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Identification of sequence-characterized amplified regions (SCARs) markers linking resistance to powdery mildew in chilli pepper (*Capsicum annuum* L.)

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Powdery mildew disease in chilli pepper caused by *Leveillula taurica* has been affecting chilli pepper grown in greenhouse and open fields. Inheritance of powdery mildew disease resistance is complex and least understood. In the present study, we identified SCAR markers using F_2 mapping populations developed from a cross between a resistant parental line 'Odisha Local' and a parental line susceptible to powdery mildew '9907-9611'. The nucleotide sequence obtained from a genome of resistant chilli pepper line 'Odisha Local' using OPA15 primer showed partial identity with RPP13 like disease resistant protein. The molecular markers developed in this study will be very helpful in chilli pepper breeding programs for powdery mildew resistance for indirect selection of the resistant plants.

Key words: Chilli pepper, powdery mildew, SCAR markers.

INTRODUCTION

Chilli pepper (*Capsicum annuum* L.) is an economically important spice crop that is widely cultivated in India as well as in tropical and sub-tropical countries. Its primary use is for culinary purposes, as a spice added to various dishes and sauces. Some varieties are commercially cultivated for capsaicin. India is the largest exporter of chilli as it exported 0.4 million tones of dry chilli in 2016-17 (Spices Board, 2018).

Chilli pepper is susceptible to many fungal and bacterial diseases affecting yield. Among fungal diseases, powdery mildew caused by *Leveillula taurica* (LEV.) is an obligate fungal plant pathogen belonging to the ascomycetes, which infects various vegetable crops, resulting in very significant yield losses and quality deterioration. The incidence of the powdery mildew disease in Chilli pepper has been showing an upward trend in both open field and protected net-houses worldwide (Jinkwan et al., 2017). It is one of the important diseases causing up to 80% loss in yield due to severe defoliation and reduction in photosynthesis resulting in less number of fruits and affecting quality of marketable yield (Mathur et al., 1972; Sivaprakasam et al., 1976; Gohokar and Peshney, 1981). The disease appears as white powdery coating on ventral side of leaves and

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correspondingly produces yellow patches on dorsal side. It usually spreads to branches of plants and fruits resulting in dropping of affected fruits.

The fungi causing powdery mildew is epiphytic and *L. taurica* is an endophytic fungus, which makes chemical control difficult (Elad et al., 2007). Therefore, developing powdery mildew disease resistance in chilli pepper is one of the main objectives of breeding programs (Jinkwan et al., 2017).

Phenotypic screening by assays is used commonly in breeding programs (Ottoman et al., 2009); however, they are expensive, laborious, inefficient and time consuming. Closely linked molecular markers to resistance genes can help breeders overcome these difficulties. Molecular markers are very effective and efficient mean in plant breeding for indirect selections and introgression of traits in certain genotypes. Thus, the identification of markers linked to genes controlling resistance/tolerance to biotic and abiotic stresses plays an important role in plant breeding programs. Therefore, globally, the main goal of pepper breeders is to develop disease resistant varieties or planting material (Jinkwan et al., 2017).

Random amplified polymorphic DNA (RAPD) technique was developed by Williams et al. in 1990 by using random primers which allows quick construction of genetic maps or the saturation of genomic regions of interest (Paran and Michelmore, 1993). RAPD technique is easy, using less quantity of DNA. However, RAPD has some limitations and its results are not always reproducible; it shows dominant inheritance and cannot be converted to codominant markers (Mishra, 2014), and is sensitive to changes in reaction conditions (Paran and Michelmore, 1993). To remove these limitations, Paran and Michelmore (1993) had developed sequence-characterized amplified regions (SCARs) as PCR based molecular markers. SCAR is a genomic DNA fragment that is identified by PCR amplification using a pair of specific oligonucleotide primers (Paran and Michelmore, 1993). SCAR markers have many advantages over RAPD marker. They are less sensitive to reaction condition (Paran and Michelmore, 1993). PCR amplification of the SCARs is reproducible and easy to score (Weng et al., 1998). Therefore, analysis using SCAR markers are fast, easy and very straightforward. The aim of the present study was to identify SCAR markers linked to powdery mildew gene that would help breeders in indirect selection for the trait in efficient and fast manner.

MATERIALS AND METHODS

Field screening and disease evaluation

In rainy season of 2012, an experiment was conducted to screen 19 chilli pepper genotypes against powdery mildew in field conditions. The observations were recorded at 15 days interval starting from 60 to 180 days after transplanting using 0-9 disease scale (Mayee and Datar, 1986) (Table 1). The per cent disease index (PDI) was calculated as per the formula given by Wheeler (1969):

$$\text{PDI} = \frac{\text{Sum of numerical values grades}}{\text{Number of plants observed}} \times \frac{100}{\text{Maximum disease rating}}$$

Plant materials

Parental lines “Odisha Local” and 9907-9611 highly resistant and susceptible to powdery mildew, respectively were selected for the present experiment. The F_1 plants were derived from a cross between “Odisha Local” and 9907-9611. F_1 hybrid was self-pollinated to produce F_2 seeds in summer of 2013. In rainy season of 2013, we evaluated 199 F_2 plant populations along with parental lines for *L. taurica* disease resistance and observations were taken till 180 days of planting using 0-9 disease scale (Mayee and Datar, 1986) (Table 1). Powdery mildew infection was scored by the appearance of mycelia growth on the leaf surface of plants and susceptible parental line also used as susceptible check (Figure 4). There was no fungal hyphae growth on powdery mildew resistant “Odisha Local” (Figure 1). In F_2 population, plants scoring zero were considered as immune, plants scored as one to seven were phenotyped as highly resistant to moderately susceptible in various classes and plant scored nine were phenotyped as susceptible. Jinkwan et al. (2017) also adapted such method to score powdery mildew disease pressure in pepper.

In SCAR marker development procedure, unequal DNA quantity from individual plants increases variation in PCR and electrophoresis quantification of alleles. Daniels et al. (1998) suggested that the DNA pooling method reduces variation, is efficient, fast reliable method to detect differences in allele frequencies and to handle large numbers of sample. To achieve this, 20 plants each were selected in highly resistant “Odisha Local” parent and highly susceptible “9907-9611” based on phenotypic scores relating to disease reaction for making two separate DNA pools, that is, resistant and susceptible DNA pool. Young, fresh and healthy leaves were collected from selected plants of both the parental lines and stored with silica gel in separate zip-lock plastic bags.

Genomic DNA extraction

Genomic DNA from susceptible and resistant parental plant tissue was extracted separately to make resistant and susceptible DNA pool, following procedure by Wang et al. (2011) and based on guanidinium thiocyanate reagent. The quality and quantity of the gDNA were analyzed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The pooled samples were used to identify putative markers for the powdery mildew resistance in Chilli.

RAPD

PCR was performed as per RAPD method described by Williams et al. (1990). The DNA pool from susceptible and resistant plant population and 200 different decamer oligonucleotide primers (Series A, B, C, D, E, F, G, K, L and BA from Operon Technologies Inc., Alameda, CA, USA) were used in PCR. The series represents different primer kits of RAPD markers. Each kit contains 20 individual 10-mer primers (supplied at a minimum quantity of 50 nmole per primer). In the RAPD technique, a single 10mer of arbitrary sequence is used as a primer in PCR to amplify genomic DNA where the sequence of the DNA is completely unknown. The reaction mixtures contained 1 × PCR Buffer (10 mM Tris pH 8.8, 50 mM KCl, 0.08% Nonidet P40; Fermentas, Lithuania), 160 µM of each dNTP, 530 pM oligonucleotide primer, 1.5 mM $MgCl_2$, 35 ng of

Table 1. Scale for Powdery mildew disease in chilli.

Grade	Symptoms and host reaction
0	Immune (I) - No symptom of powdery mildew
1	Highly Resistant (HR) - Small scattered powdery mildew specks covering 1% or less leaf area
3	Resistant (R) - Small powdery lesions covering 1-10% of leaf area
5	Moderately Resistant (MR) - Powdery lesions enlarged covering 11-25% of leaf area
7	Moderately Susceptible (MS) - Powdery lesions coalesce to form big patches covering 26-50% of leaf area
9	Highly Susceptible (HS) - Big powdery patches covering 51% or more of leaf area and defoliation occur

Figure 1. 'Odisha Local' (Powdery milder resistant) and 9907-9611 (Powdery mildew sensitive).

template DNA, 0.5 U Taq DNA Polymerase (Fermentas, Lithuania) in a final reaction mixture of 15 µl. Amplification was carried out in Biometra T1 thermal cycler programmed for 94°C for five min, followed by 39 cycles at 94°C for 1 min, 42°C for 1 min and 72°C for 1 min 30 s, terminating with a final extension at 72°C for 10 min. Amplification products were separated on 1.5% agarose gels containing 0.1% EtBr. Fragments were visualised under a UV transilluminator and archived using DigiGenius (Syn-Gene) system. Those products that were able to differentiate the studied DNA pools were isolated from agarose gel using MiniElute (Qiagen). The amplified products were cloned using TOPO TA Cloning Kit (Thermofisher) following the manufacturer's instructions and sequenced using BigDye terminator cycle sequencing kit (Applied Biosystems) using an automated DNA sequencing system (3130 Genetic Analyzer -Applied Biosystems) (Sambrook et al., 1989). Two independent clones for each set were sequenced in both orientations by using universal M13 forward and reverse primers. Nucleotide sequences were analyzed using the Contig-Express and AlignX tools available in Vector NTI software version 6.0 (Invitrogen) (Jinkwan et al., 2017; Kunkalikar et al., 2012).

Each RAPD primer was tested at least three times to ensure reproducibility of polymorphism and the banding patterns. The OPA-15 primer (Table 2) consistently yielded 1.1 Kb and 0.9 Kb amplicons in susceptible and resistant parents respectively. The amplified products were sequenced and all the obtained sequences submitted to the GenBank database (MH172153). Nucleotide sequences were analyzed using the Contig-Express and AlignX tools available in Vector NTI software version 6.0 (Invitrogen) and then compared with corresponding sequences available in GenBank (Kunkalikar et al., 2012).

Development of SCAR markers

On the basis of alignment of sequences of 1.1 Kb and 0.9 Kb DNA fragments amplified with OPA15 primers, the specific forward

Table 2. RAPD primers amplifying DNA fragments specific for resistant and susceptible pools in Odisha Local" x 9907-9611 population.

Primer	Sequence
SR1	GGTGCGGGAA
SR2	GTTTCGCTCC
OPV19	GGGTGTGCAG
OPA15	TTCCGAACCC
OPY 02	CATCGCCGCA
OPC02	GTGAGGCGTC
OPA 17	GACCGCTTGT
OPM 07	CCGTGACTC

Table 3. Primers for SCAR markers.

Primer	Sequence
OPA15-SFP	CGAATAAGGGCTTTGGCCTAATTCA
OPA15-RFP	GATTTAGTCGAGGTGCATGAAAGT
OPA15-CRP	TAYSARGCAGARYTASWRWTCCAAGT

Table 4. List of IUPAC degenerate nucleotide codes.

Codes	Nucleotide	Codes
A	Adenine	A
C	Cytosine	C
G	Guanine	G
T	Thymine (DNA)	T
U	Uracil (RNA)	U
W	Weak	A/T
S	Strong	C/G
M	Amino	A/C
K	Keto	G/T
R	Purine	A/G
Y	Pyrimidine	C/T
B	Not A	C/G/T
D	Not C	A/G/T
H	Not G	A/C/T
V	Not T	A/C/G
N	Any	A/C/G/T

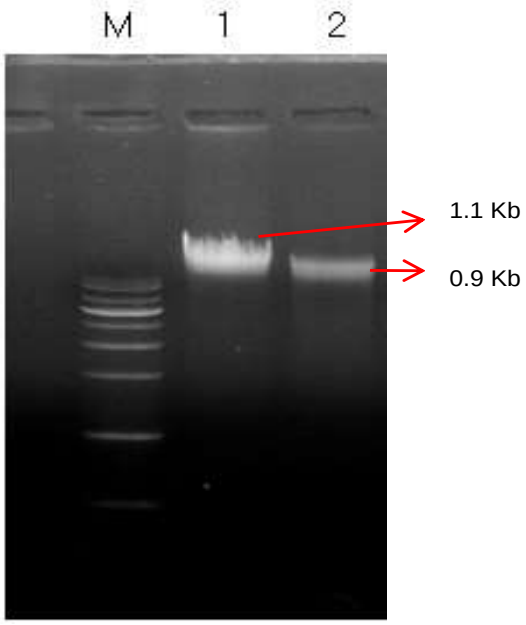

Figure 2. Amplicons of 1.1 Kb (susceptible phenotype) and 0.9 Kb (resistant phenotype)

primers OPA15SFP and OPA15-RFP specific to susceptible and resistant plants respectively along with a common reverse primer OPA-CRP (Tables 3 and 4) were designed to develop SCAR markers. These primers were used in PCR amplification of genomic DNA of "Odisha Local" and 9907-9611.

Screening F2 population using SCAR marker

Polymorphism in OPA15 SCAR markers was examined in 199 F_2 plants obtained by crossing resistant and susceptible parental lines, "Odisha Local" and 9907-9611. The genomic DNA of plants was used to obtain amplicons in primers OPA15SFP, OPA15-RFP and OPA-CRP. The plants were scored based on size of amplicons: The plants with amplicons of 0.9 Kb showing resistant phenotype were graded as "1", and with 1.1 Kb amplicon showing susceptible phenotype were graded "3". The plants showing both 0.9 Kb and 1.1 Kb amplicons were graded "2" (Figure 2). The genotypic and

phenotypic scores were analysed for chi-square test and correlation.

RESULTS

RAPD analysis

In RAPD analysis of plants in F_2 population with 200 decamer oligonucleotide primers, eight primers showed polymorphism. The OPA-15 primer was found consistent in yielding 1.1 Kb and 0.9 Kb amplicons in susceptible and resistant parents respectively.

SCAR markers

The PCR amplification of genomic DNA of "Odisha Local" and 9907-9611 using OPA15 primers yielded amplicons of 0.9 Kb and 1.1 Kb respectively (Figure 2).

The polymorphism of SCAR markers was studied in 199 F_2 plants obtained by crossing same resistant and susceptible parental lines, "Odisha Local" and 9907-9611. Out of 199 F_2 plants, 14 plants were homozygous resistant, 171 plants were segregating for the trait and 14 plants were homozygous susceptible (Figure 3).

A Chi-square (χ^2) test for goodness-of-fit (Table 6) was tested with the hypothesis of marker score aligned with phenotypic scores of the F2 population of 199 individual plants (Table 7a and b). The hypothesis was considered appropriate for a probability (P) value between 0.75 and 0.50 (Table 6). Association analysis between genotypic and phenotypic scores of F2 population was carried out. The correlation of marker scores with phenotypic scores were highly significant (r=0.623).

The nucleotide sequence obtained from a genome of resistant chilli pepper line 'Odisha Local' using SCAR-OPA15 primer showed partial identity with RPP13-like disease resistant protein (GenBank Accessions XM_016717781, XM_016717782, XM_016717784).

DISCUSSION

Identification of resistance source for powdery mildew disease caused by *L. taurica* is important in resistance breeding. Marker assisted selection is one of the most widely used applications in breeding programs (Foolad, 2007). The process reduces breeding time and allows stacking of desirable genes in an otherwise elite line. Therefore, development of molecular markers closely linked to the gene of interest for powdery mildew resistance is of high importance for breeders.

We worked to generate linked markers for molecular breeding programme. To identify resistant germplasm source of chilli pepper, a total of 19 genotypes including three commercial hybrids, eight commercial varieties, seven local collections and a susceptible check Byadgi Kaddi were screened for powdery mildew resistance in epiphytotic conditions. Lines "Odisha Local" and 9907-

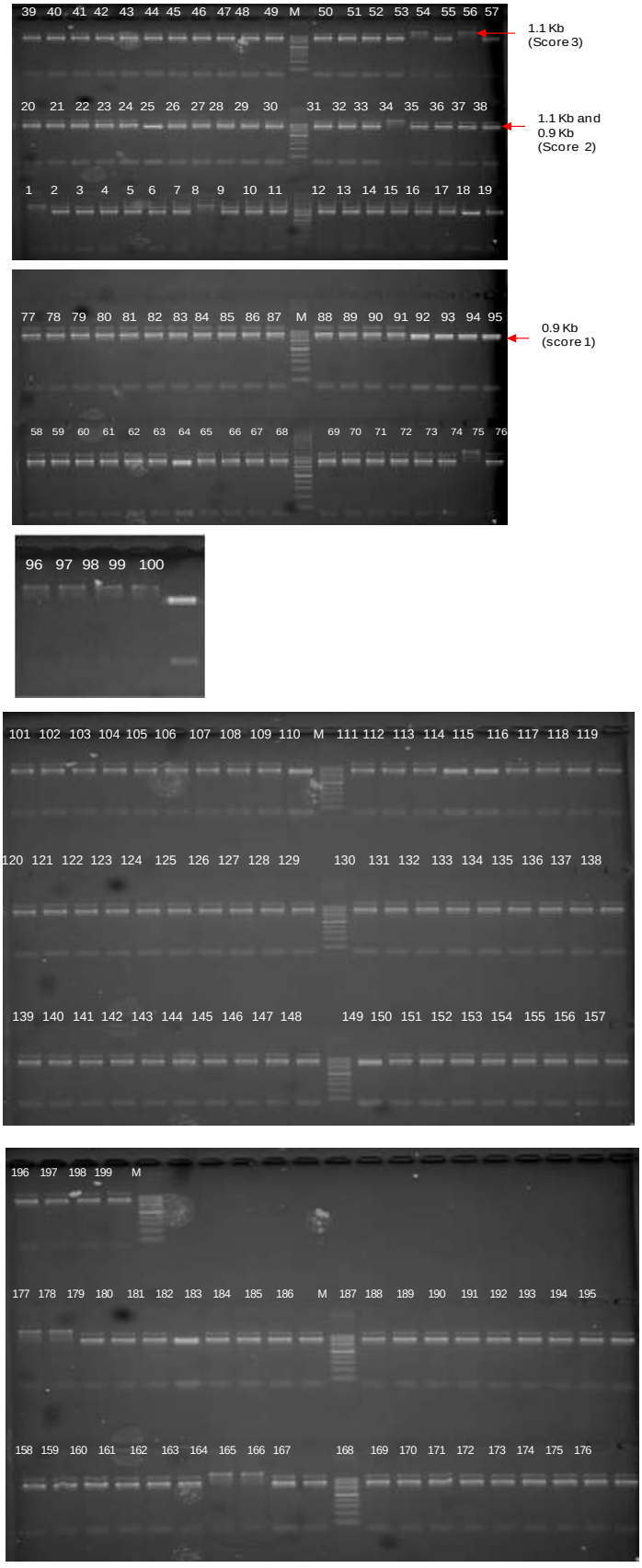

Figure 3. PCR products amplified by the SCAR primers OPA15-SFP, OPA15-RFP and OPA15-CRP. M, GeneRuler 1000 bp.

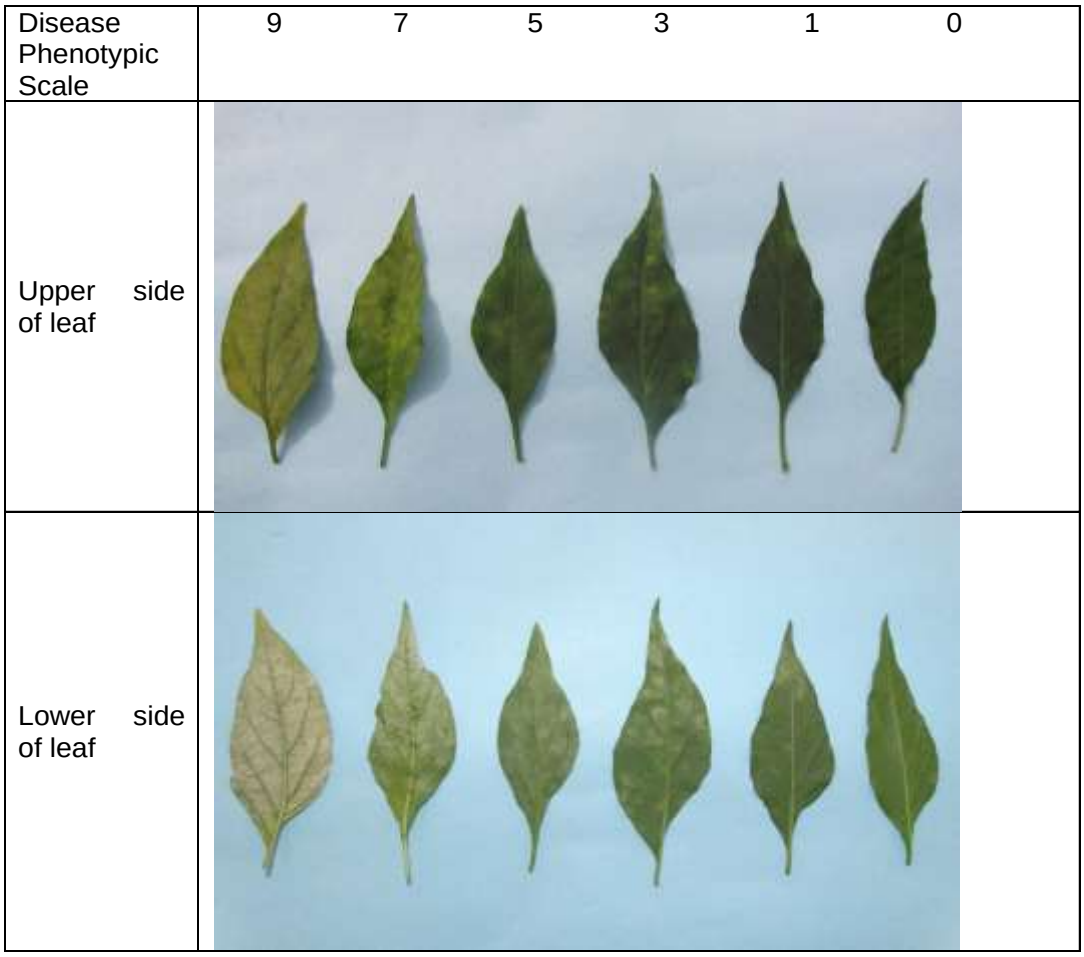

Figure 4. Powdery mildew symptoms on upper and lower leaves of Chilli plant and corresponding phenotypic scores.

9611 resistant and susceptible to powdery mildew, respectively were identified. Resistant and susceptible DNA pools from these two lines were used to further develop SCAR markers for MAS. The polymorphism of SCAR-OPA15 was also confirmed on F_2 population derived from crossing lines "Odisha Local" and 9907-9611. This molecular tool in the hands of breeders helps in indirect selection of genotypes saving time and resources in field screening.

The powdery mildew resistant 'Odisha Local' genome sequence obtained by SCAR-OPA 15 primer shows identity with allele RPP13-like protein. Bittner-Eddy et al. (2000) suggested that the RPP13 locus in Arabidopsis accession Nd-1, contains either a single gene capable of multiple isolate recognition or a group of tightly linked genes that is responsible for resistance against *P. parasitica* isolate Maks9 with localized necrotic flecks on host plant tissue and no pathogen reproduction. RPP13-like allele is implicated in conferring resistance to biotrophic fungal pathogens. The RPP13 resistance protein guards the plant against pathogens that contain an appropriate avirulent protein because of an indirect interaction with this avirulent protein. That triggers a defense system including the hypersensitive response, which restricts the pathogen growth. In contrast to other resistance proteins, RPP13-like protein works independently of ESD1 and NSD1 proteins and does not require the accumulation of salicylic acid, suggesting the existence of an independent signaling pathway (Bittner et al., 2000). ESD1 protein causes early flowering independently of photoperiod, moderate increase of hypocotyl length, shortened inflorescence internodes, and altered leaf and flower development. Also, the NSD1 gene provides instructions for making a protein that functions as a histone methyltransferase. Histone methyltransferases are enzymes that modify structural proteins called histones, which attach (bind) to DNA and give chromosomes their shape. Murthy and Deshpande (1997) reported that the genes in two different powdery mildew resistant parents in chilli pepper showed allelic differences in controlling resistance for powdery mildew at least at few loci. Shifriss et al. (1992)

Table 6. Genotypic and phenotypic scores in the F2 population.

Marker data	Powdery mildew disease grade	1	2	3	Chi Square test (P value)
	Expected genotype	**Homozygous resistant**	**Heterozygous**	**Homozygous susceptible**	-
Phenotypic data	PM Scores	0	1- 7	9	-
	Disease reaction	Immune	Highly Resistant to moderately susceptible	Highly susceptible	-
	Number of plants in phenotypic class	14	171	14	-
Genotypic data	Number of plants in genotypic class	13 (Numbers of plants with genotype *aa*, SCAR-OPA15)	172 (Number of plants with genotype *ab*, SCAR-OPA15)	14 (Numbers of plants with genotype *bb*, SCAR-OPA15)	0.0827 (0.75 -0.50)

Table 7a. Comparison of powdery mildew phenotypic and marker (genotype) scores on F_2 generation individual plants of the cross "Odisha Local x 9907-9611".

F2 Plant number	Phenotypic Score	Marker Score	F2 Plant number	Phenotypic Score	Marker Score	F2 Plant number	Phenotypic Score	Marker Score	F2 Plant number	Phenotypic Score	Marker Score
1	9	3	26	1	2	51	3	2	76	5	2
2	7	2	27	1	2	52	5	2	77	5	2
3	5	2	28	3	2	53	5	2	78	5	2
4	3	2	29	3	2	54	9	3	79	5	2
5	3	2	30	1	2	55	5	2	80	7	2
6	1	2	31	1	2	56	9	3	81	7	2
7	3	2	32	7	2	57	3	2	82	7	2
8	9	3	33	1	2	58	7	2	83	5	2
9	3	2	34	9	3	59	7	2	84	7	2
10	7	2	35	3	2	60	3	2	85	7	2
11	7	2	36	1	2	61	1	2	86	5	2
12	7	2	37	1	2	62	1	2	87	5	2
13	7	2	38	7	2	63	1	2	88	5	2
14	7	2	39	5	2	64	0	1	89	7	2
15	7	2	40	5	2	65	1	2	90	7	2
16	0	2	41	1	2	66	1	2	91	7	2
17	3	2	42	3	2	67	5	2	92	0	1
18	0	1	43	3	2	68	5	2	93	0	1
19	1	2	44	3	2	69	5	2	94	0	1
20	1	2	45	7	2	70	1	2	95	0	1
21	3	2	46	3	2	71	1	2	96	9	3
22	3	2	47	5	2	72	5	2	97	9	3
23	1	2	48	3	2	73	5	2	98	9	3
24	3	2	49	5	2	74	7	2	99	9	3
25	0	1	50	1	2	75	9	3	100	0	1

showed that, the disease resistance expression in doubled-haploid variety HV-12 was due to the restriction in pathogen infection, its colonization and leaf defoliation. The inheritance of resistance to powdery mildew in *C. annuum* involves several loci, which demand stronger selection in generations to get homozygosity (Blat et al., 2005). The mode of inheritance for powdery mildew resistance in chilli pepper is complex and inheritance study has also indicated dominant type of resistance to powdery mildew (Anand et al., 1987). This type of durable polygenic resistance is more difficult to be overcome by pathogenic strains (Van der Plank, 1968).

Table 7b. Comparison of powdery mildew phenotypic and marker (genotype) scores on F_2 generation individual plants of the cross "Odisha Local x 9907-9611".

F2 Plant number	Phenotypic Score	Marker Score	F2 Plant number	Phenotypic Score	Marker Score	F2 Plant number	Phenotypic Score	Marker Score	F2 Plant number	Phenotypic Score	Marker Score
101	3	2	126	1	2	151	3	2	176	7	2
102	1	2	127	7	2	152	3	2	177	9	3
103	1	2	128	5	2	153	3	2	178	9	3
104	3	2	129	5	2	154	7	2	179	3	2
105	1	2	130	3	2	155	7	2	180	3	2
106	1	2	131	3	2	156	3	2	181	7	2
107	1	2	132	1	2	157	3	2	182	0	1
108	1	2	133	3	2	158	1	2	183	5	2
109	1	2	134	5	2	159	3	2	184	3	2
110	0	1	135	7	2	160	1	2	185	3	2
111	1	2	136	3	2	161	7	2	186	1	2
112	1	2	137	5	2	162	3	2	187	1	2
113	1	2	138	5	2	163	5	2	188	3	2
114	0	1	139	3	2	164	9	3	189	7	2
115	0	1	140	1	2	165	9	3	190	7	2
116	3	2	141	1	2	166	3	2	191	7	2
117	7	2	142	1	2	167	3	2	192	7	2
118	3	2	143	1	2	168	3	2	193	5	2
119	5	2	144	1	2	169	3	2	194	3	2
120	5	2	145	7	2	170	5	2	195	3	2
121	1	2	146	1	2	171	1	2	196	3	2
122	5	2	147	1	2	172	7	2	197	3	2
123	3	2	148	1	2	173	7	2	198	1	2
124	1	2	149	0	1	174	5	2	199	1	2
125	3	2	150	1	2	175	3	2			

Jinkwan et al. (2017) investigated a powdery mildew disease inheritance in two F2 populations VK515 and PM Singang. The authors revealed that the single dominant locus PMR1 is responsible for inheritance of powdery mildew. One SCAR and five SNP molecular markers were identified in PMR1 locus.

The high heritability of disease resistance shows that the powdery mildew infection reaction in general is not so much influenced by environmental conditions. It was also observed by Blat et al. (2005).

The genome of chilli pepper is large and complex with high genetic variability. The SCAR-OPA15 markers developed in this study could be tested to identify breeding lines with different genetic background for resistance to powdery mildew. Our findings contribute to continuous improvement and generation of new chilli pepper hybrids.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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