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Development of a coupling-phase SCAR marker linked to the powdery mildew resistance gene '*er1*' in pea (*Pisum sativum* L.)

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Abstract Pea powdery mildew is one of the major constraints in pea production worldwide, causing severe seed yield and quality loss. The resistance is governed by a single recessive gene *er1* in majority of resistant cultivars, but *er2* and *Er3* have also been reported. The objective of the study was to find out tightly linked sequence characterized amplified regions (SCAR) markers to *er1* gene using NILs. A total of 620 random amplified polymorphic DNA (RAPD) markers were screened for length polymorphism between seven sets of NILs. The 880 bp polymorphic band of the tightly linked RAPD marker OPX 04_{880} was cloned, sequenced and a SCAR marker ScOPX 04_{880} was developed. In a population of completely classified 208 F_2 plants (supported by phenotypic data from 208 $F_{2:3}$ and 4,390 $F_{3:4}$ families) ScOPX 04_{880} was linked at 0.6 cM in coupling phase with *er1* gene in the order ScOPX 04_{880}–*er1*–ScOPD 10_{650}. ScOPX 04_{880} will correctly differentiate homozygous resistant plants from the susceptible accessions with more than 99 % accuracy. In combination with repulsion phase marker ScOPD 10_{650}, ScOPX 04_{880} can help in an error free marker-assisted selection.

Keywords Marker-assisted selection · Near-isogenic lines · Pea · *Pisum sativum* · Powdery mildew · SCAR marker

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Introduction

The pea powdery mildew is caused by an obligate ecto-parasitic biotrophic fungus *Erysiphe pisi var. pisi* D.C. (Braun 1980; Cook and Fox 1992), and has been known as one of the major constraints in pea production globally. Dixon (1978) identified powdery mildew as the greatest threat to the crop of dry pea. This disease occurs worldwide (Kraft and Pfleger 2001; Dixon 1978), and in India it is the most serious disease of pea. Powdery mildew alone can reduce 25–50 % of seed yield (Kumar and Singh 1981;

Reiling 1984) and also the seed quality of pea (Rathi and Tripathi 1994). Dry weather conditions favour the disease development, but do not assure an epidemic. In India, due to severe economic consequence of powdery mildew attack, All India Coordinated Pulse Improvement Project (AICPIP) made a decision in 1982 that no powdery mildew susceptible line would be tested in the coordinated trials (Sharma 1997).

Hammarlund (1925) reported four recessive genes for powdery mildew of field pea, but it was not confirmed by further studies. Later, Harland (1948) isolated some resistant plants from local Peruvian material. His F_2 and F_3 data perfectly fitted to 3:1 segregation ratio, susceptible being dominant over resistance. He designated this gene as *er*. The resistance was controlled by a single gene in homozygous recessive condition. This was later supported by Pierce (1948), Yarnell (1962), Cousin (1965), Marx (1971), Saxena et al. (1975), Singh et al. (1983), Mishra and Shukla (1984), Gupta (1987), Sarala (1993), Timmerman et al. (1994), Rakshit (1997), Vaid and Tyagi (1997), Janila and Sharma (2004), and Srivastava and Mishra (2004). However, the inheritance of powdery mildew has not been totally unambiguous. Several workers have reported digenic segregation (Heringa et al. 1969; Sokhi et al. 1979; Kumar and Singh 1981; Tiwari et al. 1997). In these studies genes governing resistance to *E. pisi* were reported to be controlled by recessive genes *er1* and *er2*. However, *er2* has not been confirmed in the present sets of NILs during our earlier studies. There has been limited mention of this gene in linkage studies. Katoch et al. (2010) recently reported that they have mapped *er2* to LGIII. Overwhelming evidence favors one-gene (*er1*) control of powdery mildew resistance in pea (Sharma 2003; Sharma and Yadav 2003; Srivastava and Mishra 2004). This may be partly due to the nature of resistance governed by *er2*. While *er1* offers complete resistance against the powdery mildew pathogen by preventing pathogen penetration, *er2* mediated resistance is primarily based on reduced penetration rate complimented by post-penetration hypersensitive response leading to cell death (Fondevilla et al. 2006; Katoch et al. 2010).

Development of molecular markers for *er1* gene was thought to be essential for pyramiding it with *er2* and *Er3* in single cultivar, or in a backcrossing program to convert a susceptible variety into resistant one. This is a durable gene and since the last five decades there has been no report of the resistance break-down conferred by *er1*. Scores of attempts were made in the past to find molecular markers linked to the *er1* gene. Many workers tried to develop random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) derived sequence characterized amplified regions (SCAR) markers. Timmerman et al. (1994) developed a RAPD-derived SCAR marker ScOPD 10_{650} in Canadian germplasm. This marker was reported to be linked at 2.1 cM in repulsion phase. Subsequent validation studies in two different mapping populations placed ScOPD 10_{650} at 3.6 cM (Rakshit 1997) and 3.4 cM (Janila and Sharma 2004) from *er1*. Both reported distances are large for efficient marker-assisted selection (MAS). The other two closely linked SCAR markers, ScOPO 18_{1200} and ScOPE 16_{1600} reported by Tiwari et al. (1998) were not found to be polymorphic by Janila and Sharma (2004), and did not differentiate resistant and susceptible lines of Indian origin. Several new marker systems have been reported to be linked to various powdery mildew resistance genes in pea as *er1* (Ek et al. 2005; Pereira et al. 2010), *er2* (Katoch et al. 2010) and *Er3* (Fondevilla et al. 2008).

Herein we describe our experiment that led to development of a tightly linked SCAR marker to the powdery mildew resistance gene (*er1*) in pea, using advanced generation (BC_9) near-isogenic lines (NILs).

Materials and methods

Plant materials

NILs, developed at the Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi, India, through nine cycles of backcrossing with their respective recurrent parents, were used in the present study (Table 1). The parents for generation of mapping population were selected based on their pedigree and sources of resistance (PMR)/susceptibility (PMS) to powdery mildew.

Crosses and phenotypic scoring

A set of seven crosses were attempted during winter of 2000, at IARI, New Delhi involving NILs viz., HFP 4 $(PMS)^{Bonneville}$ × HFP 4 (PMR); HFP 4 $(PMS)^{PG\ 3}$ × HFP 4 (PMR); PG 3 $(PMR)^{HFP\ 4}$ × PG 3 (PMS);

Table 1 NILs, their donor and recipient parents used in the present investigation

S. no	NIL	PMS/PMR donor parent	Recipient parent
1	HFP 4 (PMS)$^{\text{Bonneville}}$	Bonneville (PMS)	HFP 4 (PMR)
2	HFP 4 (PMS)$^{\text{PG 3}}$	PG 3 (PMS)	HFP 4 (PMR)
3	PG 3 (PMR)$^{\text{HFP 4}}$	HFP 4 (PMR)	PG 3 (PMS)
4	DMR 11 (PMS)$^{\text{KPSD 1}}$	KPSD 1 (PMS)	DMR 11 (PMR)
5	T 163 (PMR)$^{\text{DMR 11}}$	DMR 11 (PMR)	T 163 (PMS)
6	L 116 (PMR)$^{\text{DMR 11}}$	DMR 11 (PMR)	L 116 (PMS)
7	Pusa 10 (PMR)$^{\text{DMR 11}}$	DMR 11 (PMR)	Pusa 10 (PMS)

NILs are written with the PMS/PMR donor source as indicated in superscripts

PMR powdery mildew resistant, *PMS* powdery mildew susceptible

DMR 11 (PMS)$^{\text{KPSD 1}}$ × DMR 11 (PMR); T 163 (PMR)$^{\text{DMR 11}}$ × T 163 (PMS); L 116 (PMR)$^{\text{DMR 11}}$ × L 116 (PMS) and Pusa 10 (PMR)$^{\text{DMR 11}}$ × Pusa 10 (PMS).[1] About 50–60 buds per cross were emasculated to get sufficient number of F_1 seeds. It was assumed that there are no reciprocal differences for powdery mildew resistance (Singh 1984). The PMR (*er1er1*) line was chosen as female parent in all the crosses. The crossed as well as selfed seeds from both parents were harvested. Simultaneously, young unopened leaves and growing stipules were collected from each parent of all the seven crosses for parental polymorphism study.

Disease phenotyping and zygosity testing for *er1* locus in the mapping population

Plots of 5 m row length with 65 cm row spacing and 30 cm spacing between plans were planted in the field. To get error free disease reaction for the powdery mildew, all the seven F_2 populations were sown in late December, 2001. This facilitated natural epidemic of the disease at IARI, New Delhi. A single row or five row plot of susceptible variety L116 was planted every fourth row and on the boarder (five rows) of each population. Dusting of the infected plant material from L116 was also done on alternate days from 30 days after sowing till maturity for uniform spread of the inoculum. The infected foliage surface was totally covered with a white powdery mass of the fungus and the infection spread to all aerial parts of the plant, including stems and pods. Tissue beneath the infected areas in the susceptible plants turned brown, followed by the production of black fruiting bodies (cleistothecia). The resistant plants were free from infection or infection was localized in very small patches only on the foliage (stipules and leaves), but it never spread to the stem, peduncle and pods. The plants with stem, peduncle and pods free from the infection symptoms were categorized as resistant, while plants with infection on leaves, stem, stipules and pods were categorized as susceptible. Disease phenotyping was conducted according to Janila and Sharma (2004), and Srivastava and Mishra (2004). There were clear cut differences between resistant and susceptible plants with no ambiguity, based on the scoring criteria mentioned above.

The entire F_2 population of the cross PG 3 (PMR)$^{\text{HFP 4}}$ × PG 3 (PMS) was advanced to F_4 generation through F_3 by harvesting single plants separately. Unequivocal phenotyping was obtained by screening of the F_3 and F_4 families. This testing dissected the heterozygosity/homogygosity of the susceptible plants at *er1* locus and also ruled out disease escape of any susceptible plants. The F_3 families were screened at the hot-spot location for powdery mildew during 2003 at off-season nursery Dalang Maidan, Lahaul Spithi, Himachal Pradesh State, India. All the 208 F_2 plants were represented as 208 F_3 families. The population was grown in two-row plots of five m length comprising at least 20 plants per family along with resistant (HFP 4) and susceptible (PG 3) parents at every fifth row. A total of 4,390 plants in the F_3 population were screened. Seeds were harvested from each of the F_3 families to raise F_4 families that were screened again at IARI, New Delhi during winter 2003/2004. Presence of significant number of resistant plants in a particular F_3/F_4 family was taken to infer heterozygosity at *er1* locus for the corresponding F_2 plant.

DNA isolation and quantification

The DNA was extracted from individual 208 F_2 plants, from unopened leaves and young stipules using the

[1] Superscript indicates the donor parent for powdery mildew susceptible (PMS) or resistant (PMR) trait.

cetyl methylammonium bromide (CTAB) protocol (Doyle and Doyle 1990) with few modifications as use of 1 % PVP and 4 % mercaptoethanol in the extraction process. DNA was also isolated from the PMR (15 plants) and PMS (15 plants) pairs of NILs, and was bulked together as resistant and susceptible bulks, respectively. Quantification was performed by running DNA samples on 0.8 % agarose gels along with known quantity of uncut λ DNA. Quantification was also made based on UV absorbance at 260 nm using a spectrophotometer. The ratio of absorbance at 260–280 nm gave the purity of extracted DNA. The samples were considered pure, if the ratio was between 1.8 and 2.0.

PCR analysis

We used RAPD marker system for SCAR marker development. A total of 620 random, 10-mer RAPD primers procured from Operon Technologies, Inc. (Alameda, CA, USA) were used for establishing polymorphism among seven pairs of NILs. These primers included OPA 01-20, OPB 01-20 to OPZ 01-20, OPAA 01-20 to OPAC 01-20, OPAE 01-20 and OPAK 1-20. RAPD analysis was done as conducted in Williams et al. (1990). The 25 µl reaction mixture contained 200 µM of dNTP mix (dATP, dTTP, dGTP, dCTP, all in precisely equal molar concentration), 0.2 µM of a given 10-mer primer, 0.5 units of Taq polymerase assay buffer (Genetix) and 25 ng of genomic DNA in 1× Taq polymerase assay buffer (Genetix). The reaction was carried out in Perkin Elmer 9600 thermal cycler (Barnstead Thermolyne Corpn., Iowa, USA) programmed for one cycle at 92 °C for 4 min for initial template denaturation, followed by 44 cycles at 92 °C (denaturation) for 30 s, 37 °C (annealing) for 30 s and 72 °C (template extension) for 1 min, and one cycle at 72 °C for 7 min for final template extension. The amplified fragments of DNA were resolved by electrophoresis on 1.4 % agarose gel stained with ethidium bromide, and photographed under transmitted UV light (302 nm) using gel documentation system. The primers exhibiting polymorphism between near-isogenic parents were repeated four times to select only reproducible primers. Such reproducible primers were used to screen the F_2 population and analyzed for their segregation patterns.

In addition to RAPDs, previously reported SCAR markers were also used to screen parents. Three SCAR primers viz., ScOPD 10_{650}, ScOPO 18_{1200} and ScOPE 16_{1600} were synthesized. The primer ScOPD 10_{650} was reported to amplify a 650 bp band in the resistant cultivars (Timmerman et al. 1994). The primers ScOPO 18_{1200} and ScOPE 16_{1600} were reported to amplify 1,200 and 1,600 bp bands in susceptible and resistant parents, respectively (Tiwari et al. 1998). All three SCAR markers were studied for polymorphism. PCR were performed as discussed above for RAPD analysis with suitable modifications in the annealing temperatures.

Linkage map construction

Polymorphic markers identified by NIL marker analysis were used to screen the entire 208 F_2 mapping population of the PG 3 $(PMR)^{HFP\ 4}$ × PG 3 (PMS) cross. A linkage map using the candidate markers and the already reported polymorphic marker was constructed using computer programme MAPMAKER/EXP Ver. 3.0 (Lander et al. 1987), using Kosambi's mapping function (Kosambi 1944).

SCAR marker development

The parent PG 3 was used to amplify the most closely linked RAPD band of 880 bp using the primer OPX 04_{880}. The band of interest was precisely demarcated by giving a brief exposure to long wave UV. The band was then sliced out using a sterile scalpel. The eluted band was purified using Millipore filter kit obtained from M/S Millipore Inc., USA, following the manufacturer's instructions. The purified band was quantified on 0.8 % agarose gel. It was then used as a template for its re-amplification using the same primer. TA cloning strategy was employed using pGEM®-T Easy Vector system. The standard reaction consisted of 2× rapid ligation buffer- 5 µl, pGEM®-T Easy Vector-1 µl (50 ng); PCR product:1–3 µl; T_4 DNA ligase- 5 µl; deionized water to a final volume of 10 µl. The insert to vector molar ratio was kept between 3:1 and 8.2:1. The reaction mixture was mixed by pipetting, and incubated at 4 °C overnight. Transformation was carried out using high efficiency competent cells (DH-5α strain of *Escherichia coli*) following the protocol described by Sambrook and Russell (2001). The recombinant plasmid DNA from *E. coli* was isolated using Qiagen's QIAprep® Miniprep kit following the manufacturer's directions.

About 500 ng (1 µl) of plasmid was treated with six units of *Eco*RI enzyme and digested for 1 h at 37 °C. The insert size was then checked on 1.4 % agarose gel, by comparing with DNA molecular weight marker. Sequencing of the recombinant plasmids was done by taking one representative sample from each of the four classes of clones. Sequencing was performed on megaBACE-1000 sequencing machine, using M13 universal primers, at the Rice Genome Sequencing Project, IARI, New Delhi.

Designing of the SCAR primers

The sequence data generated from all four classes of clones were subjected to BLAST search (Basic Local Alignment Search Tool; http://www.ncbi.nih.gov/BLAST/). This programme was used for alignment of unique sequence and for retrieving vector and the 10-mer RAPD primer sequences. The primers were designed using the software "Primer 3" available at the URL http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi and "Web Primer" from the URL http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer for construction of suitable sets of primers. The primers were synthesized by M/S Qiagen Operon, Cologne, Germany. Each primer was supplied as a lyophilized salt with quantity ranging between 450 and 570 µg (13–19 OD). The primers were re-suspended in 1 ml of sterile $T_{10}E_1$ buffer at 4 °C for 48 h aliquoted in different tubes and stored at −20 °C.

Standardization of SCAR reaction

Based on the average T_m of the forward and reverse primers of each set, an annealing temperature gradient was set up. The annealing temperature gradient ranged between −5 and +6 of the respective average T_m for different sets of primers. The PCR conditions in 25 µl of reaction mixture were maintained as follows: 200 µmol of dNTPmix (dATP, dTTP, dGTP and dCTP in exactly equal concentration) (Promega); 25 µg of each primer; 1 unit Taq polymerase (Promega) and 25 ng of genomic DNA in 1× Taq polymerase assay buffer (Promega). Amplification was carried out in 35 cycles of 3 min of initial denaturation at 92 °C, 1 min for primer annealing at 58–67 °C for different sets of SCAR primers, 2 min of template extension at 72 °C, followed by 34 cycles of 1 min of denaturation at 92 °C; 1 min of annealing at 58–67 °C depending upon SCAR primer, 2 min at 72 °C. One additional profile of 72 °C for 7 min was given for final extension. The reactions were carried out in Biometra and Perkin Elmer 9600 thermal-cyclers. The amplified products were run on 1.2 % agarose gel and photographed in Gel-documentation system.

Results

Screening for polymorphism among parents

All seven sets of NILs were screened pair-wise to detect polymorphism for RAPD markers. Out of 620 random 10-mer RAPD primers used (OPA 01-20, OPB 01-20 to OPZ 01-20, OPAA 01-20 to OPAC 01-20, OPAE 01-20 and OPAK 1-20), nine polymorphic primers were identified between resistant and susceptible NILs. However, based on stringent reproducibility conditions (four times), three primers retained polymorphism, out of which only OPX 04 produced a bright polymorphic band (880 bp) and retained polymorphism in the NIL set PG 3 (PMR) $^{HFP\ 4}$ × PG 3 (PMS). The RAPD primer OPX 04 (5′-CCGCTACCGA-3′) amplified a fragment of about 880 bp in the susceptible parent PG 3 (Fig. 1). The other two polymorphic primers did not produce bright and thick polymorphic band as OPX 04.

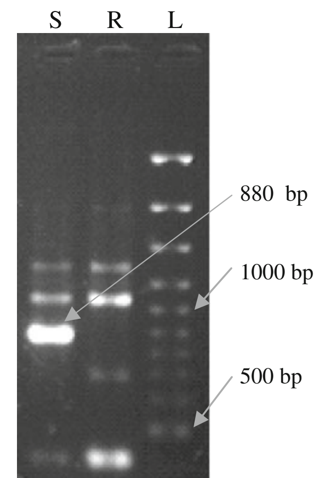

Fig. 1 RAPD marker OPX 04 showing 880 bp polymorphic band among parents of the mapping population. *S* susceptible parent PG 3, *R* resistant parent PG 3 (PMR)$^{HFP\ 4}$, and *L* 100 bp plus DNA molecular weight marker

Earlier reported *er1*-linked molecular markers viz., ScOPE 16_{1600}, OPL 06_{1900} and ScOPO 18_{1200} by Tiwari et al. (1998); and OPU 17_{1000} and OPU 02_{1100} by Janila and Sharma (2004) did not reveal polymorphism between the parents since the plant materials used in these studies were different. Out of seven sets of NILs used to study polymorphism, only one set namely PG 3 (PMR) $^{HFP\ 4}$ × PG 3 (PMS) was simultaneously differentiated by all the three polymorphic primers. The other NIL sets were either not differentiated by all the three polymorphic primers, or differentiated only by OPX 04.

SCAR marker development

Initial analysis revealed that the RAPD marker OPX 04_{880} and the SCAR marker ScOPD 10_{650} belonged to same linkage group in relation to *er1* gene. Hence OPX 04_{880} was converted to a SCAR marker. The polymorphic linked band of 880 bp, generated by the RAPD primer OPX 04 was amplified from susceptible parent PG 3, gel extracted and cloned. A total of 29 positive clones were picked up for insert check and subjected to *Eco*RI digestion. Eight clones showed insert of the right size, i.e., 880 bp. Within such clones, four different types of banding pattern were observed (Fig. 2). One representative sample from each of the four types was sequenced for primer designing and sequence homology search. Each of the four classes of clones gave four different types of sequence information. BLAST search did not yield any homology with the deposited sequences in the gene bank. Also none of the sequences shared any distinct types of repeat sequences.

Designing, synthesis and amplification check of the primers

Four Primer sets were designed from the sequence information of all the four classes of clones, using "Primer 3" and "Web Primer" software. The sense and antisense sequence details of all the four primer sets are given in Table 2. Out of the four primer sets, two (Class I & II) sets failed to distinguish between the two parents and amplified a band of 880 bp in both the parents, while Class IV primer set did not yield any amplification product. One primer set designated as ScOPX 04_{880} obtained from the Class III clone, correctly distinguished between resistant and susceptible parent (annealing temperature of 65.5 °C). This 21 mer sense (5′-CCGCTACCGATGTTATGTTTG-3′) and 20 mer antisense (5′-CCGCTACCGAACTGGTT GGA-3′) SCAR primer amplified a band of 880 bp (similar to its polymorphic RAPD counterpart OPX 04_{880}) in the susceptible parent PG 3, while no amplification was observed in the resistant isogenic parent PG 3 (PMR)$^{HFP\ 4}$. This marker, thus, behaved as a dominant coupling phase SCAR marker.

Determination of the linkage relationship of the new SCAR marker

The SCAR marker ScOPX 04_{880} segregated in precisely the same fashion as the 880 bp band of the RAPD primer OPX 04. It exhibited only one recombination (plant no. 9) with *er1* in the population of 208 F_2 plants (Fig. 3). MAPMAKER analysis placed ScOPX 04_{880} linked to *er1* gene at 0.6 cM, in coupling phase, while ScOPD 10_{650} was placed at 2.2 cM from

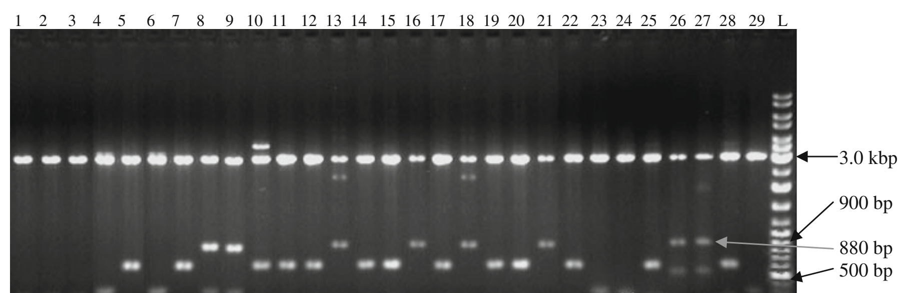

Fig. 2 *Insert* size check (880 bp) for the pGEM®-T Easy Vector with *Eco*RI digestion. The clones *8*, *9*, *13*, *16*, *18*, *21*, *26* and *27* are showing insert of the right size, i.e., 880 bp. Within these clone types, there are four distinct classes of clones. Clones *8* and *9* form class I; *13* and *18* form class II; *16* and *21* form class III; and *26* and *27* form class IV

Table 2 DNA sequence information of different SCAR primers tested and the amplification pattern on parents of the mapping population

Class	Primer sequence (5′ → 3′)	Length	Annealing temperature	Amplification		Band size
				S	R	
Ia	CCGCTACCGAAGTATTGCAAG (sense)	21	50–68 °C	+	+	880 bp
Ib	CCGCTACCGATTGATAGATAT (antisense)	21	50–68 °C			
IIa	CCGCTACCGATAATAGATTGCC (sense)	22	55–69 °C	+	+	880 bp
IIb	CCGCTACCGAGTTTATCATTAA (antisense)	22	55–69 °C			
IIIa	CCGCTACCGATGTTATGTTTG (sense)	21	65.5 °C	+	–	880 bp
IIIb	CCGCTACCGAACTGGTTGGA (antisense)	20	65.5 °C			
IVa	TAGGGTGACACCGCAGTGAC (sense)	20	53–69 °C	–	–	No band
IVb	TCATGCACGAGCCATGTATTC (antisense)	21	53–69 °C			

Class III SCAR primer, designated as ScOPX 04_{880} was synthesized from sequence information of class III clone (Fig. 3)

S susceptible parent PG 3 (PMS); *R* resistant parent PG 3 $(PMR)^{HFP4}$

er1 and 2.8 cM from ScOPX 04_{880} flanking the *er1* gene in repulsion phase (Fig. 4). The segregation pattern for these three molecular markers, and also the *er1* gene was in agreement with 3:1 ratio with non-significant χ^2 value with *P* ranging between 0.88 and 1.00 (Table 3). The presence of the amplified DNA band was taken as dominant over its absence as all these markers amplified their respective bands in the F_1 hybrids. As mentioned earlier, powdery mildew resistance was controlled by monogenic recessive gene in several studies. In the present investigation also, the F_2 mapping population always segregated in a good fit ratio of 3 PMS:1 PMR in all the crosses involving NILs.

Discussion

RAPD markers have been successfully used in the recent past to develop more reliable sequence tagged SCAR markers in crops such as oilseed rape (*Brassica napus* L.) (Mikolajczyk et al. 2008; Saal and Struss 2005), grapes (*Vitis vinifera* L.) (Kim et al. 2008; Akkurt et al. 2007), *Amaranthus* (Ray and Chandra 2009), safflower (*Carthamus tinctorius* L.) (Hamdan et al. 2008), papaya (*Carica papaya* L.) (Giovanni and Víctor 2007), *Pandanus fascicularis* (Vinod et al. 2007), faba bean (*Vicia faba* L.) (Gutierrez et al. 2007), common bean (*Phaseolus vulgaris* L.) (Park et al. 2008), sorghum [*Sorghum bicolor* (L.) Moench] (Singh et al. 2006) and wheat (*Triticum aestivum* L.) (Gupta et al. 2006). Recently Jones et al. (2009) have re-emphasized role of SCAR markers in tracking economically important genes.

This study elucidates successful use of advance generation NILs for fine gene tagging in plants. This has also been emphasized by several other workers (Martin et al. 1991; Young et al. 1988), as a more efficient tool in tagging genes with scorable phenotype than bulked segregant analysis (BSA) proposed by Michelmore et al. (1991). Bi-parental mapping populations generated by crossing NILs are more precise in mapping the target gene because it allows recombination to take place in the regions close to the gene of interest, since the rest of the genome would be homogenous. Although polymorphism between pairs of NILs may be low, but a marker, if found will have high probability to be closely linked to the gene of interest.

In the present study, seven pairs of NILs were used to screen for polymorphism. On the basis of highest number of polymorphic markers, one NIL pair was selected for development and use of mapping population for identification of molecular markers linked to the *er1* gene. This NIL pair was developed by crossing HFP 4, carrying *er1* allele in homozygous form, to the susceptible recurrent parent PG 3. In the ninth generation of back-crossing it was expected that *Er1/Er1* locus was replaced with *er/er*, while the rest of the genome was the same (>99.9 %) as that of recurrent parent PG 3. The converted NIL was resistant to the powdery mildew (as HFP 4) and was designated as PG 3 $(PMR)^{HFP\ 4}$.

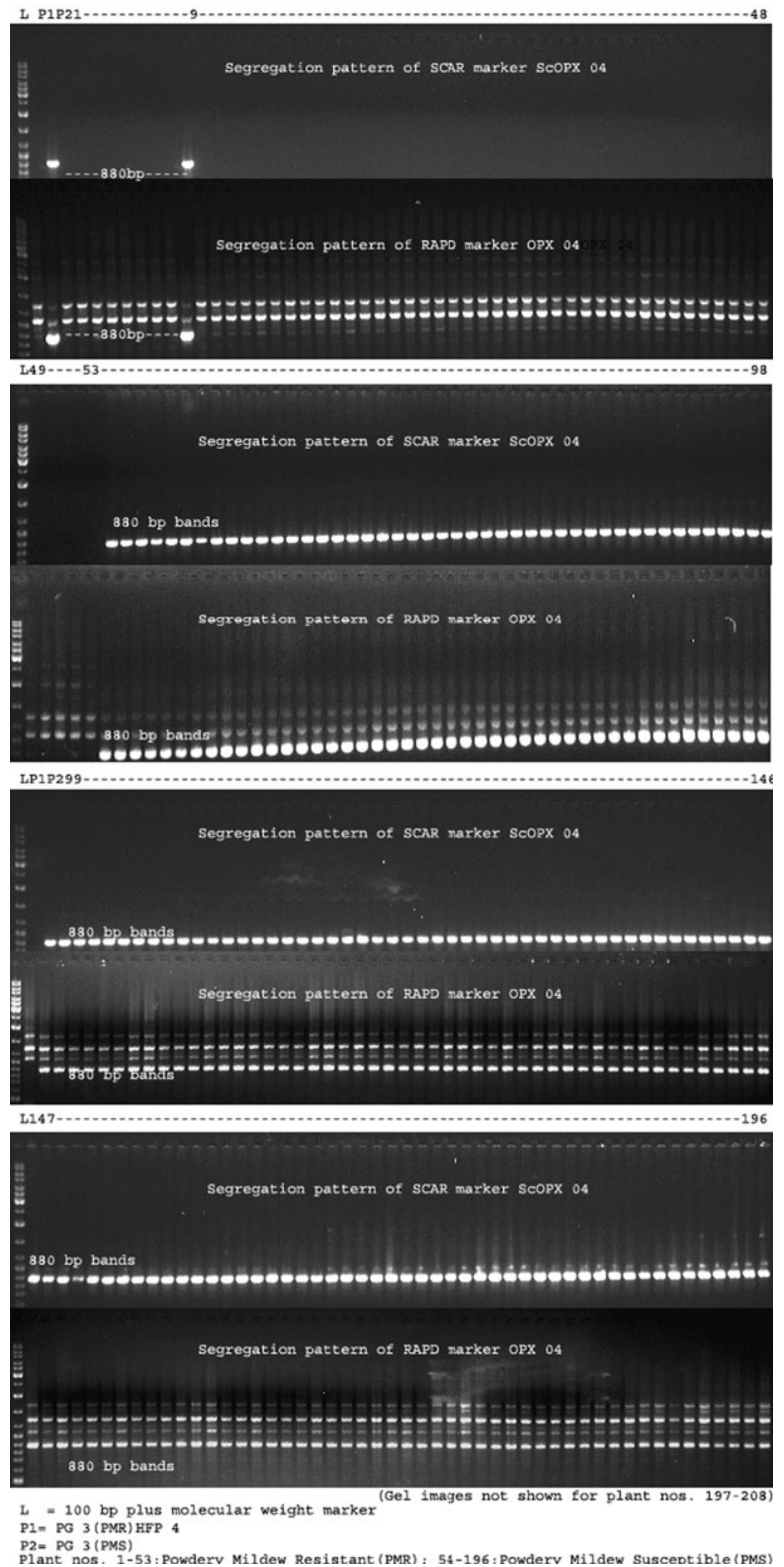

Fig. 3 Comparative segregation pattern of the RAPD (OPX_{880}) derived SCAR marker $ScOPX_{880}$ in F_2 mapping population of the cross PG 3 $(PMR)^{HFP\ 4}$ × PG 3 (PMS)

SCAR marker development

Sequencing of multiple clones of the 880 bp informative amplicon obtained with the random primer OPX 04 revealed presence of four different types of clones. No sequence homology between Class I and IV clones (Fig. 2) might be due to amplification of non-target regions of the genome by the RAPD primer or co-migration. Such random 10-mer sequences have a probability to find unlinked regions of the genome once every 1.048 Mb. It was also unusual not to find any repetitive elements in these sequences. This may be due to small sequence length (sampling error) and/or due to sequences from the functional region of the genome, known to carry very little repetitive elements.

Lack of polymorphism for Class I, II and IV SCAR primers was on expected lines. Although RAPD primer sequence was present in Class I and II primers, there was no sequence homology to the *Er1* locus, and may represent sequence complementarity with regions unlinked to the *Er1* locus. Class IV SCAR primer set was a clear case of co-migration due to non-complimentary amplification as no RAPD primer sequence could be located. This is a common phenomenon observed with RAPD primers due to their low annealing temperature (37 °C in the present study) and short primer length.

On the other hand, polymorphism and co-segregation with the powdery mildew disease for Class III SCAR primer sets derived from Class III clones may be due to presence of sequence homology to the *Er1* locus.

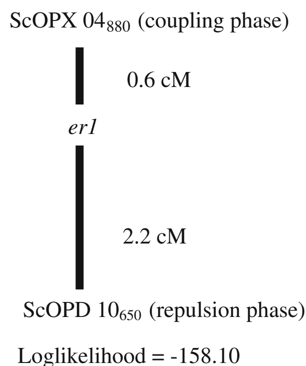

Fig. 4 Linkage map of *er1* gene with SCAR and RAPD markers

Mapping of the *er1* gene with SCAR markers

Comparable segregation pattern and band sizes of SCAR and RAPD markers may be due to inclusion of RAPD primer sequence at the 5′ ends of the sense and antisense primers. It is worth mentioning that ScOPX 04_{880} appeared at a distance of zero cM from *er1* gene in multipoint analysis, whereas pair-wise analysis (two point cross) placed it at 0.6 cM. In multipoint analysis, the lone recombinant of ScOPX 04_{880} was taken as an error (with "error detection on" command in MAPMAKER) and, therefore, was shown at zero cM distance (data not presented). Hence, the map distance of 0.6 cM obtained from pair-wise analysis appears to be more realistic (Table 4). The composite linkage map is presented in Fig. 4. In this map ScOPD10_{650} maps at 2.2 cM, very close to the 2.1 cM map distance reported by Timmerman et al. (1994), but is different from Rakshit (1997) and Janila and Sharma (2004)

Table 4 Pair-wise joint segregation analysis of molecular markers and *er1* gene

Marker	Pair-wise distance (cM)	Pair-wise LOD value
er1–ScOPX 04_{880}	0.6	48.34
er1–OPX 04_{880}	0.6	48.34
er1–ScOPD 10_{650}	2.2	37.12
ScOPX 04_{880}–ScOPD 10_{650}	2.8	3.79

Table 3 F_2 segregation of molecular markers in the cross PG 3 (PMR)$^{HFP\ 4}$ × PG 3 (PMS)

Marker	Cross			No. of F_2 plants with the marker			χ^2 (3:1)	*P*
	[PG 3 (PMR)$^{HFP\ 4}$] × [PG 3 (PMS)]			Present (S)	Absent (R)	Total		
er1	*er1er1*	*Er1er1*	*Er1Er1*	155	53	208	0.026	0.88
ScOPX 04_{880}	−	+	+	156	52	208	0.000	1.00
OPX 04_{880}	−	+	+	156	52	208	0.000	1.00
ScOPD 10_{650}	+	+	−	155	53	208	0.026	0.88

who reported ScOPD10_{650} at distance of 3.7 and 3.4 cM, respectively, from *er1*. Such minor discrepancies in map distance may arise due to differences in genetic architecture. In fact markers mapped in a particular cross rarely give the same map distance in other crosses. In present investigation SCAR markers ScOPE 16_{1600} and ScOPO 18_{1200} reported by Tiwari et al. (1998), and RAPD markers OPU 17_{1000} and OPU 02_{1100} reported by Janila and Sharma (2004) did not reveal polymorphism. This may be attributed to differences in the primer binding sites due to evolution of the lines at different geographical locations under varying kinds of selection pressure. The implications of such results may warrant development of molecular markers which are anchored to the polymorphic regions of the genome which remain conserved by pedigree and origin. The SCAR marker developed in the present study need to be verified in the other materials with different genetic background for its suitability in gene pyramiding and MAS.

ScOPX 04_{880} as a dominant coupling phase marker can precisely identify *er1er1* plants with more than 99 % precision. On the other hand, ScOPD 10_{650} would amplify in susceptible carriers as well, thereby limiting its use in germplasm screening. Therefore, the major limitation of a less tightly linked repulsion phase SCAR marker ScOPD 10_{650} can easily be overcome by the use of new tightly linked SCAR marker ScOPX 04_{880} identified in the present investigation. Extremely reliable MAS can be carried out by combining the two flanking SCAR markers ScOPX 04_{880} and ScOPD 10_{650} for BC_nF_1 and germplasm screening with almost 100 % accuracy. Presence of two bands (of 880 and 650 bp size) will indicate heterozygosity at *Er1* locus, while only one band of 650 bp will indicate homozygous resistant plants, and solitary 880 bp band will suggest homozygous susceptible plants.

RAPD-derived SCARs can circumvent the problems associated with the SCARs generated from AFLP bands that typically range between 50 and 400 bp. Such short sequences in many cases do not reveal polymorphism upon conversion to SCAR, and may need inverse PCR (I-PCR) or PCR walking strategy to generate polymorphism (Negi et al. 2000). It is easier to convert RAPD markers to SCAR markers as the former generate fragments in the size range of 500–1,500 bp (Barret et al. 1998), obviating the need for I-PCR or PCR walking. The same has been demonstrated successfully in the present study. The SCAR marker linked to the *er1* gene developed in the present study, and the other reported marker systems could be used for pyramiding genes for a more broad based durable resistance against powdery mildew pathogen.

We need to better understand *er1* gene, which is thought to evolve from loss-of-function mutation of a plant-specific Mildew Resistance Locus O1 (*PsMLO1*) (Humphry et al. 2011) under natural selection pressure. We also need to understand how a recessive gene coding for a defective/malfunctioning/nonfunctional gene product, initiates a cascade of metabolic pathways that result in a near-perfect protection of the pea from *E. pisi* globally.

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