Scientist|X89X367X|7.72|Published by LMG| https://doi.org/10.5281/zenodo.7811408
https://www.thescientist.online/

Marker-based characterization for rust resistance in garden pea [*Pisum sativum* var. *hortense* L.]

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Abstract

The experiment was conducted during *Rabi* season of 2015-16 and 2016-17 at Vegetable Research Centre, GBPUA&T, Pantnagar to find out the best suitable markers for identification of rust resistance in pea (*Pisum sativum* var. *hortense* L.). Four validated SSR markers *viz*., AA446, AA505, AD146 and AA416 associated with the identification of rust-resistant and susceptible lines have been used on five parents (Pant Uphar, PSM-4, NDVP-250, Nepal Pea and PEVAR-6) susceptible to rust and one rust resistant parent *i.e*. Arka Ajit and their five F_1's. From the PCR banding pattern of these SSR markers it was found that two markers *viz*., AA446 amplified a band of ~ 400 bp which was present in the resistant parent and F_1's and marker AA505 amplified a band of ~ 140 bp in resistant parent and F_1's. Thus, these two markers were able to distinguish among susceptible and resistant parents and their F_1's whereas two markers *viz*., AD146 and AA416 could not able to distinguish among the susceptible and resistant parents and their F_1's.

Key word- Pea (*Pisum sativum* var. *hortense* L.), SSR marker, Rust resistance, PCR

Introduction

Pea (*Pisum sativum* L.), a member of the family Fabaceae is an important vegetable crop grown in temperate and subtropical areas of the world. It is native to Europe and West Asia, while its wild prototype came from Ethiopia. It is a highly nutritive legume which is used in the form of green vegetables, soup, dal etc. It is also being used to make chapatti, mixed with barley or wheat flour. Peas are a rich source of protein, carbohydrates, vitamins B_1, B_2 and C and minerals like calcium, phosphorus and iron. Pea is not only the major contributor to the much-needed proteins (15.5-39.5 %) but also has superior quality proteins compared to all other vegetables except soybean, like higher lysine content, the limiting amino acid in cereals. It is an important legume grown as a garden and field crop and it is mainly a cool-season vegetable crop. In India, it occupies 563 thousand ha area with a production of 570 thousand metric tonnes (MT) and productivity of 98.77 q/ha. Its share is about 2.97 % and 5.43 % in total vegetable production and area, respectively (NHB, 2019).

Despite its immense economic importance, the pace of genetic improvement in pea remained slow. Low yield potential of existing varieties, reduction in productivity and quality of the product has always remained a concern for breeders in the country. Selection of parents and isolation of superior pure lines having resistance against important diseases from segregating generations are the main objectives of the breeder. Besides various diseases, especially rust is the major impediment. Pea rust caused by *Uromyces fabae* Pers. de-Bary is a major disease of peas and is responsible for significant yield losses, especially in the sub-tropical regions characterized by warm humid weather conditions (Kushwaha et al., 2006). These conditions usually coincide with the reproductive phase of pea and favour rust outbreak (Kushwaha et al., 2007). In the last few years, the disease has been observed in almost epiphytotic form and could cause up to 20-100% losses in yield (Sharma, 1998; Stavely, 1991). Screening for rust severity indicated a 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 (Chand et al., 2004; Gupta, 1990; Kumar et al., 1994; Narsinghani et al., 1980; Singh et al., 1985; Xue and Warkentin, 2002).

Resistance to rust in pea is reported to be governed by a single dominant gene(Tyagi and Srivastava 1999), an oligogene (Vijayalakshmi et al., 2005) showing partial dominance along with some minor genes and 2–3 additive genes(Singh et al., 2012). Resistance to pea rust has been reported to be post haustorial type, and none of the pea genotypes is reported to be free from rust infection (Singh and Srivastava, 1985; Chand et al., 2006).

Therefore, breeding for improved high-yielding, disease-resistant cultivars suitable for different agro-climatic conditions is necessary to further enhance the production and productivity of vegetable pea. Molecular markers-based characterization of disease is an important process that provides valuable data to detect variation at the DNA level and to assess genetic diversity in garden pea.

Information regarding the association of molecular markers with rust resistance is available (Vijayalakshmi *et al.* 2005; Singh et al., 2012; Singh and Srivastava, 1985; Chand et al., 2006; Rai et al., 2011) which seems to be governed by one major and one minor QTL. Vijayalakshmi *et al.* (2005) identified two RAPD markers SC10-82_{360} and

SCRI - 71_{1000} flanking the rust resistance gene (Ruf). Barilli *et al.* (2010a) reported a major QTL for resistance to *U. pisi* in wild pea (*P. fulvum* L.) which was flanked by RAPD markers OPY111316 and OPV171078; located on linkage group 3.

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 resistance. The use of molecular markers would allow indirect selection for rust resistance independent of environmental effects[16]. Molecular markers associated with pea rust resistance would be useful in marker-assisted selection (MAS).

For the development of rust-resistant varieties, there is a need for phenotypic as well as the molecular screening of existing lines/germplasms/cultivars. Therefore, the present research has been carried out as one of the best possible ways to stabilize the productivity of pea crops by the development of rust-resistant varieties.

Material and methods

Plant Material and SSR markers

In the present study, four validated SSR markers *viz*., AA446, AA505, AD146 and AA416 (Table 1) associated with the identification of rust-resistant and susceptible lines (Singh *et al.*, 2015) have been used on five parents (Pant Uphar, PSM-4, NDVP-250, Nepal Pea and PEVAR-6) susceptible to rust and one rust resistant parent *i.e*. Arka Ajit and their five F_1's.

Methodology

The total genomic DNA was isolated from each genotype and is done by the Cetyl tri-methyl ammonium bromide (CTAB) method (Clarke, 2009) with certain modifications. For the extraction of DNA, HiPurA™ Plant DNA Isolation Kit (CTAB Method), Himedia, Mumbai was used.

Principle: CTAB is a detergent which is used to break open and solubilise the contents of plant cells. Chlorophyll and some denatured proteins are removed from green plant tissue in the organic chloroform/octanol step, and the organic phase is separated by centrifugation. Since the extract contains DNA and RNA, RNA can be removed by the addition of RNase A, the DNA is precipitated and washed in organic solvents before redissolving in an aqueous solution. The DNA recovered is not free

from contaminants such as carbohydrates, but is of suitable grade for enzyme digestion, southern blotting and analysis by PCR.

Preparation, extraction, purification and isolation of the plant sample DNA

The parents were grown and crossed in *Rabi* season of 2015-16. Parents along with their five F1's were grown in 2016-17 at VRC, GBPUA&T, Pantnagar. Fresh Leaves from the top of plants 25-30 days old were taken for genomic DNA extraction. 0.5 g of the plant tissue was taken into mortar and pestle and ground into fine powder by pouring liquid nitrogen. Then we put this fine powder into 2 ml of Eppendorf tube which contain 800µl of extraction buffer (CTAB buffer) and mixed it gently by inversion. After that, we put the Eppendorf tubes in a hot water bath pre-warmed and set them at 65˚C for 60-90 minutes with occasional mixing by inversion.

After that, an equal volume of Chloroform:Isoamylalcohol (24:1) solution was added to the tubes and then the tubes were slowly inverted for 5-10 minutes for proper mixing. Then the samples were centrifuged at 8,000 rpm for 10 minutes and the supernatant (upper phase) was transferred into fresh tubes and again treated with Chloroform: Isoamyl alcohol (24:1) and centrifugation was done in the same way.

After collecting supernatant, RNase treatment was also given by adding 2µl of RNase (10mg/ml) to each sample and by incubating at 37^{o}C for 1 hour in the water bath. After that 0.6 ml of chilled Isopropanol (-20^{o}C) was added to the supernatant and stored at 4^{o}C for 1-2 hours. Then the centrifugation was done at 10,000 rpm for 10 minutes at 4^{o}C. The supernatant was discarded and the pellets were washed with 70% ethanol and then centrifuged at 10,000 rpm for 5 minutes. After that, we discard the 70% ethanol from the tube carefully and then the pellet was air dried. 200 µl of 1x TE buffer was added into the tube to dissolve the pellet and then the tubes or samples were stored at 4^{o}C. DNA was quantified by using nanodrop and agarose gel electrophoresis.

Table- 1 List of SSRs primers used for DNA amplification

S. No.	Primer Code	Forward primer (5´ 3´)	Reverse primer (5´ 3´)
1	AA446	TTAGCTTGCAGCCCACTC	ATCCGACCCATGGATTTA
2	AA505	ATTCACACGCGCCCA	CAATTAAGCCCTCATCCAGA
3	AD146	TGCTCAAGTCAATATATGAAGA	CAAGCAAATAGTTGTTTTGTTA
4	AA416	TTACTGTTACTTTGCGACATCA	ATAGTGTCGAAATTTTCCATCC

Note: All SSR primers were procured from Integrated DNA Technologies Inc. (U.S); New Delhi.

Primer dilution

For primer dilution, we used double distilled and autoclaved water to dilute the primers at a concentration of 10 pmol.

PCR reaction and DNA amplification

The DNA sample extracted from parental lines and their F_1's was amplified using the polymerase chain reaction (PCR) by using the protocol of Loridon *et al.* (2005) with few modification reaction conditions of primers, template DNA, $MgCl_2$, dNTPs and Taq polymerase. The optimum concentrations of various components were standardized for 20 µl reaction volume. The SSR-PCR amplification was performed using a 96-well gradient thermal cycler (Applied Biosystems). During the process, several thermocycling programmes involving varied denaturations, annealing and extension protocols were used and the protocol which gave intense, discrete and well discrete and well-dispersed bands were used. PCR amplification was carried out in thin-walled 0.2ml PCR tubes containing 20 µl reaction mixtures having the following components (Table 2).

Table 2 Reagents with their concentration and quantity used for a single PCR reaction

S. No.	Reagent	Single tube(20µl)
1	DNA template* (50 ng/µl)	4.0
2	Triple distilled water	9.74
3	Taq buffer with 15 mM MgCl2 A (10X)	3.0
4	dNTPs mix (2.5 mM each)	0.6
5	Primer Forward (50 ng/µl)	1.0
6	Primer Reverse (50 ng/µl)	1.0
7	Taq polymerase (3U/µl)	0.66

*Template DNA was added as the last component while preparing the reaction mix.

PCR tubes containing the reaction mix were gently vortexed and subjected to the thermal profile given in table 3. The amplification reaction was carried out in a gradient Thermocycler.

Table 3 Thermocycler's profile

S.No.	Step	Temperature (°C)	Duration (Minutes)
1	Initial Denaturation	95	5
2	40 cycles each of		
i.	Denaturation	94	1
ii.	Annealing	55	1
iii.	Extension	72	2
3	Final Extension	72	7
4	Hold at 4 °C		

Results and discussion

From the PCR banding pattern of these SSR markers it was found that two markers *viz*., AA446 amplified a band of ~ 400 bp which was present in the resistant parent and F_1's (Plate 1) and marker AA505 amplified a band of ~ 140 bp in resistant parent and F_1's (Plate 2) thus these two markers were able to distinguish among susceptible and resistant parents and their F_1's whereas two markers *viz*., AD146 and AA416 could not able to distinguish among the susceptible and resistant parents and their F_1's (Plate 3 and 4, respectively).

Conclusion

Pea rust caused by *Uromyces fabae* Pers. de-Bary is a major disease of peas. It is responsible for significant yield losses,, especially in the sub-tropical regions where warm and humid weather conditions prevail (Kushwaha et al., 2006). These conditions usually coincide with the reproductive phase of pea and favour rust outbreak (Kushwaha et al., 2007). One of the best possible ways to stabilize the productivity of pea crops is to grow rust-resistant varieties. Rust severity is greatly influenced by the environment during infection initiation and disease development. In phenotype-based screening, it is often difficult to detect the presence of individual resistance genes, as it is influenced by the developmental stage and environmental conditions. Thus, the use of molecular markers tightly linked to pea rust resistance would allow indirect selection for rust resistance independent of environmental effects (Rai *et al.*, 2011). Hence, these two (AA446 and AA505) markers could be used for marker-based characterization of rust resistance in garden pea. These results are in close agreement with those reported by Singh *et al.* (2015), and Upadhyay *et al*. (2017).

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to the Dean, College of Agriculture, GBPUA&T, Pantnagar, Uttarakhand, India for infrastructure and support. Hirdesh Yadav is obliged to University Grant Commission (UGC), India for providing NFO-OBC Fellowship during his Ph.D. (File no. F./2014/NFO-2014-15-OBC-UTT-1208/(SA-III/Website).

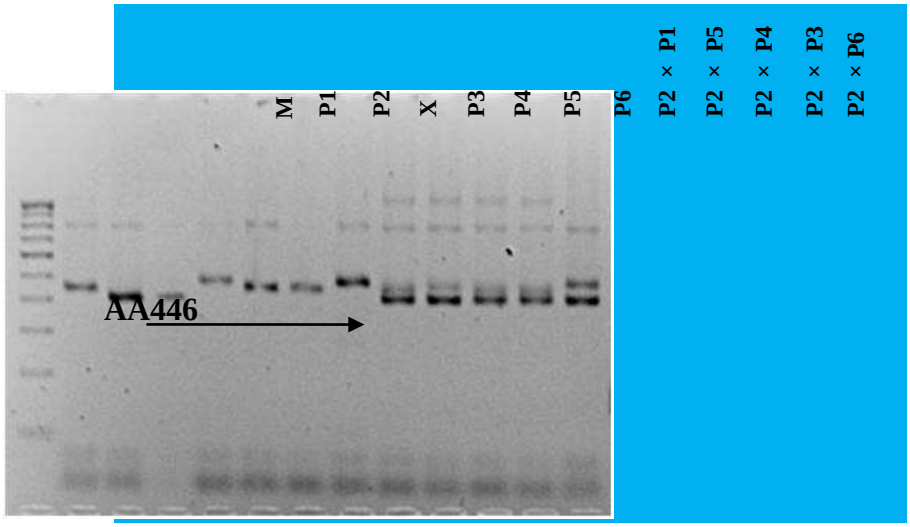

Plate 1: PCR banding pattern of the SSR marker (AA446) on parents and their F_1's.
(P1=Pant Uphar, P2=Arka Ajit (resistant), P3= NDVP-250, P4=PSM-4, P5=Nepal Pea, P6= PEVAR-6)

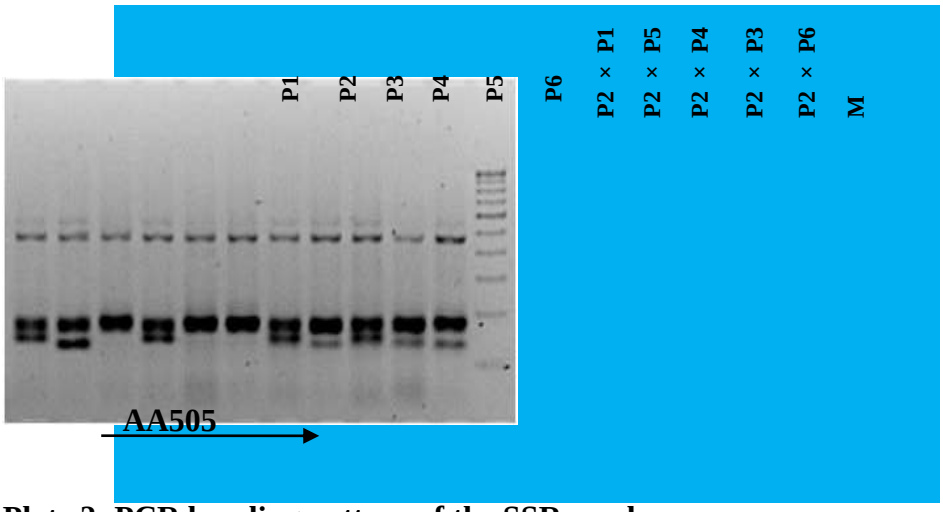

Plate 2: PCR banding pattern of the SSR marker

(AA505) on parents and their F_1's.

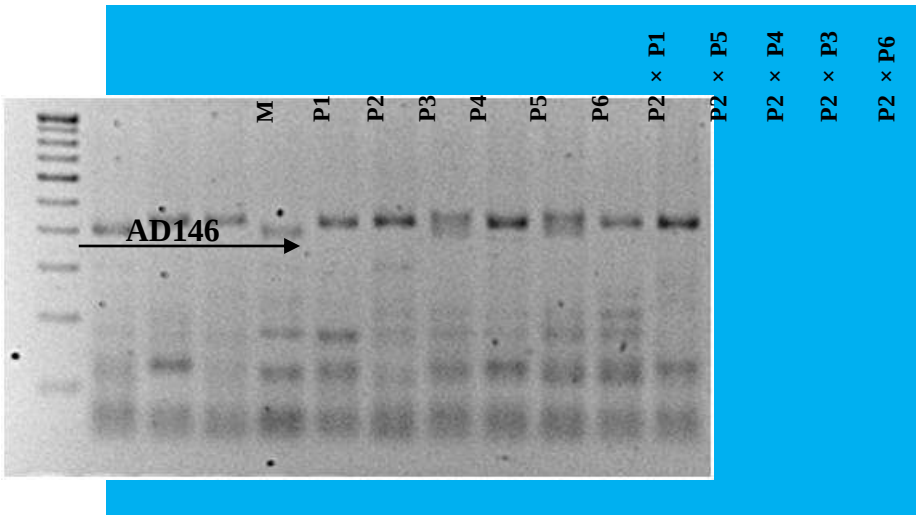

Plate 3: PCR banding pattern of the SSR marker (AD146) on parents and their F_1's.

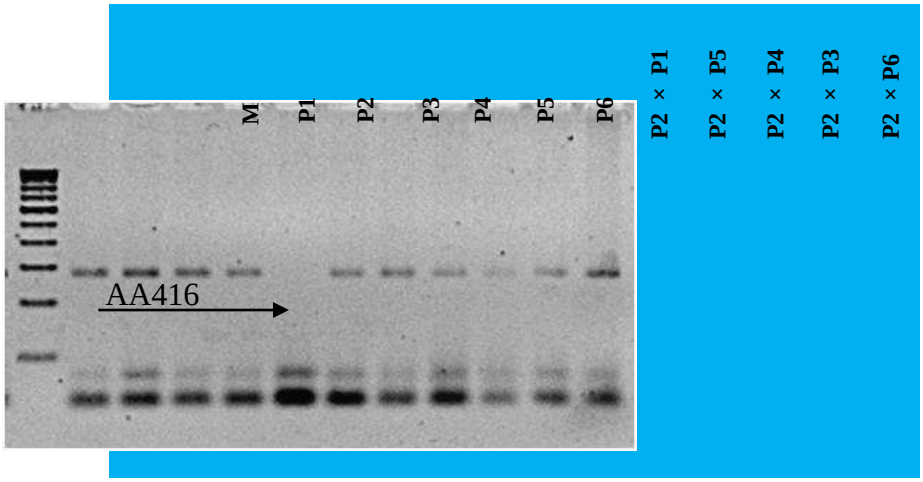

Plate 4: PCR banding pattern of the SSR marker (AA416) on parents and their F_1's.

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