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Mapping of Quantitative Trait Loci for scab resistance in Apple (*Malus × domestica*)

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Abstract

Scab caused by *Venturia inaequalis* (Cke.) Wint. is the most important fungal disease of apple. Fungicide application is a widely practiced method of disease control. The use of chemicals is however, cost intensive, tedious and ecologically unsafe. Development of genetic resistance and breeding of resistant cultivars is most reliable and a safest option. One such source of scab resistance happens to be the variety ‘*Shireen*’, released from SKUAST-Kashmir. However, till date the nature of resistance and its genetic control has not been characterized. The aim of this research was to elucidate the genetic basis of scab resistance in *Shireen*. The present study helped us to identify two quantitative trait loci (QTLs) on chromosome 2 and 8 and six potential CDGs for the polygenic resistance in ‘*Shireen*’. The genomic region corresponding to the mapped QTLs in LG 2 and LG 8 of ‘*Shireen*’ was examined for candidate genes possibly related to scab resistance using *in silico* analysis. The QTLs mapped in the genetic background of *Shireen* are the novel QTLs and may be transferred to desirable genetic backgrounds and provide opportunities for isolation and cloning of genes apart from their utility in order to achieve durable resistance to scab.

Key words: Apple scab, resistance gene, Linkage map, QTL, Gene Expression

Introduction

Apple scab caused by the fungal pathogen *Venturia inaequalis* (Cke.) Wint. is a catastrophic disease that causes huge economic losses worldwide. With most commercial apple cultivars being susceptible to the disease, the production losses in highly infected orchards may go up to 70% or more. Scab is a serious problem in temperate regions with cool, moist weather in early spring. Depending upon the weather conditions and availability of fungal inoculum, a multiplex of 20 to 30 fungicide applications are used for the control of disease [1]. Intensive fungicide use is costly and has ecological and human health concerns. Therefore, development of resistant cultivars and their targeted deployment is effective and environment friendly option for management of the disease. Genetic resistance towards apple scab is complex. The loci underlying this resistance involve both complete as well partial resistance loci; hence leave a 'great deal of grey area' between these two extremes [2]. Complete resistance is governed by a major resistance gene(s) and provides high protection to the host by inhibiting the pathogen reproduction. It involves a typical gene-for-gene interaction. Major genes facilitate strong directional selection on pathogen isolates that can lead to resistance breakdown, as illustrated with the *Rvi6* (*Vf*) scab R gene [3]. In contrast, partial resistance confers a reduction rather than complete arrest of the disease, and is controlled by Quantitative Trait Loci (QTLs). Partial resistance is presumed to be more durable than complete resistance as there is relatively low selection pressure on pathogen isolates [4], and therefore, becomes an attractive alternative for controlling apple scab.

QTLs associated with apple scab have been mapped on 10 different linkage groups (LGs) [4]. However, the nature of these QTLs remains largely unknown, in spite of the availability of whole genome sequence assembly of *Malus x domestica*. It is mostly due to a large number of candidate defense genes located inside the confidence interval of the identified QTLs. The molecular mechanism underlying such resistance can further be validated through expression studies on individual basis [5]. 'Shireen' is a scab-resistant variety released from SKUAST-Kashmir [6]. However, till date the information on the nature of resistance and its genetic control is lacking. In contrast, *StarKrimson* is popular high yielding apple variety known for its excellent quality but is highly susceptible to apple scab disease. Therefore, these two varieties with contrasting response to scab were crossed to develop an F₁ mapping population with *StarKrimson* as female. The choice of mapping population is critical for successful construction of genetic map and in part depends upon the

breeding behavior of the species concerned. Self-incompatibility and the long juvenile period of *Malus × domestica* (3-10 years) limit the development of segregating populations such as F₂, F_{2:3}, NIL or RIL. In apple F₁ mapping population derived from inter/intra-specific cross pollination of various cultivars is best suited to achieve meiotic rearrangements due to moderate to high heterozygosity prevalent across the genome. This fetches a chance to map the loci in case of tree species in a shortest possible time. Our presumption was that resistance to scab in *Shireen* is conditioned by QTLs and could be mapped in F₁ (Pseudo-backcross) population. The trait can be mapped through heterozygous marker phase near target locus as resistance is donated from wild [7] and is preserved in recipient clones.

Materials & Methods

Plant materials

The F₁ segregating population of 122 individuals derived from a cross between *Shireen* (female parent) and *StarKrimson* (male parent) was used for linkage map construction and QTL analysis. *Shireen* is a scab resistant apple variety released from SKUAST-K in the year 1995. It has been derived from Lord Lambourne × Melba × R-12740- 7A. In contrast, *StarKrimson* is popular high yielding apple variety known for its excellent quality but is highly susceptible to apple scab disease.

Artificial inoculation procedure and disease phenotyping

For resistance evaluation, two year old F₁ plants were inoculated with four races (single spore) viz., (0), (1), (2) and (1, 2) of *Venturia inaequalis*, procured from Division of Plant Pathology, SKUAST-K, Shalimar. The disease inoculum for each isolate was prepared as described by Barbara *et al.* (2008)[8]. The culture suspension was formulated at a spore concentration of 5×10⁵ conidia per ml. The conidial suspensions were applied till runoff using a manual atomizer. To ensure constant leaf wetness, the plants were placed in polythene covered structure and incubated for 48 h in dark. Disease scoring was carried out following 0-4 scale [9] and was performed at 14 days after inoculation. The reaction score of 0, 1, 2 and 3a was graded as resistant and plants showing class 3b and 4 were grouped as susceptible. The disease phenotyping assay was repeated once during next growing season.

Molecular marker genotyping

Fresh leaves of the *StarKrimson*, *Shireen* and 122 F₁ individuals were collected and ground up in liquid nitrogen. DNA was extracted using the CTAB (Cetyl-Tri Methyl Ammonium

Bromide) method as described by Murray and Thompson (1980) [10] with certain modifications. DNA quality and quantity were evaluated by using 1 % agarose gel electrophoresis and NanoDrop (Eppendorf, Hamburg, Germany). A total of 153 SSR markers were selected based on polymorphism information content (PIC) and linkage group information to survey the polymorphism between the parents. Also a set of 22 arbitrary random primers were selected based on earlier studies. The primers of selected markers were custom synthesized from Integrated DNA Technologies, USA. PCR (Polymerase chain reaction) assay was performed in 20 µl containing 25 ng of DNA, 10x PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.8 mM MgCl₂), 2 mM dNTPs (MBI, Fermentas, Lithuania, USA), 5 pmol each of forward and reverse primer and 5 U of Taq DNA polymerase (MBI, Fermentas, Lithuania, USA). PCR amplifications were performed under the following conditions: initial denaturation at 94 °C for 7 min followed by 35 cycles of 94 °C for 45 sec, 55 °C for 45 sec, 72 °C for 1 min. A final elongation step at 72°C for 10 min was included. PCR products were separated by 3.5 % agarose gel and 6% PAGE gel to detect polymorphisms.

Genetic linkage map construction

A genetic linkage map was constructed using the MAP option of ICI Mapping v4.0. A three step map approach i.e., grouping, ordering and rippling in order was used for this study. Chi square test was used to detect the marker segregation distortion. Markers with segregation distortion ($\chi^2 > 6.0$) were excluded along with redundant markers before the calculation of linkage groups. Logarithm of odds (LOD) thresholds ranging from 5 to 8 were set to divide the linkage groups. To group the markers, a minimum Logarithm of odds (LOD) threshold of 5 was applied. The position of the markers in each linkage group were obtained by considering their contribution to the average goodness of fit (mean Chi square) and the nearest neighbour fit (N.N. Fit) value. Based on these criteria, markers were removed or added to each linkage group and calculations redone until the best fit and order was obtained.

QTL analysis

QTLs underlying scab resistance were mapped using QTL IciMapping 4.1.0.0 (<http://www.isbreeding.net>) with the inclusive composite interval mapping module. QTLs with additive and dominance effects were examined with the method ICIM-ADD. The map distance (cM)

was converted by recombination fractions using the Kosambi function. The parameters for ICIM-ADD were set as a 1.0 cM scan step, a 0.001 probability used for stepwise regression analysis, 2.5 used for the LOD threshold score for significant QTLs for each trait. The phenotypic variances explained (PVE) by individual QTLs were also obtained using ICIM. QTLs were named according to the recommended nomenclature system by Rosaceae Executive Committee (RasEXEC) along with the Rosaceae International Genomics Initiative (RosIGI).

Development of new molecular markers

To delineate the marker interval flanking the QTL, qRVI.SS-LG2.2019, the sequences of flanking SSR markers, CN584771 and CN857442 were BLASTed against the ‘Golden Delicious’ genome sequence version 1.0 contig [11]; http://www.rosaceae.org/gb/gbrowse/malus_x_domestica] to identify the physical position of the markers on the reference genome. The sequences of contigs between the two markers were downloaded. The downloaded sequences in a FASTA format were uploaded in Primer3 (www.primer3plus.com) to design the primers.

Expression analysis of candidate genes

To analyze the candidate genes related to scab resistance, we investigated the expression pattern of six candidate genes namely, *enhanced disease susceptibility 1 protein*, *metallothionein3-like protein*, *lipoxygenase*, *lipid transfer protein*, *Allene oxide synthase 2 coding gene* and *a peroxidase 3* in parents and resistant and susceptible bulks using q-PCR.

Total RNA extraction and cDNA synthesis

Leaf tissues challenged by the inoculum mix of four races of *V. inaequalis* were harvested from the youngest leaves of 10 resistant and 10 susceptible individuals randomly selected from the 122 ‘Shireen’ x ‘StarKrimson’ seedlings at 96 hpi, for making the resistant and susceptible bulk. Leaf tissues were also harvested from the two parents, *Shireen* and *StarKrimson*. Total RNA was extracted from leaf tissues of both resistant and susceptible plants, by adding 1 ml of trizol (Life technologies, CA, USA) to 100 mg of crushed sample, following manufacturer's instructions. RNA was purified by adding *DNase I* (Fermentas, Thermo Fisher Scientific, US) to avoid any DNA contamination. Three biological replicates were generated for each. cDNA synthesis was carried out using Thermo Scientific Revert

Aid First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific, US) using oligodT primers. The first strand cDNA was synthesized by using manufacturer's instructions. The cDNA synthesized were stored at -20°C. Real-time qPCR amplification and detection was conducted using SYBR Green 1 qPCR master mix (Fermentas Inc.) on a Biorad CFX96. The primers of actin gene were used as an internal control to ensure that equal amounts of cDNA used in all the reactions. Dissociation curves were run for all of the primers used in this study to determine the presence of any non-specific amplification. For each reaction three technical replicates were analyzed for qRT-PCR analysis. The PCR reaction was carried out using the following cycle conditions: an initial denaturation at 95 °C for 10', followed by 40 cycles of denaturation at 95 °C for 15", annealing at 52-60 °C for 30" and extension at 72 °C for 30". After obtaining the Ct value for each reaction, the relative expression was calculated by using the $\Delta\Delta C_t$ method described by Applied Biosystems (fold change = $2^{\Delta\Delta C_t}$). Individual ΔC_t values from each technical replicate of the qRT-PCR experiments were subjected to the ANOVA procedure at a statistically significant level of $P < 0.05$. The biological level of significance for differential gene expression was set at the minimum level of two fold change between resistant bulk and susceptible bulk. Heat map was generated using Heat map 3 package of R software (<http://cran.r-project.org/web/packages/gplots/index.html>).

Candidate gene mining

In order to navigate for the presence of candidate defense responsive genes present in the genomic region corresponding to the QTL interval, a search was done *in silico* using Genome Database for Rosaceae (GDR).

Cluster analysis

Cluster analysis of the F_1 individuals, using binary data generated by markers was conducted using UPGMA method with the help of Graphical Geno Typing (GGT 2.0) software [12]. The similarity matrix was generated using Jaccards coefficient and clustering was done using UPGMA (Unweighted Pair Group Method using Arithmetic Averages). Tree diagram was constructed using Tree plot options in available in GGT 2.

Results

Phenotyping for response to *Venturia inaequalis*

The individual F₁ plants along with the parents, *StarKrimson* and *Shireen* were evaluated for disease reaction against four monoconidial races of *V. inaequalis*, during the years 2017 and 2018. Disease scoring was performed on 0-4 scale based on which the F₁ individuals were grouped into four distinct categories (Supplementary Fig. S1). Out of 122 F₁s, 32 F₁s expressed immune or hypersensitive (0) response, while 49 were found resistant (1-2) and 10 showed moderate resistance (3a). The disease score of 3b and 4 was recorded for seven and 24 F₁ individuals, respectively. The mean of disease score of 3.49 was noted across the population with continuous distribution pattern (Table 1; Supplementary Fig. S2). The disease data exhibited the skewness and kurtosis of 0.08 and -1.63, respectively.

Marker polymorphism survey between the parents *StarKrimson* and *Shireen*

Polymorphism survey was carried out between the two parents, *Shireen* and *StarKrimson* using 153 genome wide Simple sequence repeat (SSR) markers of which 40 were found polymorphic between the parents with the percentage of polymorphism at 26.14%. These 40 markers showed heterozygous-homozygous polymorphism where one of the parents amplified heterozygous allele against another carrying homozygous allele at marker loci and vice-versa (Table 2). Therefore, were used for genotyping and linkage analysis (Fig. 1). Of 40 heterozygous polymorphic SSRs, 15 belonged to EST-SSRs and 25 originated from genomic DNA sequences, referred to as g-SSRs. The number of heterozygous polymorphic markers for chromosomes varied from one for chromosome 6, 13 and 14 to four for chromosome 1, 2, 5 and 11. Chromosomes 3, 4, 7, 10, 12 and 17 had two polymorphic markers, each. Chromosome 8, 9 and 15 were analyzed with three markers each.

Genotyping of mapping population and construction of molecular map

The individuals of mapping population were screened using 40 markers showing heterozygous-homozygous polymorphism. The markers were evaluated for the degree of segregation distortion using χ^2 -test (Supplementary Table S1). Segregation pattern of these 40 markers was compared with the expected 1:1 ratio. None of the polymorphic markers showed a significant deviation from expected 1:1 segregation, hence no exclusions were necessary. The 40 markers were assigned to 16 linkage groups (LGs) (Supplementary Fig. S3) with genetic map spanning across 2454.54 cM length with an average distribution of 61.36 cM per marker.

Anchoring random markers on Linkage groups

After the genotyping was performed using simple sequence repeat markers, the parental polymorphism was detected using additional 21 random which included 13 RAPD and eight ISSR markers. The additional markers were used for genotyping in order to improve the

genome coverage. Out of 21 markers, eight RAPD and two ISSR markers were found polymorphic between two parents while rest were monomorphic. Together, these 10 polymorphic markers yielded 49 alleles, 18 of which were polymorphic. Thus an average number of 1.8 alleles per locus were recorded between *Shireen* and *StarKrimson* with 36.7% polymorphism. Of the 18 polymorphic fragments 11 were detected in *Shireen* and 7 in *StarKrimson*. The random markers were run on the population along with previously mapped SSR markers which helped to anchor them to the corresponding linkage groups based on the co-segregation pattern revealed on the population.

QTL analysis

The average phenotypic data of two years and the genotypic results of F₁ mapping population were used for QTL analysis through ICIM (Inclusive Composite Interval Mapping). QTLs with LOD (Logarithm of Odds) ratio above 2.5 at 1000 permutations were recognized to be significant. Based on these criteria, two significant QTLs, qRVI.LG2.2019 and qRVI.SS-LG9.2019 were identified on chromosomes 2 and 8. qRVI.SS-LG2.2019 explained 39.04% of the phenotypic variation (PVE%) with a LOD value of 7.67 and an additive effect of 3.57 in the *StarKrimson* × *Shireen* population (Table 3; Fig. 2). The resistant allele for the qRVI.SS-LG2.2019 was contributed by *Shireen*. This QTL region on proximal end of linkage group 2 ranged from 1.97 to 4.47 Mbp and was flanked by the SSR markers CN584771 and CN857442.

Marker CN857442 located on chromosome 2:4489514 (Unlocalised scaffold) amplified two fragments of 200 and 180bp in *Shireen* and only one fragment of 180bp in *StarKrimson*. Overall, genotyping revealed two groups in the segregating population viz., Group-I individuals carrying the 180bp allele and the Group-II comprised of heterozygous individuals carrying both the alleles. Out of 122 F₁ individuals, 60 amplified 180-bp fragment and 62 were heterozygous and amplified both 180 and 200-bp fragments. Marker CN854771 located on chromosome 2:1987284 (Unlocalised scaffold) amplified two alleles of 150 and 100bp in *Shireen* and only one allele of 100bp in *StarKrimson*. The population was categorized into Group-I individuals carrying the 100bp allele and the Group-II comprised of heterozygous individuals carrying both the alleles. Out of 122 F₁ individuals, 58 amplified 100-bp allele and 64 were heterozygous and amplified both 150 and 100-bp alleles.

qRVI.SS-LG8.2019 explained 57.91% of the phenotypic variance (PVE%) with a LOD value of 4.99 and an additive effect of 4.34 in the *StarKrimson* × *Shireen* population (Table 3; Fig. 2). The resistant allele for the qRVI.SS-LG8.2019 was contributed by *Shireen*. This

QTL region ranged from 0.05 to 2.19 bp on of the apple reference genome and was flanked by the SSR markers ch01d09 and ch03a08. Marker ch03a08 located on chromosome 8:51667 (Unlocalised scaffold) amplified two alleles of 200 and 190 bp in *Shireen* and only one allele of 200 bp in *StarKrimson* . Overall, genotyping revealed two groups in the segregating population viz., Group-I individuals carrying the 190bp allele and the Group-II comprised heterozygous individuals carrying both the alleles. Out of 122 F₁ individuals, 60 amplified 200-bp allele and 62 were heterozygous and amplified both 180 and 200-bp alleles. Marker ch01d09 located on chromosome 8:2194713 (Unlocalised scaffold) amplified two alleles of 150 and 175 bp in *StarKrimson* and only one allele of 150 bp in *Shireen*. The population was categorized into Group-I individuals carrying the 150bp allele and the Group-II comprised of the heterozygous individuals carrying both the alleles. Out of 122 F₁ individuals, 58 amplified 150-bp allele and 64 were heterozygous and amplified both 150 and 175-bp alleles.

Narrowing Down qRVI.SS-LG2.2019 region

The rate of recombination was studied for the CN584771 - CN857442 interval carrying qRVI.SS-LG2.2019 that spans 2.5 Mb region. The two flanking markers showed the recombination frequency of 20.4%. Further 23% of plants were susceptible in double heterozygote individuals and 21 and 25% plants showed susceptible reaction with the heterozygous phase at right and left marker, respectively (Supplementary Fig. S4). This indicated that there was a scope to further narrow down the QTL region using additional markers. Therefore, in an attempt to narrow down the marker interval CN584771 - CN857442 flanking the QTL, qRVI.SS-LG2.2019 on chromosome 2, four new STS markers were designed from the contigs spanning the region (Supplementary Table S2). STS markers were validated on the parents, *Shireen* and *StarKrimson* among which, only a single marker named SABA-2 was found polymorphic.

Genotyping F₁ population using STS marker Saba-2

The polymorphic marker SABA-2 was used for genotyping of F₁ population. SABA-2 amplified two alleles of 240 and 300bp fragment size from resistant parent and only 240bp allele from susceptible parent with variable segregation pattern across F₁ individuals (Supplementary Fig. S5). The marker SABA-2 showed lack of polymorphism between resistant and susceptible bulks. This is expected for a quantitatively governed trait and, therefore, indicates a QTL effect present within marker interval, SABA-2- CN857442. The SABA-2 marker mapped inside the marker interval at a distance of 1.53 Mbp and 0.97 Mbp from flanking markers CN584771 and CN857442, respectively (Supplementary Fig. S6).

The QTL region of 2.5-Mbp was narrowed down to a 0.97 Mbp interval with the introduction of marker SABA-2.

Expression profile of candidate resistance genes in a segregating F₁ population

The major effect loci, QTLs or polygenic clusters underlying resistance are largely influenced by other non-allelic transcription factors or pathway genes. Therefore, activity of few pathway genes was validated in response to *V. inaequalis* races in terms of the gene expression between two parents and also between resistance and susceptible bulks those constituted based on the mapped QTLs, qRVI.SS-LG2.2019 and qRVI.SS-LG5.2019. To find out the association between resistance loci and pathway genes, the bulk segregant analysis (BSA) was carried out. Bulks were constituted through pooling of RNA following representative sampling strategy of segregants in a population on the basis of phenotype. The expression of parents and resistant and susceptible bulks was judged as per expectations under, BSA. The expression pattern of six candidate genes namely, *enhanced disease susceptibility 1 protein (EDS1)*, *metallothionein3-like protein (MT3)*, *lipoxygenase (LOX)*, *lipid transfer protein (LTP)*, *Allene oxide synthase 2 coding gene (MdAOS_2)* and *a peroxidase 3 (PX3)* was studied through q-PCR based assay. Mean CT values for each gene were calculated in both the resistant and susceptible parents and bulks.

***Enhanced disease susceptibility 1(EDS1)*:** In our experiment, *EDS1* encoding protein showed an increased expression in both *Shireen* (resistant parent) as well as in resistant bulk. The expression level of *EDS1* in *Shireen* was 16.43 fold higher compared with the susceptible parent '*StarKrimson*', while in resistant bulk it was 12.88 fold higher. Very low *EDS1* expression was observed for 'susceptible bulk', corresponding to 2.96 times the expression in '*StarKrimson*'.

***Lipid transfer protein (LTP)*:** The *LTP* gene showed significantly higher expression (105.18 fold) in resistant bulk, while in *Shireen* it was 5.60 fold higher compared with the susceptible parent '*StarKrimson*'. Very low *LTP* expression was observed for 'susceptible bulk', corresponding to 0.91 times the expression in '*StarKrimson*'.

***Lipoxygenase (LOX)*:** The *LOX* gene showed significantly higher expression (190.46 fold) in resistant bulk, while in *Shireen* it was 22.84 fold higher compared with the susceptible parent '*StarKrimson*'. Relatively low *LTP* expression was observed for 'susceptible bulk', corresponding to 6.23 times the expression in '*StarKrimson*'.

***Allene oxide synthase 2 coding gene (MdAOS2)*:** *MdAOS2* showed an increased expression in both *Shireen* (resistant parent) and resistant bulk. The expression level of *MdAOS2* in *Shireen* was 14.12 fold higher compared with the susceptible parent '*StarKrimson*', while

in resistant bulk it was 11.00 fold higher. Very low *EDS1* expression was observed for ‘susceptible bulk’, corresponding to 1.98 times the expression in ‘*StarKrimson*’.

Peroxidase 3 (PX3): The *PX3* gene showed significantly higher expression (56.49 fold) in resistant bulk, while in *Shireen* it was 15.14 fold higher compared with the susceptible parent ‘*StarKrimson*’. Very low *LTP* expression was observed for ‘susceptible bulk’.

Metallothionein 3 (MT3): The *MT3* gene showed significantly higher expression (25.40 fold) in resistant bulk, while in *Shireen* it was 6.06 fold higher compared with the susceptible parent ‘*StarKrimson*’. Very low *LTP* expression was observed for ‘susceptible bulk’ corresponding to 1.29 times the expression in ‘*StarKrimson*’.

For all the six candidate genes, a significant change in expression (more than two fold) was observed for ‘*Shireen*’ (resistant parent) and resistant bulk as compared to ‘*StarKrimson*’ (susceptible parent) and susceptible bulk, at a significance level of $P < 0.05$ (Supplementary Table S3). Therefore, the differential expression of these genes in parents (*Shireen* and *StarKrimson*) and in bulks constituted suggests the effect of the mapped loci on up and downstream pathway genes which together condition the resistance response. Expression profiles of the candidate genes are presented in Fig. 3, along with their annotation in plant defence response according to the literature (the other possible roles of these CDGs in the general plant metabolism are not presented here). The dynamic expression change of the CDGs are shown in the heatmap (Fig. 4).

***In silico* mining of genes within QTL region, qRVI.SS-LG2.2019**

The *in silico* analysis within the genomic region delimiting the mapped QTL qRVI.SS-LG2.2019 was performed for mining of candidate R-gene loci. The analysis was carried out using Genome Database for Rosaceae (GDR) (<http://www.rosaceae.org/>) targeting Golden Delicious linkage group (LG) 2 and 8 assembly [11]. In total, 325 genes were predicted within a 2.5-Mbp genomic region (from 1.97 to 4.47 Mbp) between the markers flanking the QTL qRVI.SS-LG2.2019 on LG 2. Based on the sequence and predicted gene function, 57 of 325 genes were found specific for resistance to pathogens (Supplementary Table S4). Of these 57 resistance specific genes, 27 coded for proteins belonging to the nucleotide-binding site leucine-rich repeat (TIR-NBS-LRR) class of R-genes. On LG 8, 224 genes were predicted within a genomic region of 2.14 Mbp between the markers flanking the QTL qRVI.SS-LG8.2019. On the basis of sequence and predicted gene function, 36 genes out of 224 were found to be resistance specific (Fig. 5). Out of these 36 resistance specific genes, 15 belonged to TIR-NBS-LRR class of R-genes.

Diversity analysis

Diversity analysis was performed to find out the recombinants among F₁s which are more similar to *Shireen* and carry the QTL so that some of these which are resistant may be further propagated and maintained through grafting. The allelic profile generated across 122 F₁s and two parents was used for cluster analysis. The Jaccard's coefficients were derived and used for construction of similarity tree following UPGMA method with the help of Graphical Geno Typing (GGT 2.0) software [12] (Supplementary Fig. S7). Based on information generated, the F₁s got separated into five major clusters with 44 F₁s in cluster I, 62 in cluster II, 45 in cluster III, 14 in cluster IV and 10 in cluster V with #23 and #84 F₁ as root. 76 F₁s were found to cluster with the susceptible parent '*StarKrimson*' in Cluster II and 44 F₁s with the resistant parent '*Shireen*' in cluster I. Out of 44 F₁s in cluster I 30 were found resistant and are, therefore, the ideal ones.

Discussion

Shireen is a scab resistant apple variety released by Sher-e-Kashmir University of Agricultural Science and Technology of Kashmir (SKUAST-K), India in the year 1995 [6]. It was developed from a cross between Lord Lambourne × Melba × R-12740-7A. Therefore, by descent, *Shireen* is expected to carry scab resistance loci derived from the Russian Seedling R12740-7A [13]. As supported by Le Van *et al.* (2011)[14] characterization of successful cultivars and varieties for scab resistance, besides the information on genetic basis is going to be of prime importance at least in relation to prevalent fungal races present in the region. Molecular markers can reliably detect the presence of gene in target genotypes, though, the mere presence of resistance specific allele in a given germplasm line is not an unambiguous proof to conclude that the line carries a gene of interest. Simple way to test the hypothesis would be to evaluate the mapping population for establishment of marker-trait association which guides us to estimate the gene location.

Mapping QTLs for scab resistance

To improve the accuracy of QTL mapping by reducing the background noise, utmost importance has to be devoted for the precise phenotyping screening [15]. Reliable mapping of candidate QTLs rests on accuracy of phenotypic information. Phenotyping was, therefore, exercised using specific isolates for screening of individual plants for response to scab. Presently, the F₁ progenies exhibited significant phenotypic variance for the disease reaction. Frequency distribution of disease reaction followed a continuous pattern and fitted into normal distribution curve, as expected for a quantitatively inherited trait. The findings were in agreement with those of Soufflet-Freslon *et al.* (2008) [16], who also observed the

frequency distribution to be continuous with skewness towards resistance.

Analysis of co-segregation pattern of the marker loci with that of phenotypic information is important for detecting the genomic regions of important characters (QTL). One of the approaches followed is that of CIM (Composite Interval Mapping) [17]. CIM combines interval mapping with linear regression and includes additional genetic markers in the statistical model in addition to an adjacent pair of linked markers for interval mapping. The main advantage of CIM is that it is more precise and effective at mapping QTLs compared to single-point analysis and interval mapping, especially when linked QTLs are involved. The only molecular tool available for the dissection of a complex quantitative trait into recognizable genetic units or blocks is the QTL analysis. Xu *et al.* (2000)[18] reported that the association of the favourable or unfavourable QTL alleles of quantitative traits give rise to extreme phenotypes of quantitative traits, while intermediate phenotype preponderance usually indicates allelic dispersion. It is very difficult to differentiate phenotypically the genetic stocks with QTL allelic dispersion as they displays similar phenotype. The information of QTL allelic status and their linked molecular markers can greatly increase the selection efficiency of individuals. In the present study, the resistance to scab in *Shireen* was characterized as quantitative inheritance and we identified two QTLs, qRVI.SS-LG2.2019 and qRVI.SS-LG8.2019 on LG2 and LG8 of the *StarKrimson* × *Shireen* linkage map with the mixture of four races of *Venturia inaequalis*. qRVI.SS-LG2.2019 explained 39.04% of the phenotypic variance, where it was flanked by the SSR markers CN584771 and CN857442. It was located on the centromere of chromosome 2, which is a resistance gene rich-region. Earlier Calenge *et al.* (2004) [19] too mapped a scab resistance QTL to chromosome 2, but at location different from the presently identified location. In present study the SSR marker CH02b10 linked to major scab resistance gene *Vr* was mapped 24.5 Mbp and 22.03 Mbp below the flanking markers CN584771 and CN857442 respectively. Hulbert *et al.* (2001)[20] mapped the *Vr* gene at 7.8 cM from the SSR marker CH02b10. CH02b10 has also been mapped 1 cM above the microsatellite marker CH02c06 in the ‘Fiesta’ × ‘Discovery’ progeny [21], which is close to the likelihood peaks of the QTL detected on LG2 by Calenge *et al.* (2004) [22]. Further, the position of the scab resistance gene *Rvi8* was estimated at 9.5 cM from CH02b10 [23]. Bus *et al.* (2005a) [13] also mapped *Rvi2* to the distal part of linkage group 2 between the SSR markers, CH05e03 and CH02b10. Eventually, CH05e03 and CH02b10, which are linked to scab resistance fall away from the qRVI.SS-LG8.2019 interval reported here, which suggest a novel resistance interval.

Another QTL namely, qRVI.SS-LG8.2019 was mapped between ch01d09 and ch03a08 and explained phenotypic variance of 57.91%. No other resistance genes, for scab or any other disease has been reported in this region. The QTLs qRVI.SS-LG2.2019 and qRVI.SS-LG8.2019 explain 39.04% and 57.91% of phenotypic variability and may therefore, be regarded as novel QTLs for scab resistance in apple. Since these two QTLs were mapped against a mixture of four virulent races of *Venturia inaequalis* prevalent in Kashmir, this further validates the robustness of the mapped QTLs. Nevertheless, more experiments should be conducted to confirm the nature of genetic resistance at these loci at sequence level which may be accomplished with the cloning of genes within the interval.

Narrowing down the qRVI.SS-LG2.2019 region and candidate gene mining

Based on the recombination frequency, the CN857442 marker was found more close to the mapped QTL, qRVI.SS-LG2.2019. In an attempt to narrow down the marker interval CN584771 - CN857442 flanking the QTL, qRVI.SS-LG2.2019 on chromosome 2, four new STS markers were designed from the contigs spanning the region. Validated of these markers on the parents, *Shireen* and *StarKrimson*, revealed only a single marker named SABA-2 polymorphic and was therefore used for genotyping of mapping population. After studying the recombination events in 122 F₁ individuals, the region was further reduced to the 0.97 Mbp with the introduction of SABA-2 and other polymorphic markers.

The GD reference sequence at the QTL region, qRVI.SS-LG2.2019 was visualized in Gbrowse and Within this region, a total of 325 genes were predicted by browsing the Genome Database for Rosaceae (GDR). On the basis of function annotation, 57 out of 325 genes were found resistance specific. In apple, 56% of RGAs are located preferentially on six chromosomes, with 14% located on Chromosome 2 [24]. This supports our results that this region where we have mapped the qRVI.SS-LG2.2019 is also rich in RGAs. Out of these 57 resistance specific genes, 27 predicted genes encode proteins belonging to the NBS-LRR disease R gene family. Since most of the classes of genes which are supposed to be related to resistance are TIR-NBS-LRR and already two major scab resistant genes which are cloned belong to the TIR-NBS-LRR and LRR-RLP and in this region where we have mapped QTL both of these are present. So, possibly these can be the candidate genes for underlying QTL.

***In silico* mining of genes within QTL region, qRVI.SS-LG9.2019**

In silico search for the presence of defense responsive genes as well as genes associated with signal transduction of biotic stresses was carried within the qRVI.SS-LG8.2019 region.

Among 224 predicted genes, it is important to highlight categories of importance which included the F-box family proteins, C₂H₂-like Zinc finger protein, Thaumatin-like protein-1, pathogenesis related Thaumatin superfamily proteins, Lipid transfer proteins, Leucine rich receptor like protein kinases, Mitogen activated protein kinase-1, oxidoreductases, Disease resistance protein (TIR-NBS-LRR class) family, Peroxidase superfamily protein and Jasmonic acid carboxyl methyltransferase. The F-box protein domains found within this region are said to contain Leucine-Rich Repeat (LRR) domains associated with pathogen responses [25]. The signaling proteins such as mitogen-activated protein kinase (MAPK) transduce the external stimuli into an intracellular host defense response. Subsequently the specific genes are activated to combat the pathogen attack. The key elements for the activation of these genes are the transcription factors such as zinc finger homeodomain proteins. Eventually, a highly toxic environment is created by massive production of reactive oxygen species (ROS) (involving peroxidase, oxidoreductases). The NBS domain is part of the larger NB-ARC domain that hydrolyses ATP and GTP and functions as a molecular switch for signal transduction after pathogen recognition. Many resistance proteins encoded by RGAs contain a leucine-rich repeat (LRR) domain, involved in protein-protein interactions and in pathogen recognitions. Proteins coded by RGAs (Resistance gene analogues) can be further classified according to the presence of the toll/interleukin-1 receptor (TIR) or other N terminal features, such as coiled-coil (CC) and BED finger (Bed). The N-terminal features are involved in downstream specificity and signaling regulation. The functional annotation suggested that these 37 genes could be candidates for qRVI.SS-LG8.2019, but their real involvement in the resistance of ‘*Shireen*’ needs a thorough investigation.

Expression profile of candidate resistance genes in a segregating F₁ population

From the present study two QTLs namely, qRVI.SS-LG2.2019 and qRVI.SS-LG9.2019 were identified from resistant parent ‘*Shireen*’. The interaction of these resistance loci with avirulence factors of *V. inaequalis* is expected to trigger the cascade of reactions which ultimately conditions final host response. Since the races used clearly differentiate the parents based on disease phenotype, a differential expression pattern of various genes up or down in the pathway, was expected to be present in parents and the derived population. Here, BSA (Bulked-segregant analysis) approach was used to find the association of the selected candidate genes with scab resistance. BSA provides a simple and effective alternative to identify molecular markers linked to target genes or QTLs affecting a trait of interest by genotyping only a pair of bulked samples from two sets of individuals with

distinct or opposite extreme phenotypes . Six CDGs (candidate defence genes) were selected aiming to identify differentially expressed genes in ‘*Shireen*’, 96 h after *V. inaequalis* inoculation, as compared to the susceptible cultivar ‘*StarKrimson*’. The first visible resistance reactions, corresponding to large areas of collapsed epidermis cells, in the host appear at 96 hpi [26]. Numerous studies demonstrated that, beside basal host resistance, the key difference between resistant and susceptible hosts is the timely recognition of the invading pathogen and the activation of plant defense genes which is accompanied by the accumulation of corresponding gene products. In fact, signaling and defense genes could be invoked as good candidates for explaining the resistance of ‘*Shireen*’. The results of the qRT-PCR confirmed the differential expression of all the six candidate genes between two parents and bulks. The comparison of resistant and susceptible bulk, showed that all the six candidate genes were significantly upregulated in resistant bulk. Thereby, supporting the hypothesis of their principal role in scab resistance. Moreover, the transcripts were clustered based on their functional patterns or similarities. As the *LOX* and *MdAOS-2* genes, encoding key enzymes of the Jasmonic acid (JA) pathway genes were found to cluster together.

Regarding the Jasmonic acid (JA) marker genes, we evaluated the expression level of two genes encoding key enzymes of the JA pathway, namely Lipxygenase (*LOX*) and allene oxide synthase 2 (*AOS-2*). The first step in biosynthetic pathway of jasmonate is catalyzed by lipxygenases and *AOS2* catalyzes the first step in the biosynthesis of JA from lipxygenase-derived hydroperoxides of free fatty acids [27]. Silencing of *LOX* gene in plants has resulted in enhanced susceptibility to some microbial pathogens [27]. In present study, both *LOX* encoding protein and *AOS2* showed an increased expression in both resistant parent as well as in resistant bulk. This increased expression may inhibit the growth of fungus by the production of fungal inhibitor oxylipin substances (e.g. hexanal and colnelenic acid) or by its own antimicrobial activity, as previously described Gusberti *et al.*, 2013[27]. A PR-protein gene (*LTP*: lipid transfer protein) showed increased gene expression in shireen and resistant bulk, at 96 hpi. Lipid transfer proteins (*LTP*) are one of the important PR proteins which act as a potential mobile signal for systemic acquired resistance (SAR) in plants. The role of LTPs has been assigned to inhibition of fungal growth rather than to inhibition of fungal germination in *Malus-Venturia* pathosystem [27]. There are also several reports on transgenic overexpression of LTP genes resulting in enhanced tolerance to pathogen infection. For instance, transgenic tobacco expressing a barley LTP gene showed enhanced resistance against *Pseudomonas syringae* pv. tabaci . In our experiment, the EDS-1 gene encoding protein was up regulated in both *Shireen* and

resistant bulk. EDS gene is necessary for the functionality and signal transduction of other resistance genes. Peroxidases (PXs) are connected to avirulent-microbe defence. The biochemical functions of PXs are the biosynthesis of lignin and suberin and to regulate the reactive oxygen species (ROS). Silencing of PX gene in plants has resulted in an increased disease susceptibility while as its overexpression has been found to enhance the plant resistance. qRT-PCR results revealed a significant differential PX3 expression between the resistant and susceptible parents as well as between two bulks. This increased expression in resistant parent and in resistant bulk may be linked to strengthening of the cell wall and consequently reducing the nutrient availability necessary for the fungal growth. Metallothioneins (MT) are proteins connected to heavy metal detoxification in stressed plants. MT protein encoding genes have been reported from apple trees. In our experiment, a differential expression for MT3 gene was found between resistant and susceptible parents and bulks. The up regulation of MT3 may inhibit fungal growth through metal ion sequestration, leading to an unsuitable habitat for fungal growth, or by decreasing the fungal enzymatic activity; thus, in both situations, an inhibition of fungal growth may be expected. In one of our studies marker based screening of F₁ derived from cross of variety Firdous and another variety Gala, was undertaken [28]. The same races were used to analyse the resistance pattern of the derived population. Since it was believed that gene *Rvi6* was present in one of the parents and is a strong gene, the study supports our findings that the QTLs identified in a different background under present experiment, are effective enough in terms of favouring ETI and are expected to show high expression and response towards prevalent isolates.

Conclusions

The study was instrumental in elucidation of genetic basis of resistance in cultivar Shireen released by SKUAST-Kashmir in 1995. The two novel QTLs in Shireen named, qRVI.SS-LG2.2019 and qRVI.SS-LG8.2019 were mapped on chromosome 2 and 8. The QTL region of qRVI.SS-LG2.2019 was narrowed down by using the newly designed marker SABA_2 which helped to further delineate the identified QTL within 0.97 Mbp segment on chromosome 2. Further a genetic map was generated using SSRs and random markers which comprised of 16 LGs. The map shall function as a basic framework to saturate molecular markers which in turn may help to map genes responsible for yield and quality in apple. RNA expression of genes viz., EDS-1, LTP, PX3, LOX, MT3 and MdAs01 was found

significantly upregulated in both the resistant parent Shireen and resistant bulk derived from F₂ population.

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Credit Author Statement

ABS and SM conceived and planned the experiments. ABS & SM Cconceptualized the research. SM, AS & ABS carried out the marker analysis. SM & ZAB carried out the disease phenotyping. SM & BAP maintained and prepared the cultures. ABS & NN helped to carry statistical analysis. KMB & IM were involved in development and maintenance of mapping population. SM, KZM, SHW & ABS did the data curation and expression analysis. SM, ABS and SHW wrote and finalized the manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1: Summary statistics of phenotypic data

Sample Size	Mean	Variance	Std Error	Skewness	Kurtosis	W-test
122	3.4919	8.2032	2.8641	0.0845	-1.6392	0.7944

Table 2: Polymorphic markers between the Shireen and StarKrimson along with chromosome location

S.No.	Name of the Marker	Chromosome	Physical Location (Mb)
1	ch05g08	1	1.97
2	cn90484	1	6.09
3	cn854771	2	1.98
4	cn57442	2	4.47
5	ch02a04	2	6.75
6	ch02b101	2	26.50
7	ch03g12z	3	0.90
8	ch03e03	3	1.13
9	gd6	4	0.15
10	ch02h11a	4	8.18
11	cn949371	5	0.05
12	cn918501	5	1.43
13	cn918509	5	14.32
14	cn862287	6	23.26
15	ch04d07	7	0.04
16	ms06g03	7	25.12
17	ch03a08	8	0.05
18	ch01d09	8	2.10
19	cn917587	8	3.76
20	cn856758	9	3.77
21	ch04G10	9	14.65
22	cn919347	9	22.45

23	ch03a04	10	22.82
24	c0731611	11	1.27
25	cn817587	12	21.91
26	ch04f10	12	31.19
27	hi01d06y	13	0.33
28	cn86871	14	292.69

Table 3: Phenotypic variability explained for mapped QTLs

Trait	QTL	Chromosome	Position	Left Marker (Mb)	Right Marker (Mb)	LOD	Phenotypic variability % (PVE)	Additive effect
Resistance	qRVI.SS-G2.2019	2	11	CN854771	CN57442	7.67	39.04	3.57
Resistance	qRVI.SS-LG8.2019	8	31	ch01d09	ch03a08	4.99	57.91	4.34

Fig. 1 Genotyping of F1 population (Shireen X StarKrimson)

M: 100 bp DNA Size standard (Fermentas, Lithuania, USA); **R:** Resistant parent 'Shireen'; **S:** Susceptible parent 'StarKrimson'; **F1s** derived from two parents with Shireen as female

Fig. 2 Graphical representation of Mapped QTLs (qRVI.SS-LG2.2019 & qRVI.SS-LG8.2019) on Linkage Group 2 and 8

(a) Position of qRVI.SS-LG2.2019; (b) LOD score of qRVI.SS-LG2.2019; (c) Position of qRVI.SS-LG8.2019; (d) LOD score of qRVI.SS-LG8.2019

Fig. 3 Expression analysis of CDGs analyzed by qRT-PCR during *V. inaequalis*–*Malus* interaction.

Blue histograms indicate response in ‘Shireen’ (Resistant Parent), red histograms in ‘StarKrimson’ (Susceptible Parent), yellow histograms in SB (susceptible bulk) and green histograms in RB (resistant bulk) at 96 hpi. EDS1: Enhanced disease susceptibility 1 protein, LOX: Lipooxygenase, MdAOS2: Allene oxide synthase 2 coding gene, PX3: Peroxidase 3, MT3: Metallothionein3-like protein, LTP: Lipid transfer protein

Fig. 4 Heat map representing expression pattern of CDGs linked to scab resistance

Red shading indicates higher expression of genes and blue shading indicates lower expression of genes to that of reference gene (Actin)

Shireen: Resistant Parent

SB: susceptible bulk

RB : resistant bulk

Supplementary Information

Table S1: Evaluation of molecular markers for degree of segregation distortion

S. No.	Marker	χ^2 -Value	S. No.	Marker	χ^2 -Value
1	ch05g08	0.161	15	ch04d07	0.374
2	cn90484	0.036	16	ms06g03	0.034
3	cn854771	0.063	17	ch03a08	0.010
4	cn57442	0.069	18	ch01d09	0.009
5	ch02a04	0.004	19	cn917587	0.025
6	ch02b101	0.082	20	cn856758	0.082
7	ch03g12z	0.009	21	ch04G10	0.023
8	ch03e03	0.064	22	cn919347	0.001
9	gd6	0.003	23	ch03a04	0.094
10	ch02h11a	0.036	24	c0731611	0.143
11	cn949371	0.192	25	cn817587	0.082
12	cn918501	0.068	26	ch04f10	0.047
13	cn918509	0.116	27	hi01d06y	0.059
14	cn862287	0.025	28	cn86871	0.019

Table S2: List of STS primer designed for narrow down the qRVLSS-LG2.2019 region

S.No	Primer Name	Sequence (5'-3')	Start (bp)	Length (ntda)	Tm	GC (%)	Product size (bp)
1	Saba-1	F:ATCTGCTCCGGGGTTCTAGT	2734	20	60.10	55.00	243
		R:TTGCACCTTAGTGCCACTTG	2976	20	59.90	50.00	
2	Saba-2	F:GACTCCGGCTGACATCATTT	3523	20	60.08	50.00	239
		R:GGGCGCAAAACATAAAGTGT	3761	20	60.00	45.00	
3	Saba-3	F:CAAAACTGGGGCACAAACT	2085	20	60.01	45.00	220
		R:AGGACACCAAATCAGCATCC	2304	20	59.93	50.00	
4	Saba-4	F:TTGAACATCATTGGGGGAAT	1357	20	59.99	40.00	211
		R:CAGGGGGTAGCGACTGTTTA	1567	20	60.12	55.00	

Table: S3 Gene expression analysis of selected CDGs by qRT-PCR in ‘Shireen’ (resistant parent), ‘StarKrimson’ (susceptible parent), RB (Resistant bulk) and SB (Susceptible bulk)

S. No.	Gene	Treatment	Mean CT	ΔCT	ΔΔCT	Fold expression
1	<i>Lipoxygenase (LOX)</i>	Shireen	25.37±0.015	1.35	-4.51	22.84
		StarKrimson	29.87±0.026	5.86	0.00	1.00
		SB	22.06±0.028	3.01	-2.85	7.23
		RB	26.33±0.020	-1.71	-7.57	190.46
2	<i>Peroxidase 3 (PX3)</i>	Shireen	24.13±0.005	-2.00	-3.92	15.14
		StarKrimson	28.68±0.005	1.92	0.00	1.00
		SB	26.24±0.036	-1.21	1.16	0.45
		RB	22.06±0.017	-3.90	-5.82	56.49
3	<i>Lipid transfer protein (LTP)</i>	Shireen	27.54±0.011	-2.01	-2.49	5.60
		StarKrimson	30.14±0.015	1.92	0.00	1.00
		SB	30.73±0.005	-1.21	-1.54	2.91
		RB	28.07±0.005	-3.9	-6.72	105.18
4	<i>Enhanced disease susceptibility 1(EDSABA_1)</i>	Shireen	28.12±0.011	1.00	-4.04	16.43
		StarKrimson	29.55±0.015	5.04	0.00	1.00
		SB	29.76±0.174	3.05	-1.99	3.96
		RB	28.76±0.115	1.35	-3.69	12.88
5	<i>Allene oxide synthase 2 coding gene (Md AOSABA_2)</i>	Shireen	27.54±0.025	0.48	-3.82	14.12
		StarKrimson	29.81±0.080	2.30	0.00	1.00
		SB	29.46±0.015	2.73	-1.57	2.98
		RB	26.58±0.005	0.84	-3.46	11.00
6	<i>Metallothionein 3 (MT3)</i>	Shireen	25.84±0.011	-3.42	-2.60	6.06
		StarKrimson	28.76±0.017	-0.82	0.00	1.00
		SB	27.46±0.312	-2.01	-1.20	2.29
		RB	24.90±0.055	-5.48	-4.67	25.40

Table S4: In silico candidate gene identification within QTL region, qRVLSS-LG2.2019

S. No.	Name	Description	Type	Position	Length
1	MD02G1026100	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:1950873..1955056 (- strand)	4,184 bp
2	MD02G102	BED zinc finger	Gene	Chr02:2011757..2014750	2,994 bp

	6500			(- strand)	
3	MD02G102 6800	Calcium-binding EF-hand family protein	Gene	Chr02:2046116..2047931 (+ strand)	1,816 bp
4	MD02G102 6900	Ubiquitin system component Cue protein	Gene	Chr02:2049041..2052049 (- strand)	3,009 bp
5	MD02G102 7100	Ubiquitin-like superfamily protein	Gene	Chr02:2060739..2062825 (- strand)	2,087 bp
6	MD02G102 7400	RabGAP/TBC domain-containing protein	Gene	Chr02:2080879..2085695 (+ strand)	4,817 bp
7	MD02G102 7500	myb domain protein 82	Gene	Chr02:2086054..2087400 (- strand)	1,347 bp
8	MD02G102 7800	zinc finger (ubiquitin-hydrolase) domain-containing protein	Gene	Chr02:2141075..2144694 (- strand)	3,620 bp
9	MD02G102 8300	blue-copper-binding protein	Gene	Chr02:2171595..2172664 (- strand)	1,070 bp
10	MD02G102 8400	transmembrane receptors	Gene	Chr02:2178701..2182725 (+ strand)	4,025 bp
11	MD02G102 8500	blue-copper-binding protein	Gene	Chr02:2190395..2193146 (- strand)	2,752 bp
12	MD02G102 8600	blue-copper-binding protein	Gene	Chr02:2193148..2194245 (- strand)	1,098 bp
13	MD02G102 8700	blue-copper-binding protein	Gene	Chr02:2200895..2202539 (- strand)	1,645 bp
14	MD02G102 9200	blue-copper-binding protein	Gene	Chr02:2260915..2261942 (- strand)	1,028 bp
15	MD02G102 9300	Glycosyl hydrolase superfamily protein	Gene	Chr02:2263308..2273822 (- strand)	10,515 bp
16	MD02G103 0100	Protein kinase superfamily protein	Gene	Chr02:2328626..2333299 (+ strand)	4,674 bp
17	MD02G103 1200	Peroxidase superfamily protein	Gene	Chr02:2455656..2456282 (+ strand)	627 bp
18	MD02G103 1600	DnaJ/Hsp40 cysteine-rich domain superfamily protein	Gene	Chr02:2463247..2465649 (- strand)	2,403 bp

19	MD02G103 1800	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:2471518..2481767 (- strand)	10,250 bp
20	MD02G103 1900	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:2482000..2490538 (- strand)	8,539 bp
21	MD02G103 2000	sphingoid base hydroxylase 2	Gene	Chr02:2502428..2502895 (+ strand)	468 bp
22	MD02G103 2100	sphingoid base hydroxylase 1	Gene	Chr02:2508587..2508742 (- strand)	156 bp
23	MD02G103 2600	sphingoid base hydroxylase 2	Gene	Chr02:2554746..2555495 (- strand)	750 bp
24	MD02G103 2700	pleiotropic drug resistance 12	Gene	Chr02:2562617..2568979 (+ strand)	6,363 bp
25	MD02G103 4500	Protein kinase superfamily protein	Gene	Chr02:2640563..2644552 (+ strand)	3,990 bp
26	MD02G103 5700	Zinc finger (CCCH-type) family protein	Gene	Chr02:2714008..2716436 (- strand)	2,429 bp
27	MD02G103 7000	Protein kinase superfamily protein	Gene	Chr02:2796503..2797870 (- strand)	1,368 bp
28	MD02G103 8400	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3146609..3152747 (+ strand)	6,139 bp
29	MD02G103 8500	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3153259..3155334 (+ strand)	2,076 bp
30	MD02G103 8700	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3157463..3159101 (+ strand)	1,639 bp
31	MD02G103 9200	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3175203..3181150 (+ strand)	5,948 bp
32	MD02G103 9300	Disease resistance protein (TIR-NBS class)	Gene	Chr02:3187739..3192961 (+ strand)	5,223 bp
33	MD02G104 0000	Leucine-rich repeat protein kinase family protein	Gene	Chr02:3249315..3252911 (- strand)	3,597 bp
34	MD02G104 0600	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3291730..3296299 (- strand)	4,570 bp
35	MD02G104 0900	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3310677..3314180 (- strand)	3,504 bp

36	MD02G104 1200	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3365886..3366920 (- strand)	1,035 bp
37	MD02G104 1300	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3366922..3370112 (- strand)	3,191 bp
38	MD02G104 1700	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3394377..3398101 (- strand)	3,725 bp
39	MD02G104 2000	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3445851..3449773 (- strand)	3,923 bp
40	MD02G104 3800	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3577537..3582627 (- strand)	5,091 bp
41	MD02G104 3900	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3582880..3584124 (- strand)	1,245 bp
42	MD02G104 4300	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3616412..3620336 (- strand)	3,925 bp
43	MD02G104 5000	protein kinase family protein	Gene	Chr02:3635262..3636965 (- strand)	1,704 bp
44	MD02G104 5100	protein kinase family protein	Gene	Chr02:3636967..3638526 (- strand)	1,560 bp
45	MD02G104 5300	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:3639466..3642649 (- strand)	3,184 bp
46	MD02G104 8200	Protein kinase superfamily protein	Gene	Chr02:3809580..3810941 (- strand)	1,362 bp
47	MD02G104 8800	Protein kinase family protein	Gene	Chr02:3838656..3844418 (+ strand)	5,763 bp
48	MD02G105 0400	Toll-Interleukin-Resistance (TIR) domain family protein	Gene	Chr02:4165147..4168730 (- strand)	3,584 bp
49	MD02G105 1200	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:4216225..4218804 (- strand)	2,580 bp
50	MD02G105 1400	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:4219483..4221488 (- strand)	2,006 bp
51	MD02G105 2200	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:4284872..4286667 (- strand)	1,796 bp
52	MD02G105 2300	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:4287287..4288396 (- strand)	1,110 bp

53	MD02G105 2800	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:4323093..4333203 (- strand)	10,111 bp
54	MD02G105 3100	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:4338781..4346208 (- strand)	7,428 bp
55	MD02G105 3600	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:4365499..4370115 (- strand)	4,617 bp
56	MD02G105 4400	disease resistance protein (TIR-NBS-LRR class)	Gene	Chr02:4386879..4389513 (- strand)	2,635 bp
57	MD02G105 5500	Disease resistance protein (TIR-NBS-LRR class) family	Gene	Chr02:4480091..4484423 (- strand)	4,333 bp

Fig. S1 Phenotyping of F₁ Progenies for Scab resistance

Representative F₁s showing variable response to scab. Scoring was done on 0-4 scale following Chevalier *et al.* (1991)

Fig. S3 Linkage map generated from population derived from StarKrimson × Shireen

Fig. S4 Frequency of recombination events within the qRVI.SS-LG2.2019 region

Fig. S5 Validation of Saba_2 marker on F1 population

M: 100 bp DNA Size standard (Fermentas, Lithuania, USA); R: Resistant parent 'Shireen'; S: Susceptible parent 'StarKrimson'; RB: Resistant bulk; SB: Susceptible bulk; F1s derived from two parents with Shireen as female

Fig. S6 Narrowing Down of marker interval CN584771 - CN857442 flanking qRVI.SS-LG2.2019

Fig.S7 Clustering of F1 progenies based on Genetic distances from the parents

Fig. S2 Frequency distribution curve of scab disease severity across population derived from Shireen × StarKrimson