.3 cM from the resistant gene, respectively. Similarly, CtR496 was situated at 7.2 cM and AFLP-376 lay at a distance of 7.9 cM from the *RCt1* locus. This is the first report of the development of molecular marker linked to anthracnose resistance locus *RCt1* in *C. annuum*.

Validation of *RCt1*-linked markers

The two closely linked markers CtR-431 and CtR-594 were further genotyped on a set of 41 chili pepper lines to verify the presence or absence of a resistant RCt1 allele (Table 3). The marker CtR-431 and CtR-594 amplified a fragment of 431 and 594 bp, respectively, in the resistant parent 'Punjab Lal' as well as in another five chili genotypes, including BS35, Pant-C1, Achar Lanka, CA-4, and Bhut Jolokia, that has been previously identified as resistant to anthracnose (Garg et al. 2013; Mishra 2017). Interestingly, both the alleles were also amplified in the *C. chinense* var. PBC932 and *C. baccatum* var. PBC80, which have been formerly identified as natural sources of resistance to chili anthracnose (AVRDC 2003). However, the remaining 32 susceptible genotypes, including the susceptible parent 'Arka Lohit' could not amply either of the *RCt1*-linked markers. Therefore, the dominant markers CtR-431 and CtR-594 could be highly useful for marker-assisted selection of *RCt1* gene in the background of these chili genotypes.

Discussion

Although a combination of different strategies, including chemical, physical, and biological approaches, has been recommended for managing chili anthracnose, the complexity of the causal pathogen and emergence of insensitive races

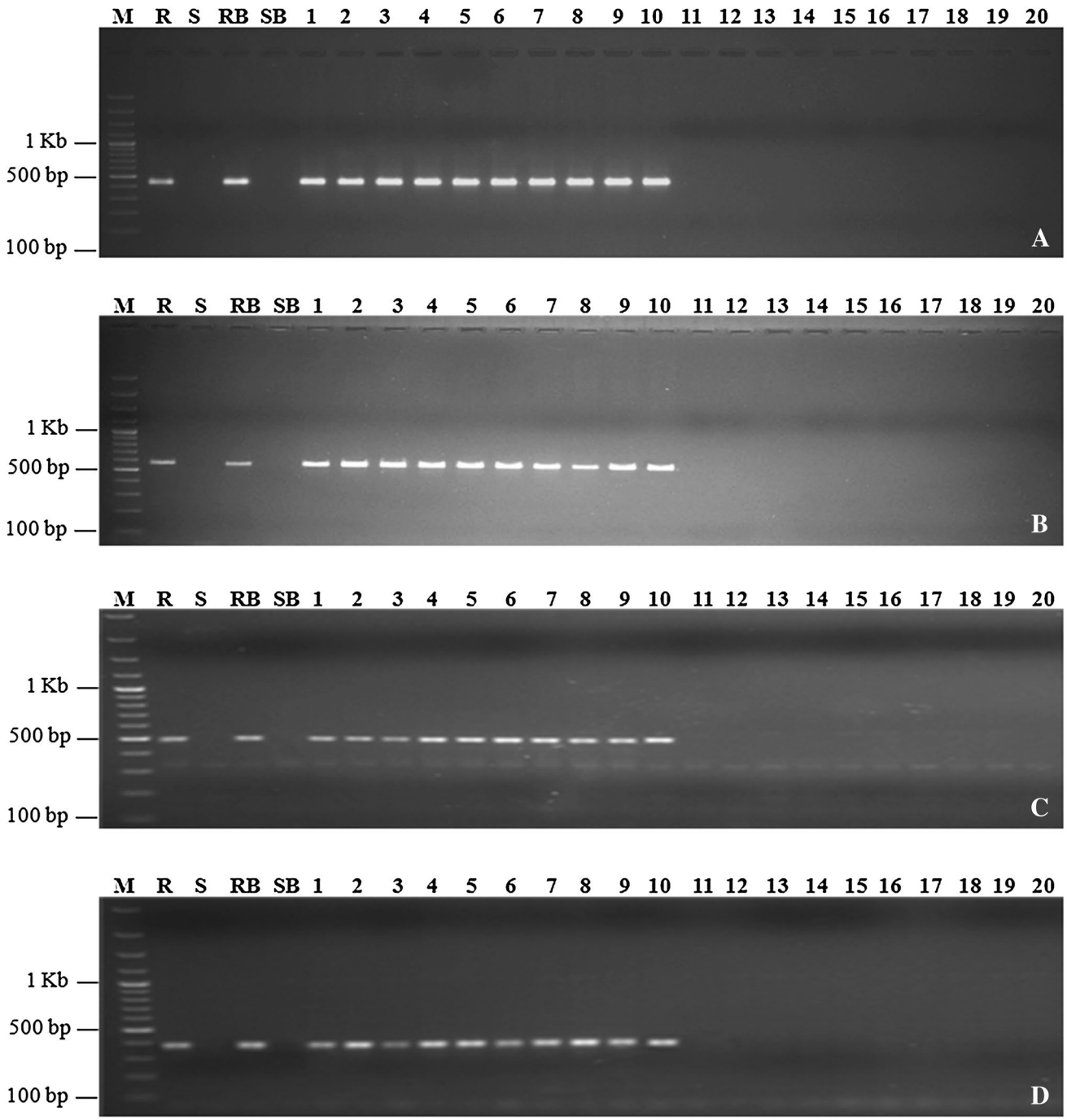

Fig. 2 Validation of STS markers CtR-431 (**a**), CtR-594 (**b**), CtR-496, and AFLP-376 in F_2 individuals either resistant or susceptible to chili anthracnose. M, molecular weight marker; *R* resistant genotype- Punjab Lal; *S* susceptible genotype- Arka Lohit, F1, F_1 individual; 1–10, F_2 individuals resistant to anthracnose; 11–20, F_2 individuals susceptible to anthracnose

makes these techniques only partially effective. Hence, the most practical, cost-effective, and safer disease management strategy involves the development of high-yielding anthracnose-resistant cultivars. Comprehensive understanding about the genetic basis of resistance in the parental genotype is a prerequisite for effective molecular tagging (Dhole and Reddy 2013). *C. annuum* cv. 'Punjab Lal' that was previously reported as a resistant genotype to *C. truncatum* infection (Garg et al. 2013; Mishra 2017; Mishra et al. 2018) was equally vindicated by disease reaction in the present study. Therefore, 'Punjab Lal' could be used for transferring resistance against *C. truncatum* into commercial high-yielding

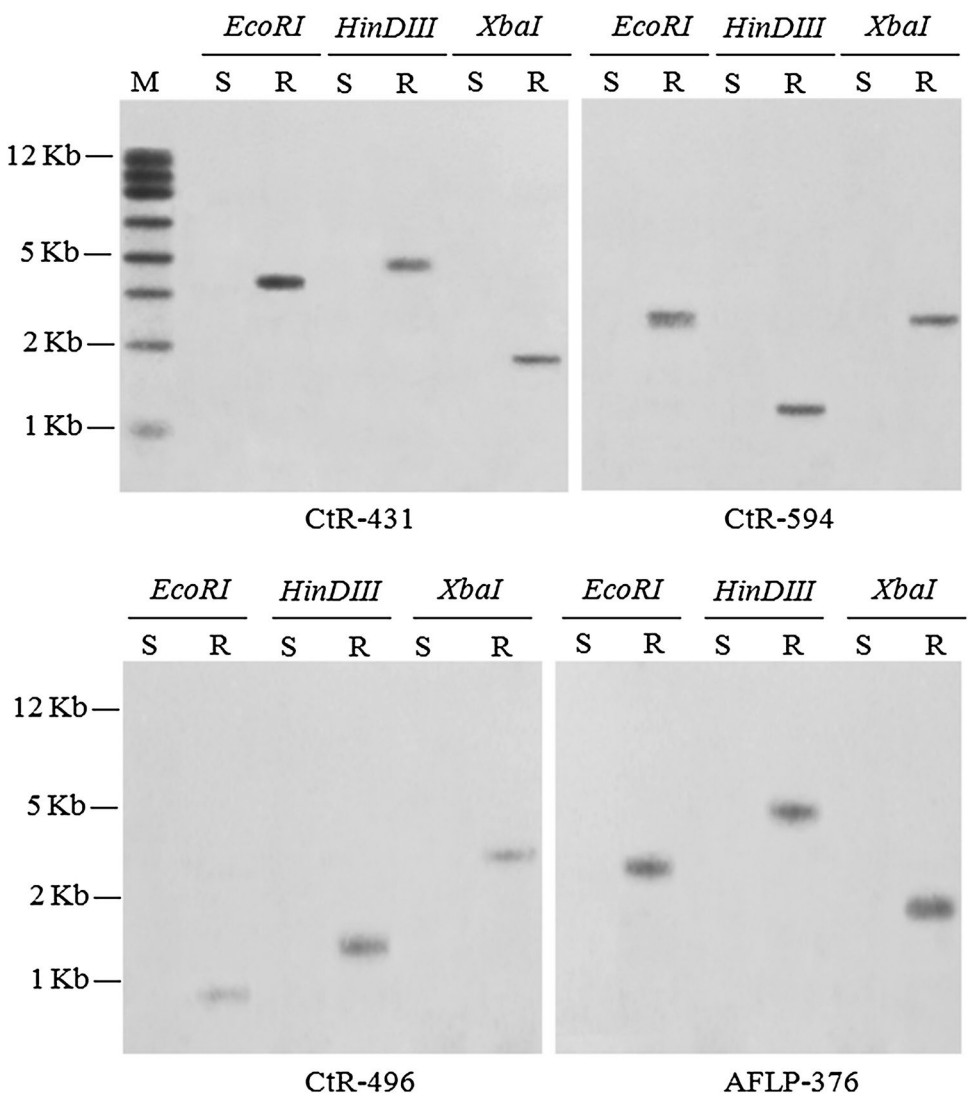

Fig. 3 Southern blot analysis of the four STS markers- CtR-431, CtR-594., CtR-496, and AFLP-376. Restriction enzymes used are *EcoRI, HinDIII* and *XbaI*. M, molecular weight marker; *R* resistant genotype Punjab Lal; *S* susceptible genotype Arka Lohit. Hybridization patterns of the genomic DNA digested with all the three enzymes revealed single band only in the resistant genotype

susceptible chili varieties. Phenotypic reaction of F_1, F_2, and BC_1F_1 population for *C. truncatum* resistance and susceptibility revealed that the resistance in 'Punjab Lal' is governed by a single dominant gene named as *RCt1*. *C. chinense* as well as the progressive lines of *C. annuum* developed from it exhibited resistance against *C. truncatum* by effect of a single recessive gene in green and red fruits (Pakdeevaraporn et al. 2005; Kim et al. 2008a, b; Mahasuk et al. 2009). In contrast, the *C. annuum* breeding lines'83-168′ and 'Chungryong' reported monogenic-dominant resistance (Lin e al. 2002). In addition, progressive lines of chili developed from a cross between *C. annuum* and *C. chinense* reported polygenic inheritance for *C. truncatum* resistance with at least one major QTL linked to infection frequency and lesion diameter (Voorrips et al 2004). These reports together with our findings from the present study suggest that inheritance of resistance to anthracnose caused by *C. truncatum* is highly variable with respect to resistance sources, pathotypes, and fruit maturity stages (Chunying et al. 2015).

Two SSR markers, an SCAR-insertion/deletion (Indel) marker and an STS marker, have been previously developed for the selection of *C. chinense* and *C. baccatum* specific fragment in progressive lines of *C. annuum* (Lee et al. 2010, 2011; Wang 2011). However, the SCAR indel and the particular STS marker are no longer reliable, since the positive PCR product tagging the resistance gene has become obvious in susceptible chili accessions (Suwor et al. 2015). Besides, the two SSR markers also failed to amplify the expected fragments in the *C. annuum* accessions used in the present study, even though the PCR reaction was successful. Therefore, a new set of reliable PCR-based markers for the identification of *C. annuum* fragments containing the gene conferring resistance to anthracnose in chili were developed in this study. Forty-four polymorphic primers including 28 ISSRs and 16 AFLPs were used to identify molecular markers associated with anthracnose resistance in *C. annuum*. Usage of ISSR in gene tagging experiments have several

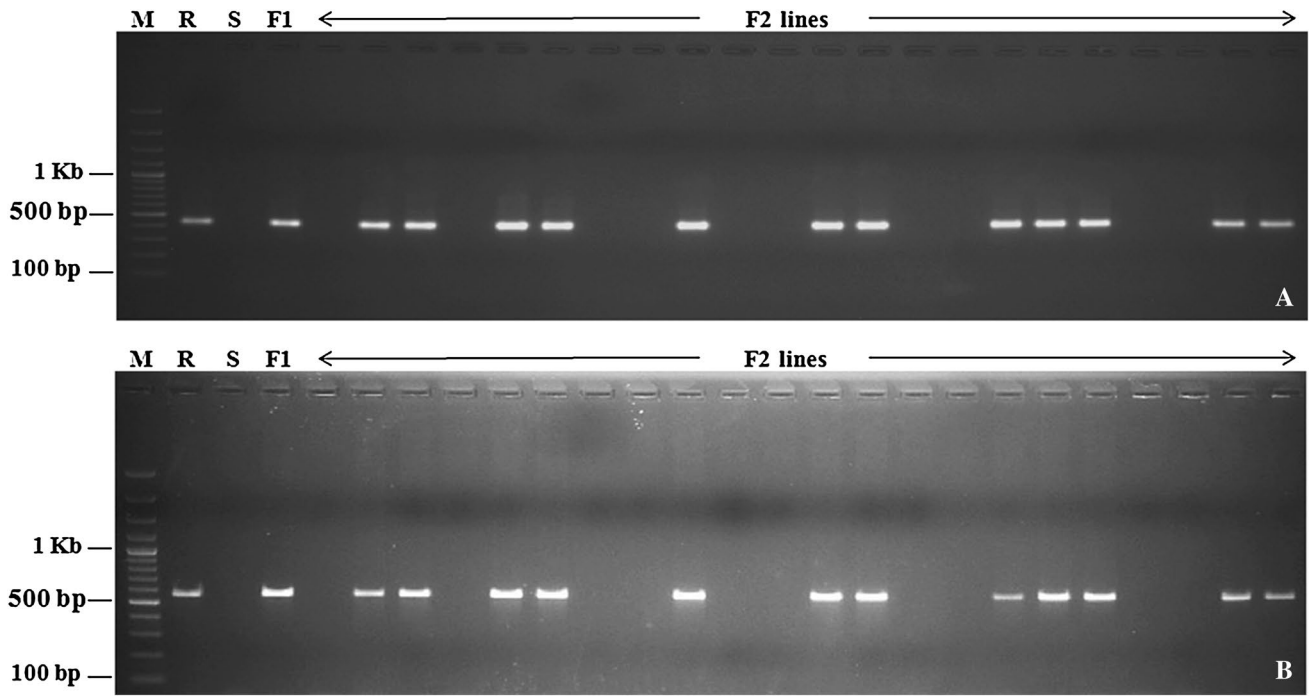

Fig. 4 Genotyping of F_2 mapping population derived from the cross Punjab Lal × Arka Lohit using closely linked markers CtR-431 (**a**) and CtR-594 (**b**). M, molecular weight marker; R, resistant genotype- Punjab Lal; S, susceptible genotype- Arka Lohit, F1, F_1 individual; F_2 lines, individuals from the F_2 populations. The details of the phenotypic expression of the F_2 genotypes are represented in supplementary table S3

Table 2 Segregation analysis of the polymorphic molecular markers with resistance locus *Rct1*

Marker	Observed phenotype		Expected ratio (R:S)	$\chi 2$	*P* value
	Resistant	Susceptible			
Observed phenotype (F_2 combined)	190	65	3:1	0.033	0.857
CtR-431	190	65	3:1	0.033	0.857
CtR-594	189	66	3:1	0.106	0.745
CtR-496	183	72	3:1	1.424	0.233
AFLP-376	183	72	3:1	1.424	0.233

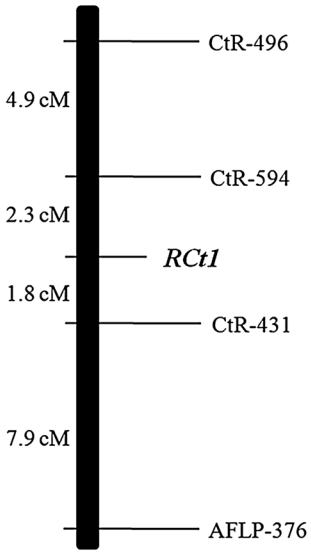

Fig. 5 The genetic linkage map of *RCt1* locus conferring resistance to Anthracnose in *Capsicum annuum*. The name of the marker and resistance gene locus are represented on the right side of the map and the estimated distances are presented on the left side

advantages such as high reproducibility, no requirement for prior sequence information, and multilocus amplification from both coding and non-coding regions (Agarwal et al. 2008). In addition, the amplification of fragments delimited by simple repeat sequences using ISSR primers is quick and easy given that there is a large abundance of SSRs in the eukaryotic genomes (Kar et al. 2014). Likewise, AFLPs are also useful in the analysis of genetic relationship between individuals in a population owing to high stability, rapid assay for detection of a wide range of polymorphism and efficient genome coverage for the creation of quick genetic maps (Chial 2008). ISSR- and AFLP-based molecular tagging of resistance genes has been carried out in multiple plant species including rice (Gowda et al. 2006), wheat (Li et al. 2007; Balta et al.

Table 3 Validation of *CtR1* linked marker CtR1-431 and CtR1-594 in different chili genotypes

Marker	CtR1-431 allele size (bp)	CtR1-594 allele size (bp)	Response
Punjab Lal carrying *CtR1* locus	431	594	Resistant
PBC80, PBC932	431	594	Resistant
Pant-C1, Achar Lanka, CA-4, BS35, Bhut Jolokia	431	594	Resistant
Arka Lohit	Null	Null	Susceptible
IC383072, EC566320, EC341075, Akola 10, Jayanti, Phule Jyoti, MI 2, Pusa Jwala, PBC 374, PBC 542, PBC 602, Dharitri, Jagannath, Black Chili, IR-8, Sadabahar, Arka Suphal, Arka Mohini, K-1, K-2, Barnali, Devil's hot, Bullet, Surya Supreme, Jwala, Teja Jhal, Kolkaati Lanka, Kacchegosani, CA-1,CA-2, CA-3, G4	Null	Null	Susceptible

2014), tobacco (Zhang et al. 2012), turmeric (Kar et al. 2014), and more recently in black gram (Gupta et al. 2015) and cauliflower (Singh et al. 2015).

Broad spectrum information about the genetics of pathogen resistance and location of resistant gene is crucial for marker-assisted selection towards the development of durable resistance in susceptible genotypes (Sharma et al. 2016). The rapid detection of highly enriched DNA polymorphism linked to anthracnose resistance *RCt1* locus was made possible using a BSA approach as have been reported earlier by several researchers (Kar et al. 2014; Sharma et al. 2016; Zou et al. 2016). In the recent times, the congregation of BSA with high-throughput genotyping approaches like microarrays and next-generation sequencing has resulted in rapid identification of genomic regions linked to desired phenotypes in numerous species (Becker et al. 2011; Kim et al. 2015; Win et al. 2016). The four putatively linked markers ($ISSR41_{1493}$, $ISSR58_{1485}$, $ISSR112_{1857}$ and E-ACA/M-CTG $_{516}$) as identified through BSA were co-segregated with the disease reactions in the F_2 individuals and showed a good fit of 3:1 ratio. A further segregation of these markers in 1:1 ratio in the BC_1F_1 population confirmed their linkage with anthracnose resistance locus. As the ISSR and AFLP marker assays are complex, expensive, and generate multiple genetic loci, it is essential that they are converted into easy and reliable STSs to increase the efficiency and effectiveness for selecting resistant genotypes. Therefore, the four putative ISSR and AFLP markers identified in the present study were subsequently converted into PCR-based STS markers, viz., CtR431, CtR-594, CtR-496, and AFLP-376. Several studies have reported the development and utilization of STS markers in molecular mapping and marker-assisted selection of disease-resistant traits in multiple species including chili (Lee et al. 2011a, b; Chunying et al. 2015), wheat (Goutam et al. 2015) and rice (Luo et al. 2016). The four STSs demonstrated specific amplification only in the resistant genotypes which was further confirmed by the development of single hybridization fragment through gel blot assay. In addition, several primer sets designed for amplifying the internal and flanking regions of STSs produced the expected PCR bands in only the resistant parent and not in the susceptible parent (data not shown). This implies that the sequences of the four STSs were only present in the resistant parent and could be localized to a chromosomal region that is tightly linked to anthracnose resistance locus *RCt1*. In addition, the STSs were arbitrary fragments and revealed no sequence similarity with any known genes, suggesting that they are probably located in the intronic or non-coding regions as has been previously reported in the other plant species (Nanda et al. 2013; Kar et al. 2014). This is interesting, because multiple introns have been identified as regulators in the eukaryotic gene expression as well as the facilitators of foreign gene expression in transgenic tissues (Jeong et al. 2006; Lu et al. 2008). A similar role for the STSs identified in the present study cannot be ruled out given their unique linkage with anthracnose resistance response. However, additional experimental validation through knockout analysis of these fragments is needed to confirm the role of such intronic sequences in the regulation of resistance gene expression.

Molecular markers identified within 5 cM from the linked locus have a greater accuracy for indirect selection through MAS as they exhibit minimum recombination frequency (Randhawa et al. 2014; Ma et al. 2015). Besides, the accuracy for selection of resistance trait could be considerably increased using a pair of markers flanking the gene of interest. Therefore, the two linked markers—CtR-431 and CtR-594—closely flanking the *RCt1* locus within a span of 4.1 cM could be recommended for resistance breeding in chili as they are expected to increase the accuracy for selecting anthracnose-resistant lines in segregating population. Successful application of MAS in plant breeding programs demands for precise identification and validation of linked markers in genotypes with different genetic background (Randhawa et al. 2014). The validation of the STS markers CtR-431 and CtR-594 linked to anthracnose resistance in different genotypes with the known anthracnose reaction revealed steady association of the markers in seven anthracnose-resistant genotypes and

absent in all the 32 anthracnose susceptible genotypes. This confirms the presence of these markers with anthracnose resistance gene in different genetic backgrounds and, therefore, could be used to speed up the introgression of *RCt1* locus in the susceptible chili variants for the development of high-yielding anthracnose resistance genotypes through MAS.

Conclusion

In conclusion, the molecular and phenotypic segregation of the F_2 and BC_1F_1 population suggested that resistance to *C. truncatum* infection in *C. annuum* cultivar'Punjab Lal' is governed by a monogenic-dominant gene *RCt1*. Although several anthracnose resistance genes and QTLs have been previously identified from allied species and progressive lines of cultivated chili, this is the first report of molecular tagging of an anthracnose resistance locus in the *C. annuum* background. *RCt1* gene is flanked by four putatively linked STS markers, two of which (CtR-431 and CtR-594) could be significantly applied in MAS with high accuracy for selection of resistant trait. As we could not amplify or link any SSR markers to the resistance locus, the chromosomal location of the *RCt1* in the *C. annuum* genome is yet to be ascertained. However, it is necessary to employ more robust and advanced DNA marker such as single-nucleotide polymorphism (SNPs) to fine map the resistance locus with more closely linked markers. Hence, the identification of new anthracnose resistance gene(s) in the *C. annuum* background together with development of tightly linked markers would be of huge significance towards development of pre-breeding anthracnose-resistant genetic lines in cultivated chili through marker-assisted breeding.

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Author contributions RM and RKJ conceived and supervised the project. RM collected samples, isolated DNA, and performed molecular marker analysis and detection of linked markers. ER and JNM cloned the STS fragments and performed marker validation. RM and RKJ interpreted the data and prepared the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors have declared that there is no conflict of interest.

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