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Molecular Screening of Pea Germplasm for Rust Disease Resistance using SSR Marker

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In order to determine the association of molecular markers with the pea rust resistance, thirty two diverse pea genotypes were phenotypically screened on the basis of disease reaction followed by molecular screening using four SSR markers - AA446 and AA505 flanking the major QTL *Qruf*; AD146 and AA416 flanking the minor QTL, *Qruf1* associated with pea rust resistance. SSR markers AD146 flanking the minor QTL, Qruf1 were able to identify one moderately resistant (Pant P 42), six moderately susceptible genotypes and four susceptible genotypes (25-30 percent disease severity) with amplified fragment of 430bp. Whereas, SSR markers AA416 flanking the minor QTL, Qruf1amplified a fragment of 280bp in four moderately susceptible genotypes, seven susceptible genotypes (25-30 percent disease severity) and one susceptible genotypes with 49.17 percent severity (HUDP1301). It was observed that most of the germplasm with disease severity of less than 30 percent showed the presence of Qruf and/or Qruf1 governing partial resistance against rust. Therefore, molecular screening of germplasm may conclude that these SSR markers (AA446, AA505, AD146 and AA416) if used together, can be effective in marker assisted selection (MAS) of pea rust resistance.

Keywords: Molecular Markers, Rust, Disease Severity, Marker Assistant Selection.

Pea is affected by a number of fungal (rust, powdery mildew, downy mildew, root rot, alternaria blight, aschochyta blight, wilt, anthracnose, cercospora leaf spot, damping off, seedling rot etc.), bacterial (bacterial blight and brown spot), nematode (cyst nematode, lesion nematode and root-knot nematode) and viral diseases (cucumber mosaic virus, pea early browning virus, pea enation mosaic, pea mosaic, pea seed borne mosaic, pea streak and pea stunt). These diseases, under the right conditions, can significantly decrease both yield and quality. Among these, the rust of pea caused by *Uromyces viciae–fabae* (Pers.) J. Schrot (syn. *Uromyces fabae* (Pers.) de Bary) is considered the most important under warm and humid conditions[1]. It has been reported from different parts of the country including eastern India[2, 3], central India[4], southern parts of India[5, 6] and from Himalayan region of Uttarakhand and Himachal Pradesh[7, 8]. In the last few years, disease has been observed in almost epiphytotic form and could cause up to 20-100% losses in yield[9, 8]. Screening for rust severity indicated wide range of variations for rust resistance in the germplasm lines of pea and none of the genotypes tested were found to be free from infection[4, 10, 2, 6, 11, 1]. Rust severity is greatly influenced by the environment during initial infection and disease development. This is the major bottleneck in screening and selection for rust

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resistance. Use of molecular markers would allow indirect selection for rust resistance independent of environmental effects[12]. Molecular markers associated with pea rust resistance would be useful in marker assisted selection (MAS). For the development of rust resistant varieties there is need for phenotypic as well as molecular screening of existing lines/ germplasms/cultivars, therefore the present research has been carried out.

MATERIALS AND METHODS

Screening of thirty two pea germplasm under natural epiphytotic condition was carried out in the field during *Rabi* season 2013-14 and 2014-15 at N.E. Borlogue Crop Research Centre (NEBCRC), G.B. Pant University of Agriculture and Technology, Pantnagar. The germplasm screening was undertaken following 'Infector row technique'. Each entry was sown with wider spacing of 30 x 10cm in 3m row with a susceptible check 'HFP-4' after every five entries and a susceptible border row for over 2 seasons (*Rabi* 2013-2014 and 2014-2015). The observation on rust severity was recorded when first symptoms appear and subsequent observations were recorded at ten days interval and final observations was recorded at 20 days before harvesting of entries. Disease severity was determined using 0-9 rating scale[13]. The genotypes were later grouped into different categories based on 0 to 9 scale of disease severity from immune to highly susceptible according to Mayee and Datar (1986) with slight modifications (Table 1.). Thereafter, molecular screening of thirty two diverse pea genotypes which were phenotypically screened on the basis of disease reaction was evaluated using four SSR markers (Table 2.) (AA446 and AA505 flanking the major QTL *Qruf*; AD146 and AA416 flanking the minor QTL, *Qruf1*) associated with pea rust resistance[12].

PCR procedure

DNA from each germplasm was extracted following the CTAB method[14]. About 100 mg of young leaf tissue was excised from aseptically grown seedlings of each genotype. PCR

Table 1. Disease severity scale showing different types of disease reaction

Rating	Description	Disease reaction
0	No symptoms on leaf	Immune (I)
1	Rust pustules small, scattering covering 1% or less of leaf area	Resistant (R)
3	Rust pustules more in number covering 1-10% of leaf area	Moderately resistant (MR)
5	Typical rust pustules covering 11-25% of leaf area	Moderately susceptible (MS)
7	Typical rust pustules covering 26-50% of leaf area. Leaf shedding	Susceptible (S)
9	Typical rust pustules covering 51% or more of leaf area. Defoliation severe	Highly susceptible (HS)

Table 2. Primers used in molecular screening (Loridon *et al.*, 2005).

Sl. No.	Primer Name	Forward sequence	Reverse sequence	PIC*	Tm© (^{0}C)
1.	AA446	5' TTA GCT TGC AGC CCA CTC 3'	3' ATC CGA CCC ATG GAT TTA 5'	0.66	55
2.	AA505	5' ATT CAC ACG CGC CCA 3'	3' CAA TTA AGC CCT CAT CCA GA 5'	0.69	55
3.	AD146	5' TGC TCA AGT CAA TAT ATG AAGA 3'	3' CAA GCA AAT AGT TGT TTT GTT A 5'	0.84	51
4	AA416	5' TTA CTG TTA CTT TGC GAC ATC A 3'	3' ATA GTG TCG AAA TTT TCC ATC C 5'	0.64	61

* PlC- Polymorphism information content, ©Tm- Annealing temperature

amplification solution was prepared using 10mM tris-HCl (pH 9.0; 1.5mM $MgCl_2$;50mM KCl and 0.01% gelatin), 0.5 mM $MgCl_2$, 200 mM dNTPs, 1.25 ìM of primer, 20 to 25ng of DNA and1unit of Taq polymerase per 25Kl reaction volume. SSR markers AA446 and AA505 flanking the major QTL Qruf and AD146 and AA416 flanking the minor QTL, Qruf1[12] were commercially synthesized and procured from Eurofins Inc., Bengaluru. The amplification reaction was carried out in thermo cycler (MyCycler®, Bio-Rad Laboratories, California). After initial denaturation at 94 °C for 5 min, cycle was repeated 40 times; denaturing at 94 °C for 1 min, annealing according to primer [15] for 1 min, extension at 72 °C for 2 min, and the final extension segment was hold for 7 min. Thereafter, PCR products were separated electrophoretically in 2% (w/v) agarose gel. Ethidium bromide solution at a final concentration of 0.5 ìg/ml was added to the agarose solution. The gel was visualized and documented using gel documentation system (Transilluminator with filter, GeNei, Benagluru).

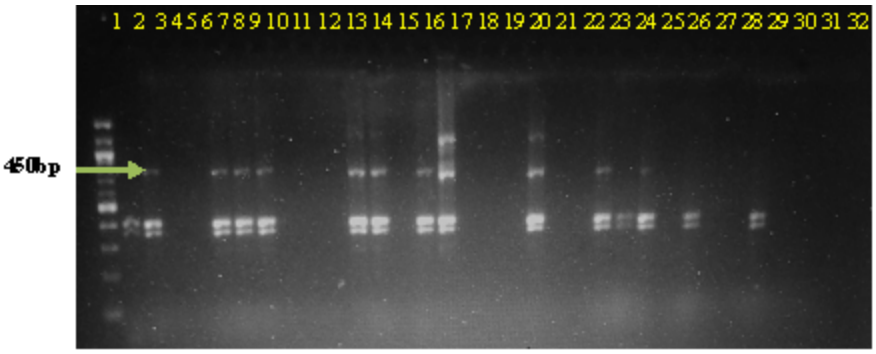
Table 3. Molecular screening of selected germplasm for rust resistance in pea.

S. No.	Germplasm	Disease severity (%)	Disease reaction	[1]Qruf		[2]Qruf1	
				AA446 (450bp)	AA505 (140bp)	AD146 (430bp)	AA416 (280bp)
1.	Pant P 244	8.17	MR	+	+	_	_
2.	Pant P 42	8.50	MR	+	+	+	_
3.	KPMR 522	42.50	S	_	_	_	_
4.	HUVP 1	55.83	HS	_	_	_	_
5.	HFP 530	30.00	S	+	+	_	+
6.	HFP 1016	25.83	S	+	+	+	+
7.	HFP 9907	21.67	MS	+	+	+	_
8.	KPMR 925	45.00	S	_	_	_	_
9.	VL 202	43.33	S	_	_	_	_
10.	Pant P223	27.50	S	_	+	+	+
11.	VL 59	21.67	MS	+	_	+	+
12.	Pant P 222	19.17	MS	+	+	_	_
13.	Pant P 217	10.33	MS	_	+	+	+
14.	Pant P 213	12.17	MS	+	+	_	+
15.	VL 58	13.17	MS	+	_	_	_
16.	KPMR 853	47.50	S	_	_	_	_
17.	HUDP 1302	22.50	MS	_	+	+	_
18.	HUDP 1209	25.83	S	_	+	+	_
19.	RFP 2009-2	19.17	MS	+	_	+	+
20.	RFP 2009-3	39.17	S	_	_	_	_
21.	HUDP 1301	49.17	S	_	_	_	+
22.	KPMR 851	25.83	S	+	+	_	+
23.	KPM 928	25.83	S	+	+	+	+
24.	HUDP 15	20.83	MS	+	+	+	_
25.	IPFD 13-14	25.83	S	_	+	_	+
26.	IPF 10	30.83	S	+	+	_	+
27.	IPFD 5-19	23.33	MS	_	_	_	_
28.	IPFD 99-13	44.17	S	_	_	_	_
29.	IPFD 11-5	25.83	S	+	+	_	_
30.	IPFD 12-2	45.83	S	_	_	_	_
31.	IPFD 13-4	39.17	S	_	_	_	_
32.	HFP-4 (check)	65.00	HS	_	+	_	_

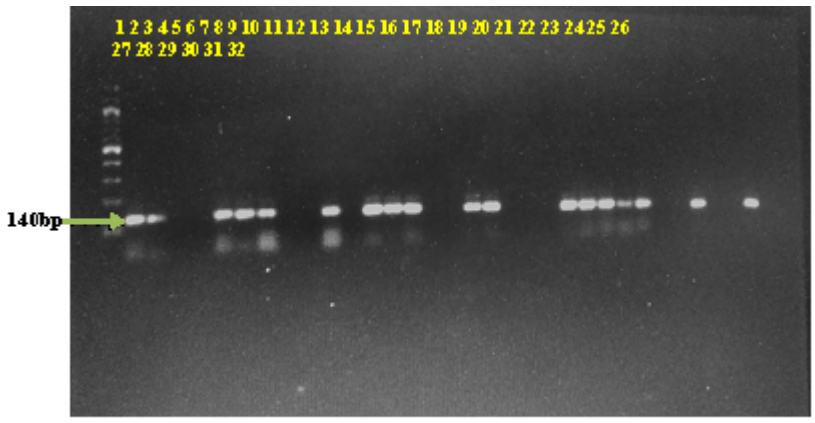
[1]Qruf flanked by SSR markers AA446 and AA505 (Rai *et al*., 2011), [2] Qruf1 flanked by SSR markers AD146 and AA416 (Rai *et al*., 2011), + indicates presence of a band and " indicates absence of the specific band

RESULTS AND DISCUSSION

Among 32 pea genotypes screened, none of them were found resistant to rust disease during both the seasons. Further, in our search, none of the genotype was found to be completely resistant to the rust disease, which was in agreement with earlier reports[2, 16] although these reports were based on the screening of limited genotypes. Only two genotypes showed moderate reaction with 1-10 per cent disease severity (Pant P 244 and Pant P 42). Maximum numbers (18) of genotypes fall under susceptible category followed by moderately susceptible (13) and highly susceptible category (2). Thus, two genotypes showing moderately resistant reaction can be integrated with reduced number of fungicidal spray to obtain maximum yield with minimal rust severity. Screening for rust severity indicated wide range of variations for rust resistance in the germplasms lines of pea and none of the genotypes tested were found to be free from infection[4, 10, 2, 6, 11, 1]. Pal *et al*[17] screened a total of 292 accessions of pea (*Pisum spp.*) under field conditions for resistance to rust and he found only three accessions PJ207508, PJ222117, and EC109188 which was resistant to rust. Kumar *et al*[6] used area under disease progress curve (AUDPC) to depict the overall disease stress that the plants were subjected to and described the pea varieties Pant P8, HUP 8063, KPMR 22 to possess good level of partial resistance. Likewise, Chand *et.al*[18] screened 345 accessions, out of which forty-four genotypes were evaluated for disease intensity. Wide range of variation was found for those traits. The genotypes Pant P 11, FC 1, HUDP 16, JPBB 3 and HUP 14 appeared as slow rusting genotypes. Similarly, Mishra *et al*[19] evaluated 107 genotypes of field pea against rust (*Uromyces viciae-fabae)*, out of which genotypes P 9-77, P 2432; P2572 and P 2930 were found resistant, whereas 27 exhibited



PCR banding pattern of the SSR marker AA446 flanking the major QTL 'Qruf'



PCR banding pattern of the SSR marker AA505 flanking the major QTL 'Qruf'

Plate 1. PCR banding pattern of the SSR markers AA446 and AA505 flanking the major QTL 'Qruf' in selected pea germplasm

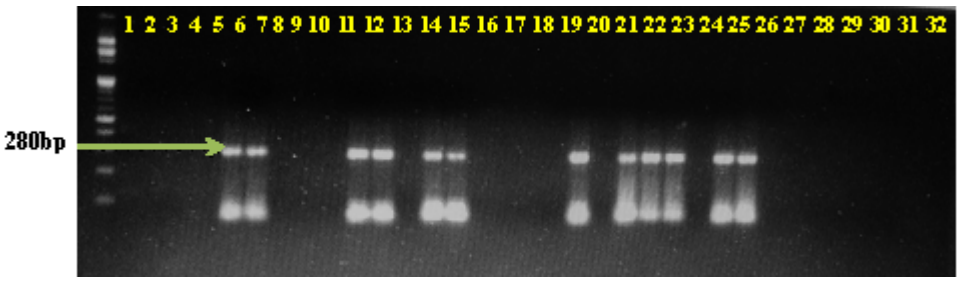
moderate reaction. Barilli *et al*[20] evaluated 2759 pea accessions for resistance against *Uromyces pisi* (Pers.) Wint. All accessions in his experiment displayed a compatible interaction (high infection type) both in adult plants under field conditions and in seedlings under growth chamber conditions, but with varying levels of disease reduction. The identified resistance was based on reduction of disease severity with no associated host cell necrosis, which fits the definition of Partial Resistance. No complete resistance or incomplete resistance based on hypersensitivity was observed by them.

Severity of rust is greatly influenced by the environment during infection initiation and disease development. This is the major bottleneck in screening and selection for rust resistance. Thus, use of molecular markers would allow indirect selection for rust resistance independent of environmental effects.

SSR marker AA446 flanking the major QTL Qruf amplified a fragment of 450 bp in two moderately resistant (Pant P 244 and Pant P 42), seven moderately susceptible and six susceptible genotypes with 25-30 percent disease severity. Whereas, SSR marker AA505 flanking the major QTL Qruf amplified a fragment of 140 bp in two moderately resistant(Pant P 244 and Pant P 42), six moderately susceptible, nine susceptible genotypes (25-30 percent disease severity) and one highly susceptible genotypes(HFP-4) (Table 3 and Plate 1).

SSR markers AD146 flanking the minor QTL, Qruf1 were able to identify one moderately resistant (Pant P 42), six moderately susceptible genotypes and four susceptible genotypes (25-30 percent disease severity) with amplified fragment of 430bp. Whereas, SSR markers AA416 flanking the minor QTL, Qruf1amplified a fragment of 280bp in four moderately susceptible genotypes, seven susceptible genotypes (25-30 percent disease severity) and one susceptible genotypes with 49.17 percent severity (HUDP1301) (Table 3 and Plate 2).

It was observed that most of the germplasm with disease severity of less than 30 percent showed the presence of Qruf and/or Qruf1 governing partial resistance against rust. Therefore, molecular screening of germplasm may conclude that these SSR markers (AA446, AA505, AD146 and AA416) if used together, then it can be more effective in marker assisted selection (MAS) for pea rust resistance. Similarly, Singh *et al*[21] utilize molecular markers associated with the pea rust resistance for evaluation of 30 diverse pea genotypes using these four SSR markers. On the basis of marker allele analysis they concluded that



PCR banding pattern of the SSR markers AD146 flanking the minor QTL 'Qruf1'

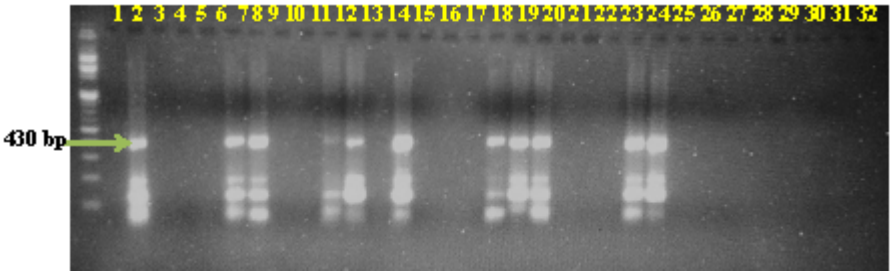


Plate 2. PCR banding pattern of the SSR markers AD146 and AA416 flanking the major QTL 'Qruf1' in selected pea germplasm

these SSR markers can be used in MAS of pea rust resistance. Vijayalakshmi *et al*[22] suggested two RAPD makers, viz., *SC10-82*360, and *SCRI-71*1000, flanking the rust resistance gene (*Ruf)* if used together, the effectiveness of marker assistant selection for rust resistance would be improved considerably. Avila *et al*[23] also identified random amplified polymorphic DNA (RAPD) markers linked to resistance gene (*Uvf-1*). This result conclude that for the development of rust resistant varieties there is need for phenotypic followed by molecular screening of existing Table.3

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