Tagging and mapping of SSR marker for rust resistance gene in lentil

(Lens culinaris Medikus subsp. culinaris)

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Lentil, as an economical source of protein, minerals and vitamins, plays important role in nutritional security of the common man. Grown mainly in West Asia, North Africa (WANA) region and South Asia, it suffers from several biotic stresses such as wilt, rust, blight and broomrape. Lentil rust caused by autoecious fungus Uromyces viciae fabae (Pers.) Schroet is a serious lentil disease in Algeria, Bangladesh, Ethiopia, India, Italy, Morocco, Pakistan and Nepal. The disease symptoms are observed during flowering and early podding stages. Rust causes severe yield losses in lentil. It can only be effectively controlled by identifying the resistant source, understanding its inheritance and breeding for host resistance. The obligate parasitic nature of pathogen makes it difficult to maintain the pathogen in culture and to apply it to screen segregating progenies under controlled growth conditions. Hence, the use of molecular markers will compliment in identification of resistant types in different breeding programs. Here, we studied the inheritance of resistance to rust in lentil using F₁, F₂ and F₂:₃' from cross PL 8 (susceptible) x L 4149 (resistant) varieties. The phenotyping of lentil population was carried out at Sirmour, India. The result of genetic analysis revealed that a single dominant gene controls rust resistance in lentil genotype L 4149. The F₂ population from this cross was used to tag and map the rust resistance gene using SSR and SRAP markers. Markers such as 270 SRAP and 162 SSR were studied for polymorphism and 101 SRAP and 33 SSRs were found to be polymorphic between the parents. Two SRAP and two SSR markers differentiated the resistant and susceptible bulks. SSR marker Gllc 527 was estimated to be linked to rust resistant locus at a distance of 5.9 cM. The Gllc 527 marker can be used for marker assisted selection for rust resistance; however, additional markers closer to rust resistant locus are required. The markers linked to the rust resistance gene can serve as starting points for map-based cloning of the rust resistance gene.

Keywords: Bulk segregant analysis, Fungal, Hybridization, Molecular marker, Phenotyping, Pulses

Lentil (Lens culinaris Medikus subsp. culinaris), domesticated in the foot hills of Southern Turkey and Northern Syria by selection within population, is an important source of protein, minerals and vitamins and plays key role in nutritional security of people in the region. Lentil is mainly grown in West Asia, North Africa (WANA) region and South Asia. In India, it is mainly grown in Madhya Pradesh and Bundelkhand region of Uttar Pradesh, and in eastern region including Bihar, West Bengal and East Uttar Pradesh. The main lentil producing countries are India, Canada, Turkey, Nepal, Ethiopia and Australia. Globally, lentil is grown in about 4.34 million hectares with production of 4.95 million tons¹. This crop suffers from several biotic stresses like fusarium wilt, rust, ascocysta blight, anthracnose, stemphylium blight and broomrape. Rust caused by Uromyces viciae fabae (Pers.) Schroet causes heavy yield losses and is the most important foliar disease in Bangladesh, Ethiopia, India, Morocco, Nepal, Pakistan and South America. Yield losses ranging from 25-100% have been reported from different countries by different workers²,³. Lentil rust is autoecious in nature and is characterized by lesions on leaves and stem, leaf drop and premature plant death⁴ Uromyces viciae fabae has worldwide distribution⁵. The symptoms of rust are visible on all aerial plant parts. The initial rust symptoms are seen in the form of yellowish white pycnidia and aecial cups on the lower surface of leaflets and on pods singly or in small groups. Oval to
circular pustules are visible on stem, pods and leaflets. The source of primary infection is infected debris with teliospores. The telia formed late in the crop season are dark brown to black, elongated and are seen on branches and stem. The dried lentil teliospore can become a source of infection the following crop season. Cloudy weather with temperature of 20-22°C and high humidity favours disease development. After severe infection plant sheds leaves and shriveled seed or no seeds are formed in pods. Based on differential reactions on different cultivars of lentil, Singh & Sokhi identified 6 pathotypes of rust in pea and sweet pea. *Vicia, lathyrus* and *Pisum* are important sources of inoculums and pathogenic variants. There is inconsistency in number of races reported for this disease by different scientists. Rust resistant lentil varieties are available, and most studies on genetics of rust resistance in lentil have revealed that resistance is monogenic and dominant. However, single dominant gene for rust resistance in L 2991, L 2981, L 1534, L 178 and HPCL 8868 and two dominant genes were reported in Precoz. Resistance to rust has also been reported to be controlled by two duplicate, non-allelic and non-linked dominant genes and single dominant, two independent dominant, two complimentary or by three independently inherited gene. The race specific nature of the resistance emphasizes the necessity of screening plants simultaneously or even sequentially with several races and this process is difficult or impossible. Therefore, the development of reliable and user-friendly molecular markers closely linked to resistance genes provides a useful tool to breeders for tagging, mapping and pyramiding genes. In recent years, use of DNA markers has improved the efficiency and effectiveness of breeding desirable resistances in various crops. Here, we studied the genetics of rust resistance and tried to identify molecular markers linked to rust resistance gene that have potential use in marker assisted selection.

Materials and Methods

Plant material and development of mapping population

In the present investigations, genotype L 4149 was used as resistant parent and PL 8 was used as susceptible parent. The hybridization between L 4149 and PL 8 was carried out in winter season during 2010-11 at Division of Genetics, Indian Agricultural research institute, New Delhi and the hybrid seeds of cross PL 8 x L 4149 were harvested. Sixteen F₁s of this cross were sown in the year 2011-12 in the field with both the parents sown along with as checks for identification of true hybrids. Hybridity of F₁ was also confirmed through molecular marker analysis. Four true F₁s of this cross were harvested individually to produce their respective F₂ populations. The F₂ populations were sown in the field, and the F₂ plants were harvested individually in 2012-13. The F₂ families were raised in the field to identify the homozygosity and heterozygosity of individual plants. The F₂ families were screened for rust resistance at Chaudhary Sarwan Kumar Himachal Pradesh Krishi VishwaVidyalaya, Hill Agriculture Research and Extension Centre, Dhaulakuan, Sirmour, H.P., India, the hot spot for rust in India. Among the four populations, one F₂ population of cross PL 8 x L 4149 was randomly identified for development of F₂ families and for tagging rust resistant gene in L 4149. This population comprised of 119 individuals. The seed of all 119 individual plants were grown in separate rows and observations were recorded in each row for rust susceptibility and resistance. The F₂ derived progenies expressed 34 non-segregating rust resistant plant progeny rows, 59 heterozygotes segregating for rust and 26 non-segregating susceptible plant progeny rows in 1:2:1 ratio. The results obtained were utilized for development of resistant and susceptible bulk for bulk segregant analysis. F₂ derived progenies were sown for germination, 4 weeks after sowing the young leaves were collected from individual plants of PL 8, L 4149, F₁ and F₂ population for DNA extraction.

Evaluation of populations with rust pathogen

For evaluation of test material against rust, the infector row technique of sowing two test rows alternating with spreader row of highly susceptible variety L 830 was adopted at test location Chaudhary Sarwan Kumar Himachal Pradesh Krishi VishwaVidyalaya, Hill Agriculture Research and Extension Centre, Dhaulakuan. The parents, F₁, F₂ plants were scored for disease incidence at flowering and fruiting period after 100% plants of infector row showed rust incidence during 2011-12 and F₂ during 2012-13.

DNA extraction and PCR analysis

The genomic DNA from each individual was isolated using cetyltrimethyl ammonium bromide
Polymerase chain reactions were performed in 20 μL volume containing 2.0 μL 10 × buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin); 200 μM each dNTP; 0.5 μM each of forward and reverse primers; 1U Taq DNA polymerase (PCR reagents and primers procured from Sigma-Aldrich, Spruce Street, St. Louis, USA); ~40 ng DNA and were performed in Veriti™ thermal cycler. The amplification DNA fragments were resolved on 3% metaphor™ agarose gels (Lonza, Rockland, ME USA) and 2% agarose for SSR and SRAP markers, respectively and visualized by ethidium bromide staining. The band size was obtained in comparison to a 100 bp DNA ladder (MBI, Fermentas, Vilnius, Lithuania). The polymorphic bands were identified on the basis of differences in allele size or presence/absence of marker allele visible on the gel.

Markers for identifying putative genes/ SSR and SRAP analysis

Limited number of lentil specific SSR markers has been reported. Total of 162 SSR markers and 270 SRAP markers were used in the present study. Sequence-related amplified polymorphism SRAP were reported for amplification of open reading frames. The primers are 17-18 nucleotide long consisting of core sequence (13-14 bases long, first 10-11 bases start from 5′ end followed by CCGG in forward primer and AATT in reverse primer) followed by three selective nucleotide at 3′ end. For the first 5 cycles, annealing temperature was 35°C and the following cycles were run at 50°C. The SRAP markers had been used in lentil by Saha et al.20 The PCR protocol for SSR markers consisted of one denaturation cycle at 94°C for 4 min followed by 30 cycles of 94°C for 1 min, annealing at 59-62°C (depending upon the primer) for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 8 min.

Bulk segregant analysis

Bulk segregant analysis (BSA) was used to tag rust resistance gene in lentil. In this method, two pooled DNA samples of individuals from segregating population originating from single cross are compared. Resistant bulk comprised DNA 30 ng/μL each from 12 homozygous resistant plants from F₂ population, and the susceptible bulk had DNA 30 ng/μL each from 12 homozygous susceptible plants. The polymorphic markers identified between the resistant and susceptible parents were used to genotype resistant and susceptible bulks along with the parents. The association of marker with gene(s) controlling rust resistance was established on the basis of amplification of allele, either in resistant bulk and resistant parent or susceptible bulk and susceptible parent. Later, the SSR/SRAP markers exhibiting associations with target gene were used to genotype individual F₂ plants and genotypic data was recorded on each individual plant.

Segregation and linkage analysis

The phenotypic data of disease reaction was recorded on individual F₂ plants and each of F₂ families. The segregating populations were classified in distinct classes and analyzed with the help of Chi square (χ²) test for a fixed ratio hypothesis. The hypothesis was tested at 5% level of significance. For determining the linkage between resistant gene and markers, MapMaker26 ver. 3.0 was used. Using Kosambian mapping function, map was constructed at LOD 3.0.

Results

Inheritance of rust resistance gene

Inheritance of rust resistance gene was studied in four F₂ populations derived from four individually harvested F₁s of the cross PL 8 (susceptible) x L 4149 (resistant). The parent L 4149 displayed resistance reaction with score of 1 and parent PL8 displayed susceptible reaction with score of 9. The studied F₂ populations exhibited segregation for resistant gene. The homogeneity Chi square test among the four populations further confirmed that the segregation ratio as being homogeneously 3:1 (Table 1). Analysis of the pooled population of the four F₂ populations including resistant and susceptible individuals also fitted well in 3:1 segregation ratio.

Table 1—Segregation for rust resistance gene in F₂ populations of PL 8 x L 4149

<table>
<thead>
<tr>
<th>Population No.</th>
<th>Segregants in F₂ population for rust</th>
<th>Expected ratio</th>
<th>χ²* DF</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Susceptible</td>
<td>3:1</td>
<td>0.652</td>
</tr>
<tr>
<td>1</td>
<td>90</td>
<td>25</td>
<td>3:1</td>
<td>0.698</td>
</tr>
<tr>
<td>2**</td>
<td>93</td>
<td>26</td>
<td>3:1</td>
<td>0.923</td>
</tr>
<tr>
<td>3</td>
<td>89</td>
<td>24</td>
<td>3:1</td>
<td>0.135</td>
</tr>
<tr>
<td>4</td>
<td>89</td>
<td>32</td>
<td>3:1</td>
<td>1.211</td>
</tr>
<tr>
<td>Pooled</td>
<td>361</td>
<td>107</td>
<td>3:1</td>
<td>0.40</td>
</tr>
</tbody>
</table>

*value for significance of P = 0.05; ** population used for gene tagging/mapping
Tagging of rust resistance gene in L. 4149

The parental polymorphism between resistant parental line L. 4149 and susceptible parental line PL 8 was analyzed with 162 SSR primers and 270 SRAP primers. The 33 SSR and 101 SRAP primers, polymorphic among the parental lines, were used to study the polymorphism between the susceptible bulk (S bulk) and resistant bulk (R bulk). Among these, two SSR primers Glc 527 and pbalc 219 and two SRAP primers me1_r8 and me5_em1 exhibited polymorphism among the resistant and susceptible bulk (Table 2). SSR markers Glc 527 and pbalc 219 amplified alleles of 122 & 166 bp, respectively in L.4149 in contrast to 127 & 158 bp alleles in PL8. Similarly, SRAP markers me1_r8 and me5_em1 amplified alleles of 400 & 450 bp, respectively in contrast to PL8 in which absence of band was observed.

The F2 genotypic classes obtained with the four polymorphic markers were used for linkage analysis. \( \chi^2 \) values for SSR marker pbalc 219 was observed to be non-significant and hence was not used for further linkage analysis. Linkage was detected between rust resistance gene and SSR marker Glc527 \( (\chi^2_L = 198.87, \text{2df}) \). Similarly, linkage was also detected between SRAP markers me5_em1 \( (\chi^2_L = 26.898, \text{2df}) \) and me1_r8 \( (\chi^2_L =16.04, \text{2df}) \) and the rust resistance gene (Table 3).

The polymorphic primers were used to screen the entire F2 population comprising 119 genotypes. The data generated was used for linkage analysis using MapMaker ver. 3.0. The amplification profile of parent and the individuals used for making bulks of F2 mapping population of rust with SSR marker Glc527 is presented as Fig. 1. The map of rust resistant locus in the sequence me5_me1 - Glc527 - me1_r8. The identified SSR marker Glc 527 was estimated to be 5.9 cM from the gene for rust resistance.

<table>
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<tr>
<th>Table 2—Test of the linkage between leaf rust gene and SSR markers in the F2 populations of the cross PL 8 x L 4149 of Lens culinaris.</th>
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<tbody>
<tr>
<td><strong>RR/M1Mrr/M1MrR/M2Mrr/M2M</strong></td>
</tr>
<tr>
<td><strong>( \chi^2 )</strong></td>
</tr>
<tr>
<td>GLCC 527</td>
</tr>
<tr>
<td>ME1-EM1</td>
</tr>
</tbody>
</table>

RR: homozygous-resistant; rr: homozygous-susceptible; Rr: heterozygous-resistant; M1: marker allele present in PL8; M2: marker allele present in L4149

Discussion

Lentil is important cool season grain legume grown in about -40 countries, from the Near East to the Mediterranean area, Asia, Europe and finally the Western Hemisphere, principally for its protein rich seed. This crop is important for nutritional security for resource poor farmers in South Asia. The rust caused by U. viciae fabae is responsible for considerable losses to lentils in several regions of the world including India. Lentil is grown in about 1.4 million hectares area with production of about 0.95 million tones. Rust is a major disease in the Eastern India and Northern Hills. The fungus is autoecious. Several methods for rust control have been proposed, including chemical and biological control, and systemic induced resistance, but these were found to be ineffective at the commercial level. The most economic and viable solution for resource poor farmers is to use resistant cultivars. Proper screening against rust and breeding rust resistant lentil are important for lentil breeder. Resistant cultivars are the most practical and cost-efficient method for the control of this disease. The fungus Uromyces viciae fabae is an obligate parasite and it is difficult to maintain the culture and screen against this pathogen under controlled conditions. Screening under the natural condition depends on existence of suitable

<table>
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<th>Table 3—Details of polymorphic markers between resistant and susceptible bulks</th>
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<tr>
<td><strong>SSR/SRAP</strong></td>
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<tr>
<td>SSR**</td>
</tr>
<tr>
<td>SSR**</td>
</tr>
<tr>
<td>SRAP**</td>
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<tr>
<td>SRAP**</td>
</tr>
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</table>

References indicated in superscript
The resistance to rust is race specific and there is an indication of existence of several races of pathogen. Due to the variable nature and with wide race spectrum of the pathogen, the rust resistance breeding has met with limited success. The continuous cultivation of varieties with race specific resistance in large areas increases selection pressure on the pathogen that may eventually lead to development of new races capable of infecting the previously resistant cultivar. The identification of reliable molecular markers closely linked to resistance genes provides a useful tool to breeders for tagging, mapping and pyramiding rust gene(s). In recent years, DNA markers are being used by the breeders routinely for enhancing the efficiency and effectiveness of breeding programmes. In lentil, there are only two reports on identification of molecular markers associated with rust resistance. In the first report, sequence related amplified polymorphism (SRAP) marker F7XEM4a, was estimated to be 7.9 cM from the gene for resistance; and the second report linked SSR marker Glle 106 with rust resistant gene at a distance of 10cM. These studies indicated the need for identification of closely linked markers for marker assisted selection.

The aim of present investigation was to tag and map gene(s) controlling resistance to rust in lentil. For this purpose, L 4149 was identified as resistant parent and PL 8 as susceptible parent based on screening at the hot spot, Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishwavidyalaya, Hill Agriculture Research and Extension Centre, Dhaulakuan, Sirmour, India. Using the disease reaction data of individual F$_2$ in four population’s inheritance of rust resistance was worked out. Our results revealed that single dominant gene controls the inheritance of rust resistance in lentil. The frequency of resistant and susceptible plants in 3:1 ratio strongly indicated that the resistance to rust in lentil is governed by single dominant gene. The results are in agreement with earlier reports. For tagging and mapping of resistant gene, F$_2$ population was developed. The homozygosity of F$_2$ individuals was assessed by screening of F$_2$ population against rust at the hot spot. SSR markers are co-dominant, polymorphic and reproducible. SRAP marker system is efficient, simple marker system with reasonable throughput rate, discloses numerous co-dominant markers, allows easy isolation of bands for sequencing and targets open reading frames. The system is useful for map construction, gene tagging, genomic and cDNA fingerprinting, and map based cloning. We identified SSR marker Glle 527 linked with gene for rust at estimated distance of 5.9 cM. Glle 527 has previously been reported on linkage group 824. This is one of the earliest reports on tagging and mapping of closely linked SSR marker with rust resistance gene in lentil. The identified SSR marker Glle 527 is co-dominant will ensure speedy introgression of the rust resistance gene into susceptible agronomically superior cultivars via marker-aided selection. The markers linked to the rust resistance gene will also serve as starting points for map-based cloning of the rust resistance gene.
This is one of the early reports on tagging and mapping of SSR markers for rust resistance in lentil. This marker can be used for marker assisted selection for development of rust resistance in lentil.

Acknowledgement
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