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Development of SCAR marker for *Phytophthora* resistance in black pepper (*Piper nigrum* L.)

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Abstract

Foot rot disease caused by *Phytophthora capsici* is one of the major production constraints in black pepper (*Piper nigrum*) in India. RAPD profile of moderately resistant and susceptible lines indicated the presence a unique band of 360 base pair in moderately resistant lines with the primer OPA-01. The DNA fragment associated with these lines was cloned, sequenced and converted into a SCAR marker. The SCAR primer was tested on plants that were classified as moderately resistant and susceptible. SCAR primers amplified DNA invariably from resistant plants only. The sequence of the unique band matched with stress related gene sequences reported in several eukaryotes including *Arabidopsis thaliana*.

Keywords: black pepper, *Phytophthora, Piper nigrum*, resistance, SCAR marker.

Abbreviations: AFLP=Amplified length polymorphism; CTAB=Cetyl trimethyl ammonium bromide; dNTP= Deoxyribonucleotide triphosphate; EDTA=Ethylenediaminetetraacetic acid; HSP=Heat shock protein; ORF=Open reading frame; PVP=Polyvinylpyrillidone; QTL=Qualitative trait loci; RAPD=Random amplified polymorphic DNA; RFLP=Restriction fragment length polymorphism; SCAR=Sequence characterized amplified region; TE=Tris Ethylenediaminetetraacetic acid.

Introduction

Phytophthora capsici Tsao (Tsao 1991) is one of the most serious threats to black pepper (*Piper nigrum* L.) cultivation in India (Anandaraj 2000). None of the existing black pepper genotypes are resistant to this disease (Ravindran *et al*. 2000). Several moderately resistant lines (initially termed as tolerant) have been identified that includes open pollinated and hybrid progenies. One of the lines moderately resistant to *P. capsici* has been tested in the field and released as IISR-Shakthi. Several molecular markers such as RAPD, AFLP and RFLP are used to locate the resistant genes or QTL (Paran & Michelmore 1991; Williams *et al*. 1993; Vandemark *et al*. 2000). The present study was an effort to study the RAPD profile of moderately resistant and susceptible lines and to develop a SCAR marker for locating *Phytophthora* resistance in black pepper, which could be used for large scale screening.

Materials and methods

Plant material

About 3000 lines of black pepper germplasm have been collected and assembled at Indian Institute of Spices Research, Calicut. The germplasm has been screened and some of them have been classified as moderately resistant to *P. capsici*. The variety IISR-Subhakara (KS-27) is considered as susceptible. The moderately resistant lines namely, IISR-Shakthi, HP-780,

HP-23, HP-1, C-1090, and C-1095 were used in this study along with IISR-Subhakara for RAPD assay. For SCAR marker assay about 500 seedlings raised from 26 genotypes were used.

Screening of seedlings for Phytophthora *resistance*

Screening of black pepper germplasm against *Phytophthora* was carried out at two stages namely, on seedlings with zoospore suspension and on cuttings with stem inoculation (Sarma *et al*. 1994; Bhai *et al*. 2007). The seedlings were raised in plastic trays and the percentage germination of seeds was recorded for each line. The seedlings at 4–5 leaf stage were inoculated with zoospores at a concentration of 106 and incubated by flooding the soil. Seedling mortality was recorded after 15–30 days. For stem inoculation, black pepper cuttings at five node stage were inoculated at the third internode from the top with mycelial disc taken from 48 h old culture of *P. capsici* grown on carrot agar and covered with wet cotton wad and incubated for 72 h at 22–25ºC. The lesion size and depth of penetration were recorded. Resistance rating was given taking into account both lesion length and depth of penetration (Sarma *et al*. 1994). Resistance to *P. capsici* was measured as lesion length (mm) and depth of penetration as index in a scale of 0–4. The seedlings produced lesion length ranging from 1–120 mm within 72 h and depth of penetration ranged from the surface to rotting of the entire tissues of internode. The depth of penetration was graded as 0=No lesion, 1=Lesion confined to surface, 2=Up to 25% penetration, 3=Up to 50% penetration and 4=>50% penetration.

DNA extraction

The genomic DNA from black pepper was extracted using modified CTAB method by adding 50 mg of PVP for every 3 g of leaf sample (Mandal *et al*. 2000). Young leaf sample (3 g) was ground to fine powder in liquid nitrogen and suspended in 10 ml of pre-warmed extraction buffer (100 mM Tris-HCl, 20 mM EDTA pH-8, 1.4 M NaCl, 2% CTAB and 0.2% ß-mercaptoethanol). The suspensions were centrifuged at 10,000 rpm for 10 min at 4°C after incubation at 60°C for 30 min. The aqueous phase was extracted with equal volume of chloroform: isoamyl alchohol (24:1) and centrifuged at 10,000 rpm for 10 min at 4°C. The nucleic acid was precipitated from the aqueous phase by adding 0.6 volume of isopropanol followed by centrifugation at 10,000 rpm for 10 min. The pellet was washed in 70% ethanol and dissolved in sterile distilled water containing 2 µl of RNase 50 µg/µl. Further purification of DNA was done with 500 µl of phenol: chloroform: isoamylalchohol (25:24:1) and precipitated by adding 0.6 volume of isopropanol following centrifugation at 10,000 rpm for 10 min. The pellet was washed in 70% ethanol and air-dried and dissolved in 50 µl of TE buffer (10 mM Tris and 1 mM EDTA pH-8). The purified DNA was quantified using Eppendorf Biophotometer.

RAPD assay

The purified genomic DNA was subjected to PCR using 15 decamer random primers obtained from Operon Technologies, California, USA (Table 1). The PC reactions were optimized for RAPD conditions in a thermocycler. Each reaction was carried out with a reaction volume of 25 µl, which consisted of 20 ng genomic DNA, 0.5 U Taq DNA polymerase, 400 µM dNTP mix, 2.5 mM $MgCl_2$, 10 pM primer and

Table 1. Nucleotide sequences of operon primers

Primer	Sequences 5' to 3'
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-05	GGGGTCTTGC
OPA-06	GGTCCCTGAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-09	GGGAACGCC
OPA-10	GTGATCGCAG
OPA-11	CAATCGCCGT
OPA-12	TCGGCGATAG
OPA-13	CAGCACCCAC
OPA-14	TCTGTGCTGG
OPA-18	AGGTGACCGT

PCR buffer. All the PCR reagents except the primer were obtained from Bangalore Genei, Bangalore. The thermal cycler was programmed for 30 cycles as follows: initial denaturation at 94°C for 5 min and subsequent denaturation at 94°C for 1 min, primer annealing at 48°C for 1 min, extension of the annealed primer for 1 min and final extension at 72°C for 15 min. The reaction products were analysed by 2% agarose gel electrophoresis. The bands were visualized by exposure to UV light and the gel was documented in a gel documentation system (Alpha Innotech).

Development of SCAR marker

The polymorphic bands obtained were scored for each black pepper line tested. A band (400 bp) appeared in all the moderately resistant lines but absent in the susceptible ones with the primer OPA-01 was eluted from the agarose gel, using Sigma Nucleotrap extraction kit for developing SCAR marker. The PCR product was ligated using the PCR-SCRIPTTM Amp cloning kit (Stratagene) and transferred into *Escherichia coli* strain DH5-a using standard molecular biology procedures (Sambrook & Russel 2001). Plasmids were extracted using alkaline lysis method and the recombinant clones were identified by restriction endonuclease digestion using the enzyme Nco 1 and also by PCR using specific primers. Selected clone was sequenced at the automated DNA sequencing facility at Bangalore Genei, Bangalore, India.

Designing of primers

Based on the sequence, primers were designed for SCAR markers. The sequences of the designed primers were: forward-5' GCCCTTCCAATAAAAGGAAC 3' and reverse- 5'CTTCGAGTTGGAGTGTGATG 3'.

SCAR testing

The SCAR primer was tested on the moderately resistant lines and on 21 genotypes that included both susceptible and moderately resistant ones. Approximately 50 ng samples of DNA were used in 25 µl reaction mixture containing 15 mM Tris HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$ 100 µM each of dNTPs and 10 pM each SCAR primer and 2.5 U of Taq DNA polymerase (Bangalore Genei, Bangalore). Annealing temperature was optimized for SCAR primers. The optimal thermocycling profiles for SCAR primers was as follows: 5 min denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 1 min at 58°C, 1 min at 72°C and a final extension step for 10 min at 72°C.

Results and discussion

Screening of seedlings

About 34% seed germination was recorded in the nursery. Susceptible seedlings died after soil drenching with zoospores within 30 days, whereas moderately resistant plants remained healthy without developing any lesion. The seedlings mortality ranged from 14% to 100% after two rounds of soil drenching (Table 2). The seedlings that remained healthy after first round of screening with zoospore inoculation were subjected to stem inoculation. The seedlings, which produced lesion length below 5 mm and penetration index 1, were considered as moderately resistant to *P. capsici* (Table 3).

RAPD assay

The genomic DNA from black pepper yielded DNA that varied from 150 to 180 µg ml^{-1} for each gram of tissue. Out of the 15 RAPD primers tested, primer OPA-01 amplified four regions out of which three were monomorphic and the remaining one was present in all the moderately resistant lines while absent in the susceptible control IISR-Subhakara (Fig 1). The specific band (~ 360 bp) that appeared in OPA-01 in the agarose gel was eluted, cloned, sequenced and converted into a SCAR marker. OPA-02 altogether amplified 5 loci out of which two were monomorphic and rest of the three showed polymorphism. OPA-03 generated the highest polymorphism, which amplified nine loci out of which two were monomorphic and the rest seven were polymorphic. Out of the four loci amplified by OPA-04, only one was polymorphic and the rest were monomorphic. OPA-09 amplified eight loci out of which three were monomorphic and the remaining were

Table 2. Germination of black pepper seeds and seedling survival after root inoculation

Variety / Accession	No. of seeds sown	Germination of seeds (%)	Survival of seedlings after root inoculation (%)
Panniyur-1	1000	15.8	39.8
Panniyur-2	1000	24.7	25.0
Panniyur-3	500	38.2	15.2
Acc. 1578	500	37.0	20.0
Acc. 4199	250	2.8	85.7
IISR-Shakthi	500	20.2	31.7
Acc. 861	750	16.1	3.3
Acc. 5271	250	26.0	13.8
Acc. 1205	250	88.0	7.3
Acc. 1597	750	44.9	1.2
Acc. 888	500	31.4	3.2
Acc. 4076	250	60.8	0.7
Acc. 959	250	50.0	13.6
Acc. 1052	250	10.0	0.0
Acc. 951	250	38.0	4.2
Acc. 1103	250	42.4	22.6
Acc. 965	250	44.8	12.5
Acc. 1319	250	35.2	45.5
Acc. 4070	250	75.6	1.6
Acc. 865	250	18.8	25.5
Acc. 1080	250	49.2	16.3
Acc. 992	250	93.6	9.4
Acc. 5097	250	43.2	0.0
HP-780	250	15.6	43.6
IISR-Subhakara	250	56.4	1.4
OPKM	500	20.8	22.1
Mean		38.4	17.9

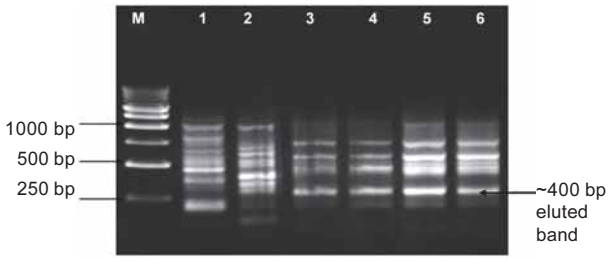

Fig. 1. A unique band present in all the moderately resistant lines to *Phytophthora* while absent in the susceptible control IISR-Subhakara. M: 1Kb DNA ladder; Lane-1: IISR-Subhakara; Lane-2: IISR-Shakthi; Lane-3: HP-780; Lane-4: HP-23; Lane-5: C-1090; Lane-6: C-1095.

polymorphic. OPA-13 amplified eight loci out of which four were polymorphic and the other four were monomorphic. OPA-18 amplified eleven loci out of which only three were polymorphic and the remaining eight were distinctly monomorphic. The other primers tried namely, OPA-05, OPA-06, OPA-07, OPA-08, OPA-10, OPA-11 and OPA-14 generated all monomorphic bands. The sequence of the unique band was aligned and matched with existing sequences in the GenBank. It matched with the sequences reported against various biotic stresses.

Table 3. Reaction of black pepper seedlings to stem inoculation

Variety	No. of seedlings tested	Range of lesion length (mm)	Range of penetration index (0–4)	Seedlings rated as moderately resistant (%)
Panniyur-1	63	4–58	1–4	3.2
Panniyur-2	62	4–60	1–4	1.6
Panniyur-3	29	4–60	1–4	6.9
Acc. 1578	8	9–30	1–4	0.0
Acc. 4199	6	15–45	3–4	0.0
IISR-Shakthi	32	5–60	1–4	3.1
Acc. 861	4	30–55	2–4	0.0
Acc. 5271	9	4–35	1–4	11.1
Acc. 1205	16	10–50	1–4	0.0
Acc. 1597	15	15–35	1–4	0.0
Acc. 888	5	24–46	4	0.0
Acc. 4076	1	25	4	0.0
Acc. 959	17	5–40	1–4	5.8
Acc. 951	4	21–45	1–4	0.0
Acc. 1103	24	3–38	1–4	16.6
Acc. 965	14	15–40	1–4	0.0
Acc. 1319	25	4–50	1–4	8.0
Acc. 4070	3	28–35	4	0.0
Acc. 865	12	11–53	1–4	0.0
Acc. 1080	20	3–41	1–4	15.0
Acc. 992	22	4–38	1–4	4.5
HP-780	17	4–50	1–4	5.9
IISR-Subhakara	2	13–27	1–4	0.0
OPKM	23	4–36	1–4	4.3

Development and testing of SCAR marker

Primer sequences and expected products

Out of the 26 plants screened, primers MA1 and MA2 amplified the specific band ~ 360 bp in moderately resistant lines only (Fig 2). Primer annealing temperature had been set at 58°C to avoid non-specific primer binding and thus spurious amplification products. The reproducibility of the bands was confirmed by running the PCR thrice. Sequences were further confirmed by cloning and sequencing of the specific band (Table 4). The SCAR primer was tested on selected black pepper seedlings. SCAR primers amplified DNA from moderately resistant seedlings only. An amplicon of 364 bp was obtained for all moderately resistant

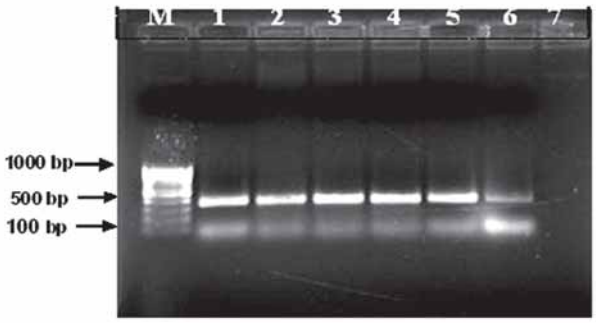

Fig. 2. The amplified products of primers MA1 and MA2. The specific band ~ 360 bp is seen in moderately resistant lines only. M: 1Kb DNA ladder; Lane-1: C-1095; Lane-2: IISR-Shakthi; Lane-3: HP-780; Lane-4: HP-23; Lane-5: C-1090; Lane-6: IISR-Subhakara.

Table 4. Testing of SCAR marker on black pepper plants

Accession / line	Presence/ absence of band	Reaction to *P. capsici* (Lesion length in mm)	Penetration index (0–4)	Disease rating (MR/S)
HP-780-16	+	4.0	1	MR
05-1319-18	+	4.0	1	MR
05-PR-III-7	+	4.0	1	MR
05-1080-1	+	3.0	1	MR
05-PR-I-6	-	43.0	3	S
05-1578-3	-	20.0	2	S
05-PR-III-26	-	26.0	4	S
05-PR-III-25	-	24.0	2	S
HP-780-16	+	4.0	1	MR
05-1080-15	-	24.0	2	S
05-1103-14	+	5.0	1	MR
05-1103-22	+	4.0	1	MR
05-992-15	+	4.0	1	MR
05-1205-3	-	38.0	4	S

Index scale: 0=No symptoms; 1=Lesion on the surface; 2=Up to 25% penetration; 3=26%–50% penetration, 4=>50% penetration; MR=Moderately resistant; S=Susceptible
The plants used are individual progenies raised from seeds

varieties (Figs. 3 & 4). The six susceptible genotypes showed no amplification for SCAR. The unique band was sequenced and submitted to GenBank (Acc. No. FM 246506).

Resistance in black pepper is genetically and physiologically complex. Screening of black pepper germplasm is done adopting stem and root inoculation techniques (Nambiar & Sarma 1979; Sarma *et al*. 1994). In this study, black pepper genotypes that were identified as moderately resistant lines were assayed with RAPD and the unique band that was obtained in moderately resistant lines was converted into SCAR marker and tested on seedlings that included genotypes showing resistant, highly susceptible and intermediate responses to the pathogen.

The DNA extraction protocol employed could yield an optimum amount of good quality DNA. It is well documented that the high content of polyphenols in black pepper reduces the quality and quantity of extracted DNA. Addition of 50 mg PVP reduced the blackening of the ground sample by binding to the high content of

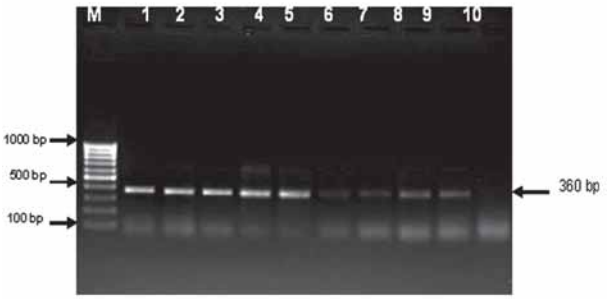

Fig. 3. The amplicons of primers MA1 and MA2 from moderately resistant lines of seedlings tested. M: 100 bp ladder; Lane 1–10: moderately resistant seedlings (05-1103-15, 05-1103-22, 05-1103-14, 05-PR-III-07, 05-992-15, 05-861-04, 05-5271-03, 05-1319-18, 05-1080-01, Negative control)

polyphenols in black pepper. RAPD primers produced clear and reproducible banding pattern in all the cultivars tested. Out of the 15 primers used, OPA–01 produced an approximately 400 bp region which was monomorphic and characteristic of all moderately resistant lines. The unique band was developed into a SCAR marker to differentiate moderately resistant and susceptible lines.

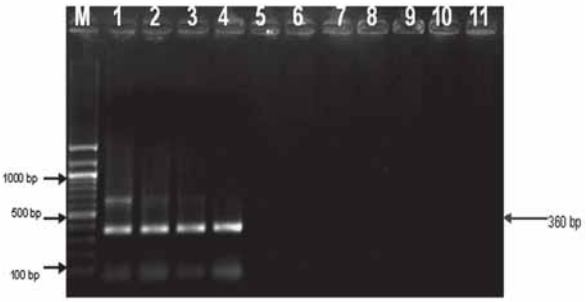

Fig. 4. PCR with SCAR marker showing amplifications in resistant lines. M: 100 bp ladder, Lane 1–4: moderately resistant lines (HP-780-16, 05-1319-18, 05-PRIII-7, 05-1080-1); Lane 5–10: Susceptible lines (05-PR-1-6, 05-1578-3, 05-PR-III-26, 05-PR-III-25, 05-1080-15, 05-1205-3)

BLAST analysis of the 360 bp sequence matched with stress-induced phosphoproteins (Hsp70/Hsp90) of eukaryotes. These synonyms are used for gene STIP1 (stress-induced-phosphoprotein1-Hsp70/Hsp90-organizing protein). Hsp's are induced in cells when exposed to different environmental stressful conditions. This suggests that genetic mechanisms, which determine the stress resistance of moderately resistant lines, are probably related to transcription of Hsp70/Hsp90-organizing genes. Numerous studies have previously documented the role of Hsp in plants and the correlation between Hsp induction and adaptation to stress in plants (Prändl *et al*. 1998), and the role of pathogenesis-related proteins (Van Loon 1997).

Protein BLAST analysis of the 360 bp sequences resulted in two ORF region consisting of 71 as well as 31 amino acids. ORF region of 39 amino acids partially matched with Acriflavin resistance protein B of *Rhodobacter* and that of 71 amino acids partially matched with retrotransposons/polyprotein of *Oryza sativa* L.

SCAR primers can be used to discriminate *Phytophthora* resistant and susceptible lines of black pepper and also can assess levels of virulence in the fungal isolates and levels of resistance in the hosts. Black pepper is a vegetatively propagated crop although a distinct sexual reproduction exists. The seedling progenies show greater variation both for agronomic traits and reaction to pests and diseases. The first *Phytophthora* resistant line IISR-Shakthi is an open pollinated progeny of a susceptible cultivar. The resistant reaction in this variety has been reported to be due to the early induction of defence genes and synthesis of PR proteins (Stephen *et al*. 2001). The moderately resistant lines such as HP-1 and HP-780 identified earlier were progenies of parents that are susceptible (Sarma *et al*. 1994). When both parents being susceptible, the hybrids showing resistance indicates involvement of polygenes in conferring resistance and some being recessive. The genetics of *Phytophthora* resistance is not clearly understood. However, the present data on segregating populations indicates that it is polygenic. The SCAR marker developed has invariably produced a band in moderately resistant lines. However, in case of a few seedling progenies, a band also appeared in the susceptible category where the lesion length was more than 5 mm and penetration index was 2 in a scale of 0–4. There is no germplasm or seedling progeny that showed a resistant reaction of 0 both for lesion and depth of penetration except *P. colubrinum* Link, a related species of black pepper so far. Hence, resistance to *P. capsici* in black pepper appears to be a complex interaction of genes and in the absence of clear resistance source in the cultivated types, only moderate resistance is identified so far. The SCAR marker developed may be useful for screening black pepper germplasm at seedling stage itself.

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