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Detection and validation of highly conserved pathogen-specific genes with putative role in mycotoxin synthesis using genome-wide insilico approach

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Abstract
The PCR-based technique was used to analyze a set of polymorphic and monomorphic DNA markers identified through experimentation on isolates of *Erysiphe pisi* pathogen. These putative DNA markers were used for a genome wide-search to detect the associated pathogen-specific gene sequences from the available draft sequence of *Erysiphe pisi* genome in public domain. The contigs in which these markers were showing homologous pairing were obtained and translated into amino-acid sequences. The sequences were analyzed for the presence of the open reading frames (ORFs) as they code for putative genes and also the domains in the sequences. The search was carried both at nucleotide and amino acid level.
A significant level of correlation among the sequences with high level of similarity at nucleotide level and high level of identity at amino acid level was observed with already detected virulence genes based on multiple sequence alignment using clustalW analysis suggesting the possibility of the same function for the identified protein sequences. The predicted gene sequences were confirmed through genomic and RT-PCR testing using DNA as well as total RNA of three isolates of *E. pisi* in infected garden pea plant. Healthy plants served as a control for the experiments.

Keywords: *Erysiphe pisi*, RT-PCR, DNA marker, pea plant, amino acids

Introduction
Powdery mildew of peas (*Pisum sativum*) is caused by the fungus *Erysiphe pisi*, an obligate biotrophic ascomycete infecting different parts of the plant including seeds, leaves, stems and pods. This disease has been shown affect a number of agronomically important plant traits, which in turn adversely affects yield and quality. Powdery mildew poses the greatest threat to crops of dried peas, which are an important source of dietary protein in many countries. This disease causes important crop damage and yield losses, especially in semiarid regions. The biological cycle of the parasite includes very well-defined stages from conidia infection to colony formation and conidia production which includes different stages like germ tube formation, appressorium development, host leaf cuticle penetration, haustoria, mycelium, and colony formation.
The powdery mildew infection in garden pea (*Pisum sativum*) is favoured by cool wintery nights associated with high humidity. Individual isolates of powdery mildew fungi typically have a very narrow host range owing to their specificity to adapt to a particular host genotype. This disease adversely affects total biomass yield, number of pods per plant, number of seeds per pod, plant height, and number of nodes. Severe infection may result in 25% to 50% yield reduction. Conidia and ascospores of *E. pisi* germinate on susceptible pea leaves and produce large, lobed primary appressoria which develop several hyphae spreading across the host epidermis. Subsequent mycelial growth depends on nutrients obtained through haustoria from epidermal cells. The first symptoms are small, diffuse, light-coloured spots on the upper surface of the lowest and oldest leaves. These lesions become covered by white, powdery fungal colonies. Mycelial hyphae produce short conidiophores on the plant surface. Conidia are usually borne singly on conidiophores and are disseminated mainly by wind to cause secondary infections. The incidence and severity of this disease can be controlled through the use of resistant cultivars. Any powdery mildew management program that includes the use of host resistance will require information on the virulence genes that

exist in the pathogen population of interest and the effective resistance genes in the host germplasm. Information related to the existence of physiological races in *E. pisi* is limited.

The research studies undertaken to study the nature of resistance in garden pea against *E. pisi* showed that resistance is governed by monogenic-recessive (*er1* and *er2*) resistance in *Pisum sativum* and monogenic-dominant (*Er3*) in the wild germplasm line, *Pisum fulum*. However, not much information is available about the pathogen *E. pisi*. An attempt is made through the study to unravel the genetic information pertaining to the pathogen.

Picture 1: Powdery mildew infection on garden pea (*Pisum sativum* L.)

The virulence of a pathogen is reported to be key player in determining the ability of isolates of *E. pisi* as virulent or avirulent. The nature and the genetic mechanisms are poorly understood. In case of *Fusarium graminearum*, another ascomycete member, mycotoxin biosynthetic genes involved in the synthesis of deoxy –Nivelanol or Nivelanol mycotoxins are reported to be involved in virulence of the pathogen. This forms the rationale of our study to carry out genome-wide search for virulence genes using in-silico analysis as well as testing them in-planta to detect their presence and confirm their in infected garden pea plants.

DNA markers provide powerful tool for mapping economically important genes. Since large scale screening to detect putative genes could be made possible through simple, rapid and cost-effective approaches, an attempt is made to screen the genes using short stretches of random and polymorphic DNA markers. Further, it also provides the opportunity for developing tightly linked phenotypically neutral genetic tags to the traits in study especially to detect introgression phenomena in plant breeding programs. DNA markers have been used to map and tag a number of disease resistance genes occurring in crop species also.

A genetic marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species. Generically it is described as a variation (Which may arise due to mutation or alteration in the DNA sequence of genomic loci) that can be observed. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (Single nucleotide polymorphism, SNP), or a long one, like micro or minisatellite markers. Molecular genetic markers can be divided into three classes: biochemical markers which detect variation at the gene product level such as changes in proteins and amino acids; cytological markers which detect variation at macro level in the chromosomes such as deletion, duplication, inversion or translocation of chromosomes. The third class of markers involve detection of micro level variation at nucleotide level due to point mutations leading to deletions- transversion and transition, duplications or substitution or insertion of nucleotides which inturn causes frame shift of the gene sequence in the genome of an organism. Markers can exhibit two modes of inheritance, i.e. dominant/recessive or co-dominant. If the genetic pattern of homozygotes can be distinguished from that of heterozygotes, then a marker is said to be co-dominant. Generally co-dominant markers are more informative than the dominant markers.

Some of the markers employed to detect variation in the genome of an organism or to compare the genome with genome of other organisms through comparative genomics is through the basic and simple markers like restriction fragment length polymorphism (RFLP), Amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR). They can be used to create genetic maps of the organism being studied. RAPD technique involves PCR based detection using arbitrary, short primers (8–12 nucleotides) which could screen the genomic DNA to search for polymorphism. By resolving the PCR amplicons through gel electrophoresis method, the DNA bands which are putative and polymorphic could be isolated. No prior information of the DNA sequence for the targeted genome is required, as the primers bind in consensus with the homologous sequences present anywhere in the whole genome. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared (It is not suitable for forming a DNA databank). Because it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species specific DNA comparison methods, such as short tandem repeats. However, the robustness of the technique to apply to any genome under study makes it a dependable technique to screen large genome sizes rapidly with less effort. RAPD technique is still has been an adapted practice to characterize and trace the phylogeny of diverse organisms.

The genome sequence of *Erysiphe pisi* which is in draft form is available in the public domain. The sequence was downloaded from NCBI website to carry out in-silico analysis to detect pathogenesis, virulence/avirulence related sequences by using genetic marker like RAPD markers. The hypothesis being tested is that these sequences would have been evolved across the members of Ascomycete members simultaneously and might be indispensable for the fungi of this group to infect and cause disease in plants. Though preliminary research findings have been reported in other members of Ascomycetes for the pathogenesis, virulence/avirulence genes, however, there are no reports yet confirming the existence of this putative pathogenesis, virulence/avirulence genes or gene loci in *E. pisi*.

Materials and Methods

The short oligo nucleotide primers used for the screening study are based on the molecular genetic studies carried out to differentiate virulent and avirulent isolates of the pathogen, *E. pisi* (Malathi Bheri & Ragiba Makandar, unpublished data). A few of the putative polymorphic DNA markers identified in the study are employed further to identify the gene contigs in which they pair based on their homologous pairing to the gene sequences in the contigs. The associated gene sequences in the contigs are analyzed to

detect the virulence related components.

In-silico analysis

Single protein function search

Initially DNA markers were taken and searched for the contigs in which they have fallen in the draft sequence of *Erysiphe pisi* which is downloaded from NCBI. Then the respective contigs are translated into protein by using NCBI translation tool. The translated sequences were subjected to protein function search using BLASTP and putative functions of the detected gene sequences are obtained.

ORF and Domain search

The ORF and Domain search was carried in the contigs in which DNA markers showed homology using the NCBI ORF FINDER TOOL. The ORF obtained from the contigs are subjected to BLASTP for searching for domains. These domains were analyzed to check if they are related to virulence function.

Phylogenetic analysis of the various domains obtained in *Erysiphe pisi*

Available domain sequence of *Erysiphe pisi* is taken from the draft sequence. BLAST search for this sequence was carried out with different members and strains of ascomycetes family. Multiple sequence alignment of nucleic acid and their respective amino acid sequence of domains were done using CLUSTAL OMEGA. Dendrograms of the domains and their corresponding proteins in different species and organism of ascomycetes were constructed using the tool Dendroscope. The sequence which were closely related at the level of nucleotide sequences, at the level of amino acid sequences and the level of both amino acids the nucleotide.

In vivo experiments

DNA Isolation

Erysiphe isolates belonging to Ascomycetes family were ground in a mortar with a pestle in liquid nitrogen and the resulting fine powder was re- suspended in 800µl of extraction buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2% CTAB, 2% β Mercaptoethanol). The crude homogenate was incubated for 1-2 hrs. At 65 ºC and then, extracted twice with an equal volume of chloroform: isoamyl alcohol (24:1), 12, 000 rpm for 15mins. The supernatant was taken in a fresh eppessndoff tube containing 0.6 volumes of absolute isopropanol and the contents were mixed gently. The samples were stored at -20 °C for an hour and centrifuged at 12,000 rpm for 15 min. The DNA pellet was washed with ice- cold 70% ethanol and the pellet was dried at room temperature and resuspended in 50 µl TE buffer. The DNA quantified by Nanodrop spectrophotometer at 260nm.

RNA Isolation

Erysiphe isolated were ground in a mortar with a pestle by using micro centrifuge then 0.5 ml of trizol reagent was added, then 200µl chloroform was added and vortexes for 5mins, and tubes were centrifuged at 10, 000 rpm for 10 mins at 4 °C. Aqueous layer was transferred in to fresh tube and 1:1 volume of isoproponal was added, tubes were kept in -20 °C for overnight, after centrifugation at 15000 rpm for 15mins, RNA forms a pellet. This pellet was washes with 70% ethanol and isopropanol at 15000 rpm for 5mins, supernatant was removed again washed with 70% ethanol and extra alcohol was removed, pellet air-dried and RNA dissolved in 50 µl of DEPC treated water.

cDNA preparation from RNA of *Erysiphe* isolates

The reaction mixture-1 contains RNA template of 1.0 µl, Oligo dT primer of 0.5, and 5X reaction buffer of 1.0 µl. this mixture was incubated at 60 °C for 15minutes to denature the secondary structure of RNA and then allowed to cool down to room temperature gradually over 10 to 15 minutes such that primers get time to anneal at the complementary site. To the contents, RT enzyme of 0.2 µl, 25 mM dNTPs of 2.0 µl and milliQ water of 3.0 µl. The total reaction mixture volume was adjusted to 10 µl; this mixture was incubated at 37ºC for 60 to 90minutes to enable activity of Reverse Transcriptase enzyme for cDNA synthesis. After cDNA synthesis, the samples were stored at -20ºC for further analysis.

Table 1: cDNA Cocktail Preparation

S. No	Components	Volume(µl)
1	Water	3
2	5X reaction buffer	1
3	dNTP	2
4	$MgCl_2$	2
5	RNase inhibitor	0.25
6	Oligo dT	0.5
7	Reverse transcriptase	0.25
8	Template RNA	1
	Total	10

PCR Components used for amplification

A total of six primers were taken out of which three are monomorphic and three are polymorphic are used for analysis. The respective RAPD primer is used to amplify the gene product for all three isolates at DNA and cDNA level for 45cycles.

Table 2: PCR Composition

S. No	Components	Volume(µl)
1	Water	8
2	Primer	3
3	DNA	2
4	Master Mix	12
	Total	25

The PCR program was as follows

- **Step 1:** 94C for 2min (Initial Denaturation).
- **Step 2:** 95C for 1min (Denaturation).
- **Step 3:** 36C for 1min (annealing).
- **Step 4:** 72C for 1min (Extension).
- **Step 5:** Goes to step 2 for 45cycles.
- **Step 6:** 72C for 10min (final extension).
- **Step 7:** Hold at 4C

PCR products were loaded on 1.2% (w/v) Agarose gel for confirmation of the amplified products with 100kb ladder as a marker and visualized by Ethidium Bromide.

In-silico analysis

Single protein function search

All thirty four short oligo nucleotide DNA sequences which act as DNA markers showed consensus with related contigs were analyzed by translating the genome sequence of the

contigs into amino acid sequence which was then subjected to protein search. The obtained putative proteins through in-silico search are listed and their function is predicted. Out of these thirty four short oligonucletide sequences, seventeen were polymorphic and seventeen were monomorphic in nature. All the proteins which showed the function related to pathogenesis, virulence/avirulence, and PRR are listed in the tables (Table 1 & 2) for monomorphic and polymorphic DNA markers respectively.

ORF & Domain Search

All thirty four DNA marker related contigs searched for ORF region and protein domains and the putative domains are listed. All the ORF regions of putative domains which show pathogenesis, virulence/avirulence, and PRR related hits assembled for both monomorhic and polymorphic DNA markers are listed in table 3 and table 4 respectively.

Phylogenetic analysis of the various domains obtained in *Erysiphe pisi*: Multiple alignments of domains in different ascomycetes organisms and their strains showed maximum level of conservancy between amino acid motifs. Phylogenies of different domains were constructed by maximum parsimony analysis at level of both nucleic acid and amino acids using Dendroscope software (Figures 1-26). Those sequences were analyzed which were aligned at one node in the tree. The phylogenetic analysis revealed the accession of ascomycetes sharing maximum similarity at both nucleotide level and amino acid level

***In-vivo* analysis**

DNA and RNA isolation was carried for different isolates of *E. pisi* along with control. The cDNA was prepared using total RNA of the samples of *E. pisi* infected leaf and non-infected or a leaf of healthy garden pea plant. The DNA and cDNA of the three isolates along with control pea leaf DNA were subjected to genomic and RT-PCR using the respective primers to check for the presence of putative genes and their expression in planta. Data carried with three such DNA markers for monomorphic and polymorphic DNA markers are being presented. The amplifie

Phylogenetic analysis of domains at nucleotide and amino acid level

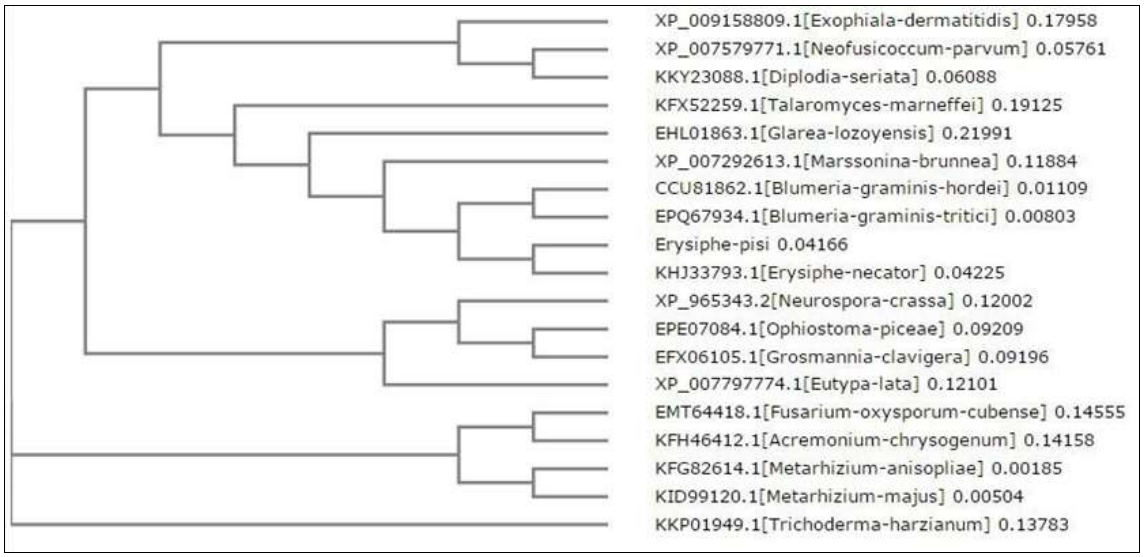


Fig 1: Phylogenetic tree of nucleic acid sequence of SAP domain of different organisms and strains of ascomycetes

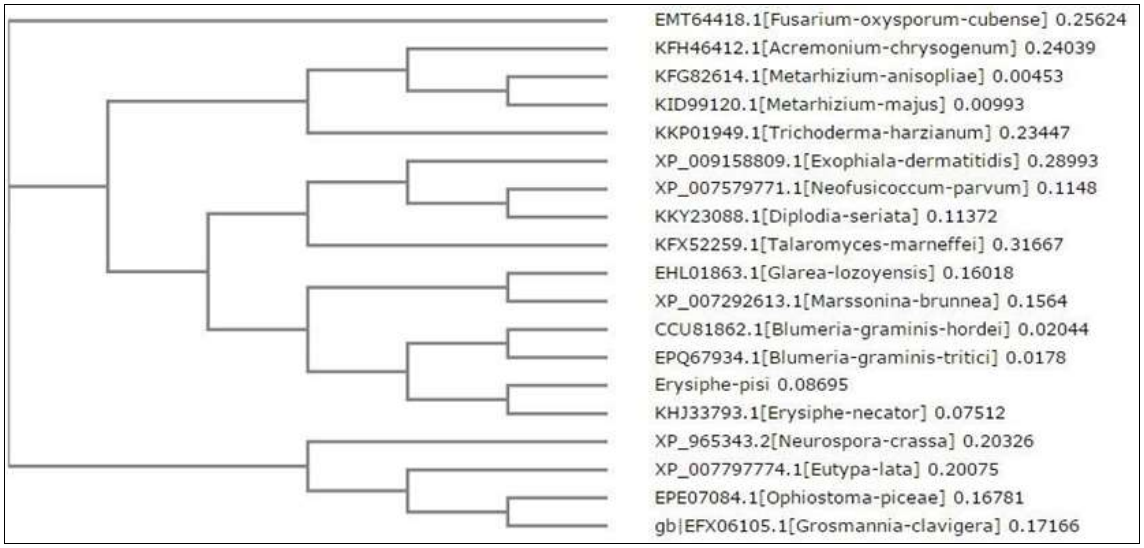


Fig 2: Phylogenetic tree of amino acid sequence of SAP domain of different organisms and strains of ascomycetes

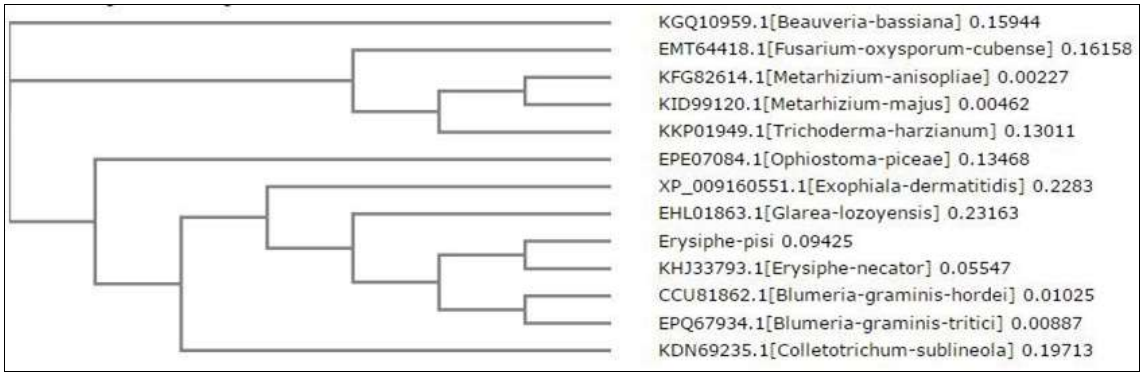


Fig 3: Phylogenetic tree of nucleic acid sequence of TAXi_N domain of different organisms and strains of ascomycetes

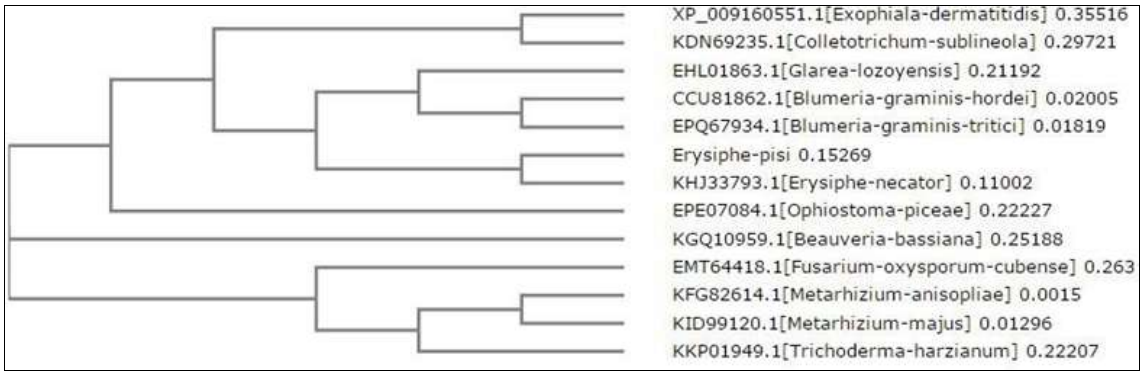


Fig 4: Phylogenetic tree of amino acid sequence of TAXi_N domain of different organisms and strains of ascomycetes

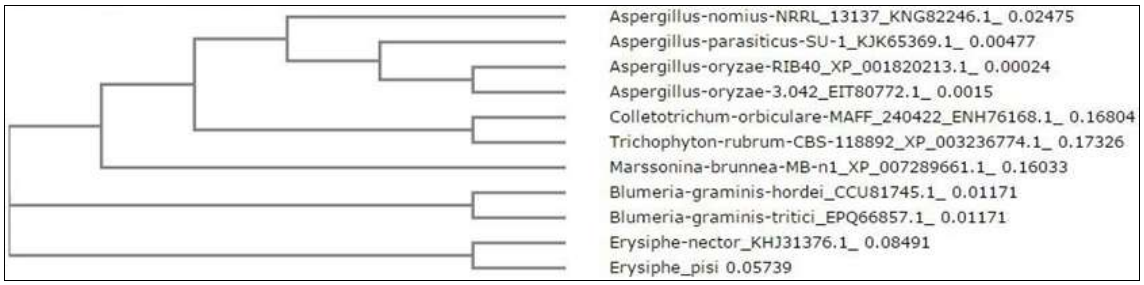


Fig 5: Phylogenetic tree of nucleic acid sequence of Ssc62 domain of different organisms and strains of ascomycetes

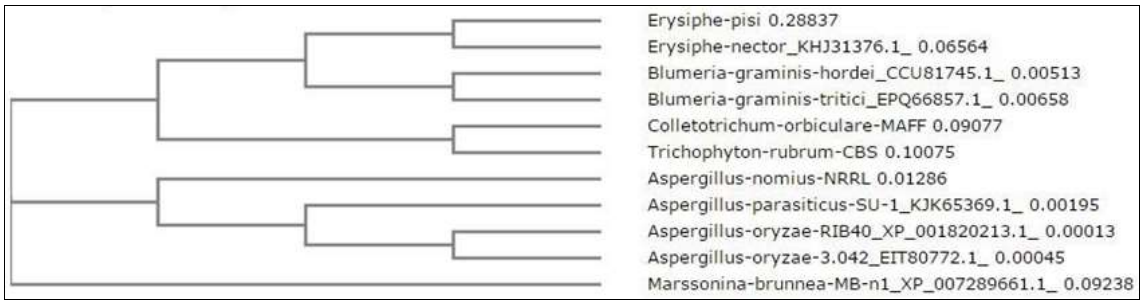


Fig 6: Phylogenetic tree of amino acid sequence of Ssc62 domain of different organisms and strains of ascomycetes

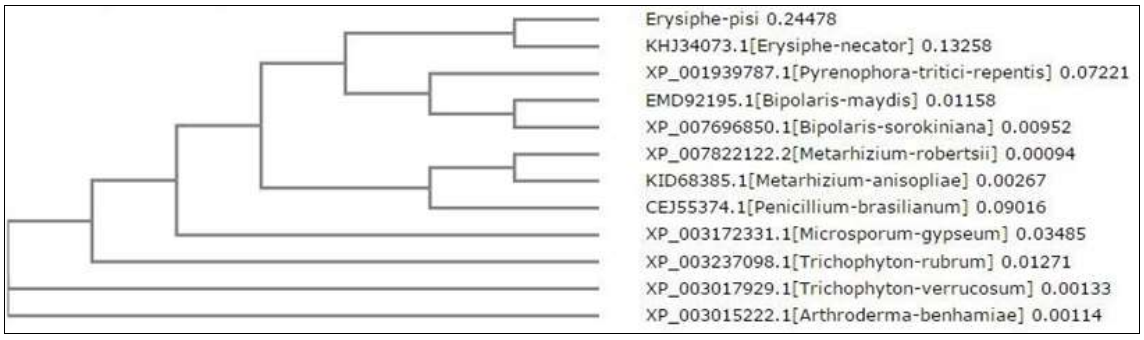


Fig 7: Phylogenetic tree of nucleic acid sequence of TRI12 domain of different organisms and strains of ascomycetes

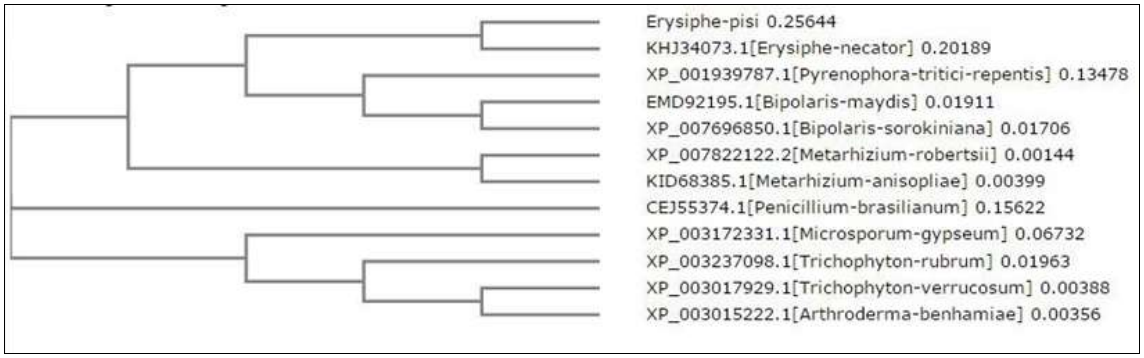


Fig 8: Phylogenetic tree of amino acid sequence of TRI12 domain of different organisms and strains of ascomycetes

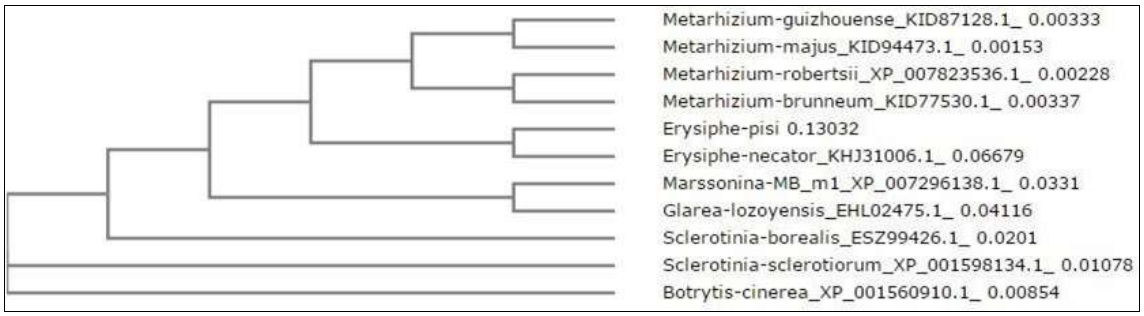


Fig 9: Phylogenetic tree of nucleic acid sequence of GST_C domain of different organisms and strains of ascomycetes

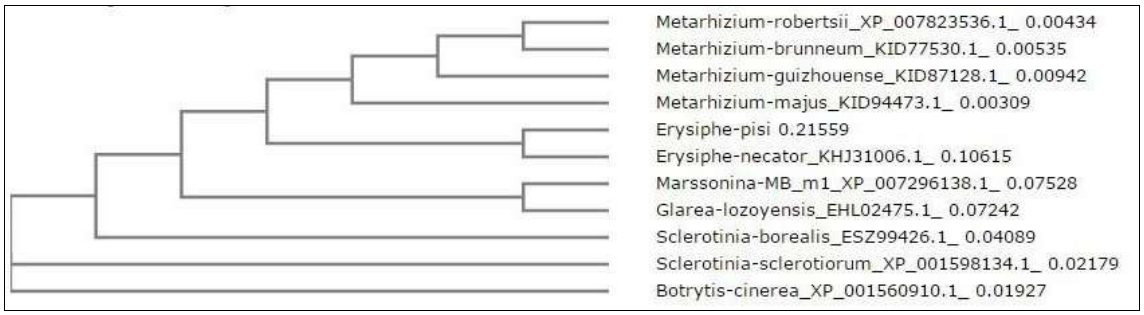


Fig 10: Phylogenetic tree of amino acid sequence of GST_C domain of different organisms and strains of ascomycetes

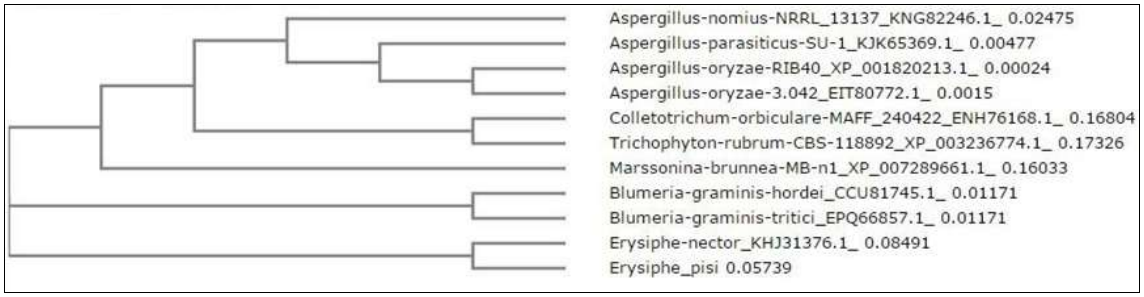


Fig 11: Phylogenetic tree of nucleic acid sequence of SLC5_6 domain of different organisms and strains of ascomycetes

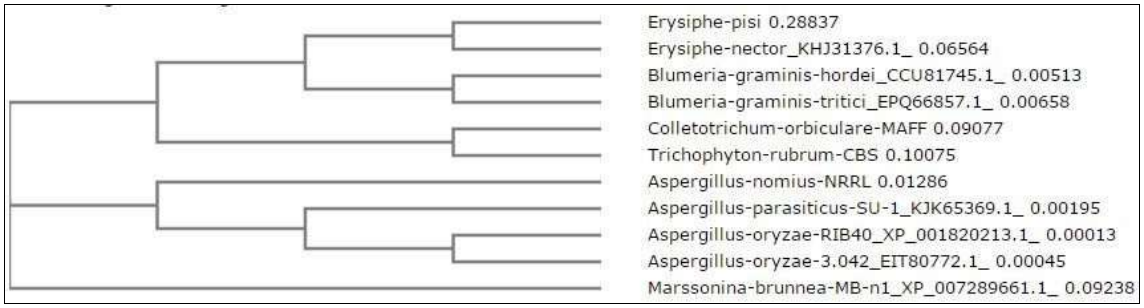


Fig 12: Phylogenetic tree of amino acid sequence of SLC5_6 domain of different organisms and strains of ascomycetes

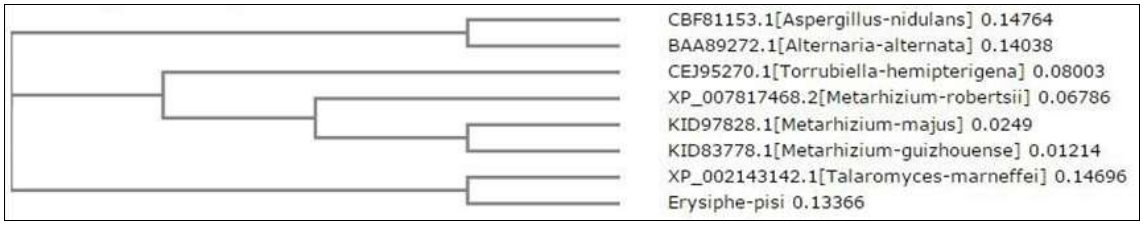


Fig 13: Phylogenetic tree of nucleic acid sequence of Pepsin_retro pepsin domain of different organisms and strains of ascomycetes

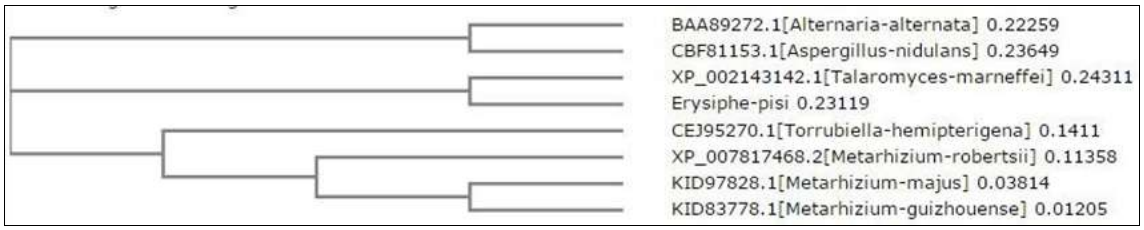


Fig 14: Phylogenetic tree of amino acid sequence of Pepsin_retro pepsin domain of different organisms and strains of ascomycetes

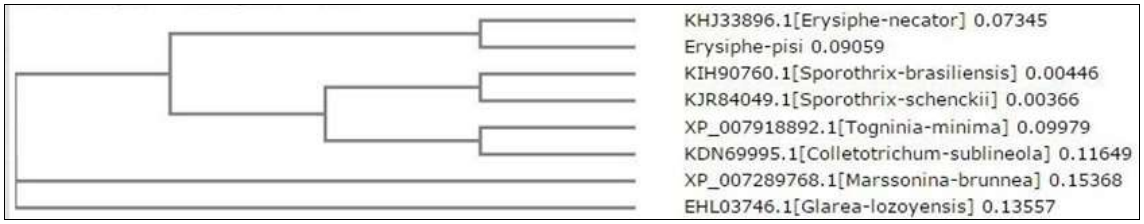


Fig 15: Phylogenetic tree of nucleic acid sequence of zbip domain of different organisms and strains of ascomycetes

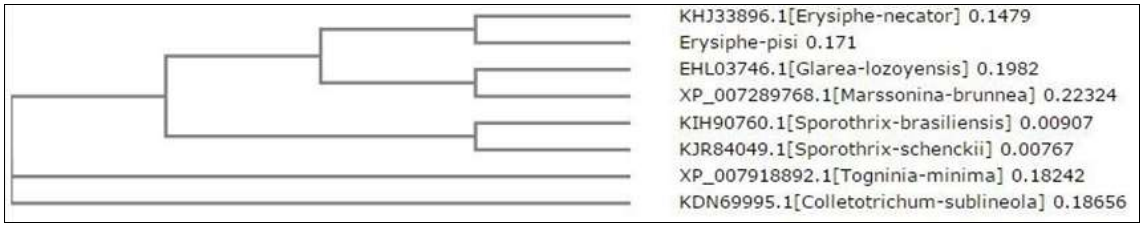


Fig 16: Phylogenetic tree of amino acid sequence of zbip domain of different organisms and strains of ascomycetes

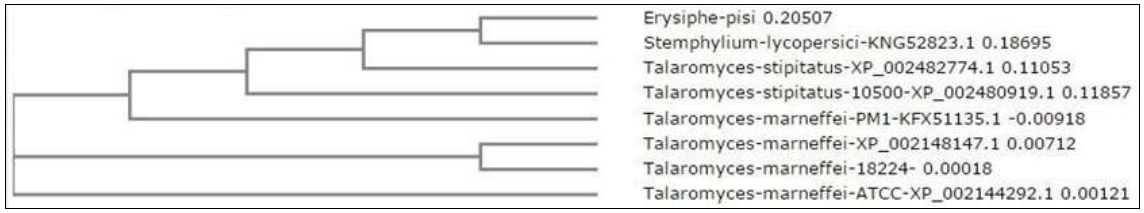


Fig 17: Phylogenetic tree of nucleic acid sequence of HTH_Trip_Tc3_2 domain of different organisms and strains of ascomycetes

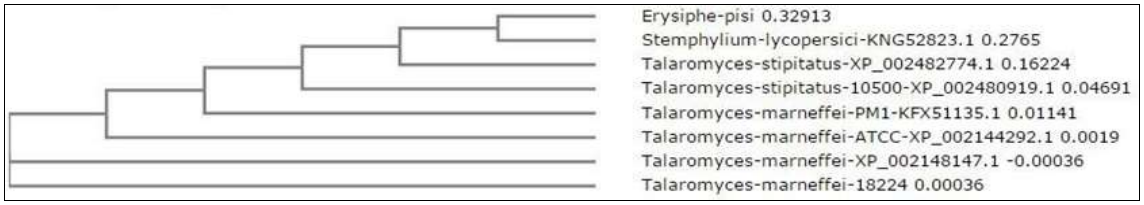


Fig 18: Phylogenetic tree of amino acid sequence of HTH_Trip_Tc3_2 domain of different organisms and strains of ascomycetes

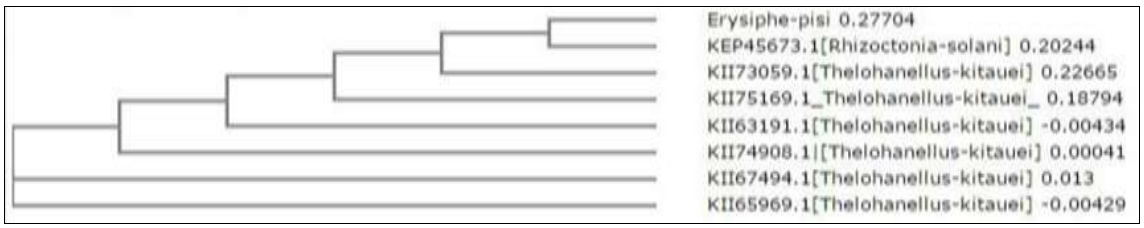


Fig 19: Phylogenetic tree of nucleic acid sequence of RNase H domain of different organisms and strains of ascomycetes

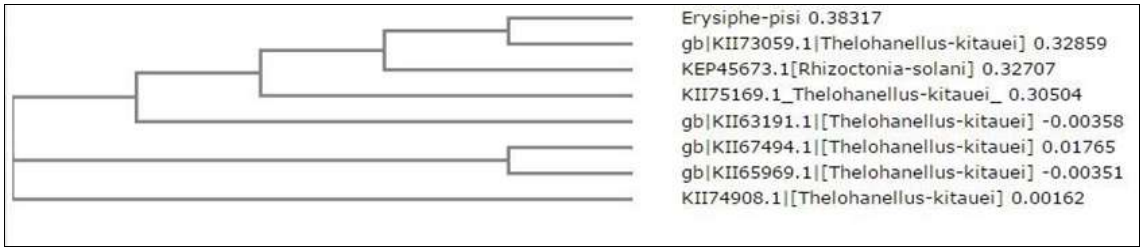


Fig 20: Phylogenetic tree of amino acid sequence of RNase_H domain of different organisms and strains of ascomycetes

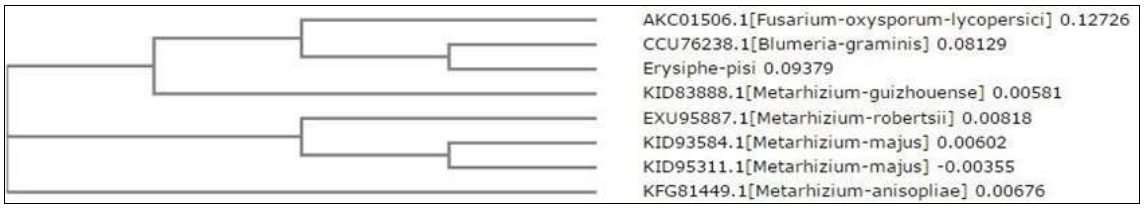


Fig 21: Phylogenetic tree of nucleic acid sequence of RT domain of different organisms and strains of ascomycetes

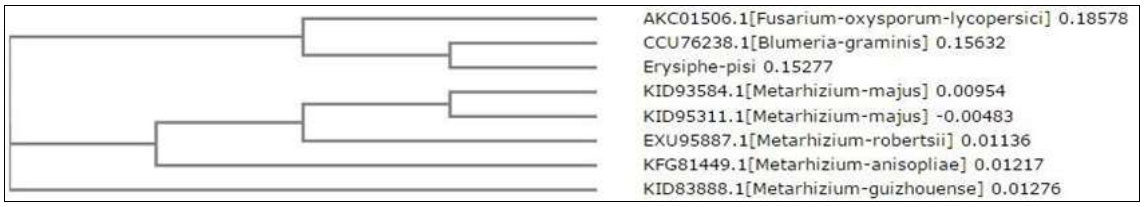


Fig 22: Phylogenetic tree of amino acid sequence of RT domain of different organisms and strains of ascomycetes

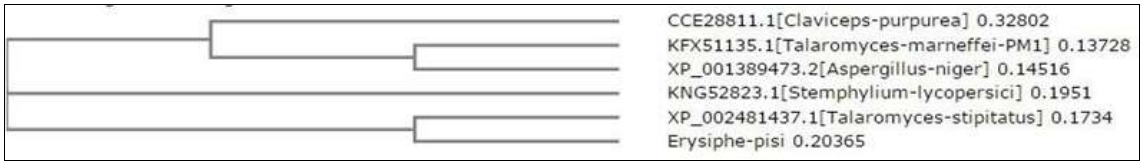


Fig 23: Phylogenetic tree of nucleic acid sequence of rve domain of different organisms and strains of ascomycetes

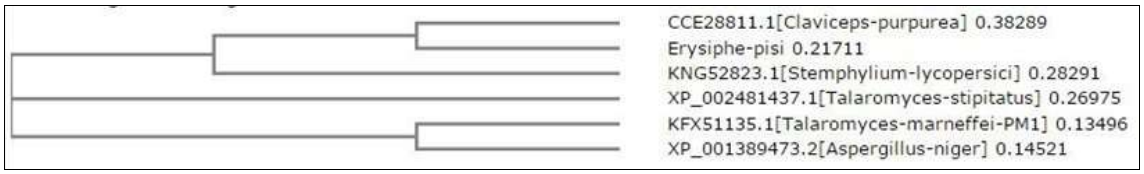


Fig 24: Phylogenetic tree of amino acid sequence of rve domain of different organisms and strains of ascomycetes

Gel images showing amplification of gene products both at DNA and cDNA levels

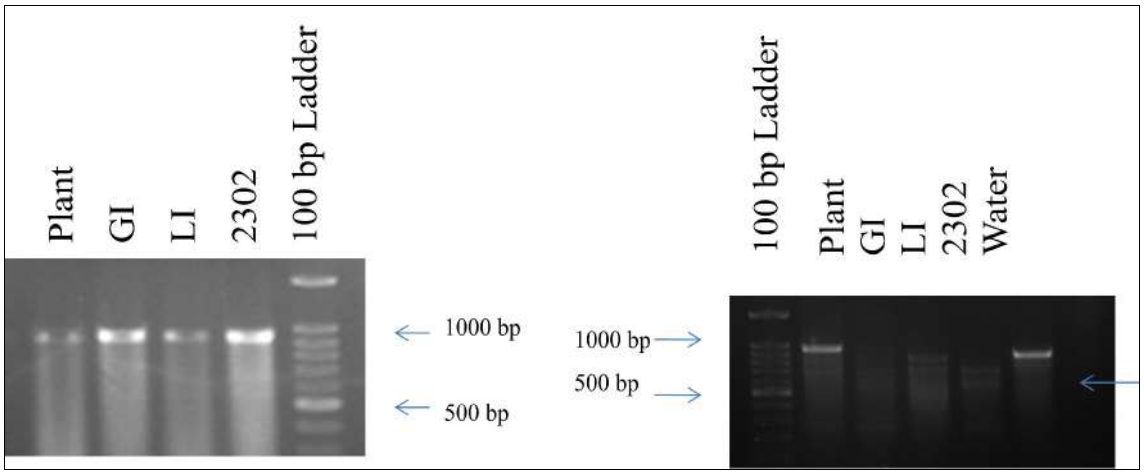


Fig 25: PCR of Monomorphic Primer number 57 at cDNA (a) and Genome (b) level

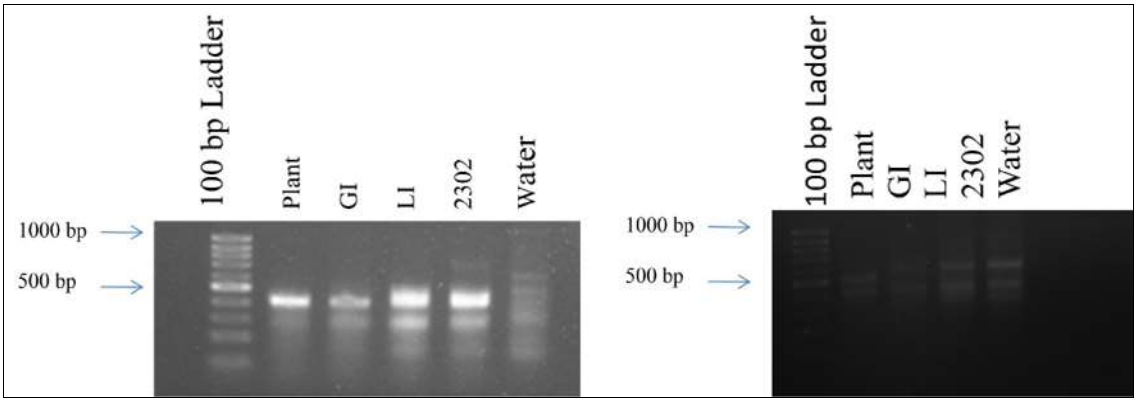
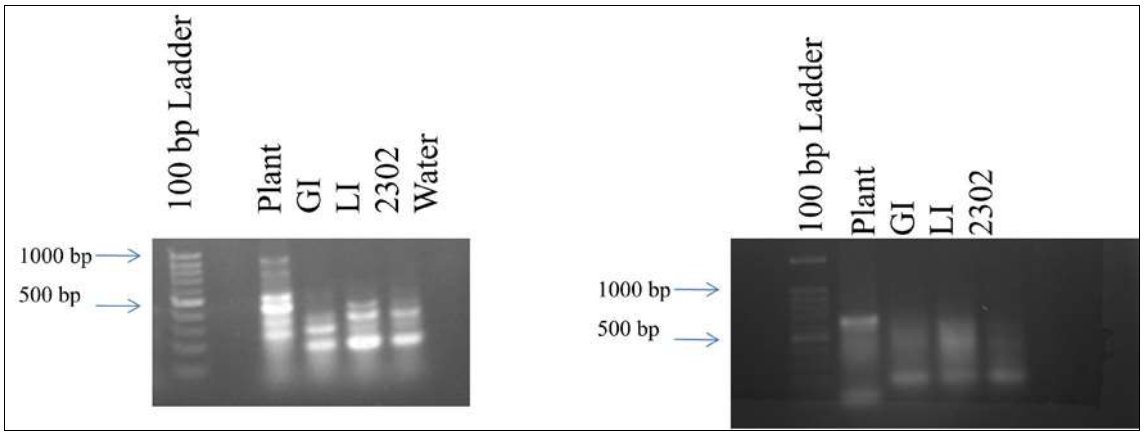


Fig 26: PCR of Monomorphic Primer number 64 at cDNA (a) and Genome (b) level



The genomic PCRs carried out using DNA isolated from three different isolates to check and validate the results obtained through in-silico based prediction confirmed that there is significant level of correlation among the in-silico based prediction and the findings obtained from studies in planta. Further the reverse transcriptase polymerase chain reaction (RT-PCR) based amplification of the gene loci in *Erysiphe pisi* infected pea showed the expression of the genes suggesting that the genes which are predicted through in-silico based studies are not only present but also expressing in plants.

Summary

The study has been undertaken to analyze a set of polymorphic and monomorphic DNA markers identified through experimentation on isolates of *Erysiphe pisi* pathogen. These putative DNA markers were used for a genome wide- search to detect the associated pathogen-specific gene sequences from the available draft sequence of *Erysiphe pisi* genome in public domain. The contigs in which these markers were showing homologous pairing were obtained and translated into aminoacid sequences. The sequences were analyzed for the presence of the open reading frames (ORFs) as they code for putative genes and also the domains in the sequences. The search was carried both at nucleotide and amino acid level.

A significant level of correlation among the sequences with high level of similarity at nucleotide level and high level of identity at amino acid level was observed with already detected virulence genes based on multiple sequence alignment using clustalW analysis suggesting the possibility of the same function for the identified protein sequences. The predicted gene sequences were confirmed through genomic and RT-PCR testing using DNA as well as total RNA of three isolates of *E. pisi* in infected garden pea plant. Healthy plants served as a control for the experiments.

Our research findings suggest that there is significant correlation between in- silico based testing and in-planta analysis for genes. Several putative domains could be identified involving in avirulence of the pathogen especially in polymorphic markers. In case of monomorphic several transposan related gene sequences were amplified. Since any eukaryotic genome is chiefly constituted (Approximately 80- 90% of the whole genome) with transposans, there is a high level of possibility of getting transposon or its related gene sequences.

We also observed several leucine rich repeat sequences, which suggest that some plant material might have got amplified with this marker. In addition, there is likelihood that similar sequences may also be present for recognition in pathogen with which it could sense and recognize a host plant. In the process, we also observed some putative gene/s involved in mycotoxin synthesis which needs further testing to show substantial evidence of the existence of the gene with proper experimentation.

The study also suggests that for screening of DNA markers it would be best to screen the DNA with the suitable primer through genomic PCRs and then the derived sequence could be utilized to design a set of primers. These primers further could be used to screen a number of isolates simultaneously by converting the DNA marker into a SCAR marker instead of amplifying the cDNA directly with the DNA marker based primers. This will help in overcoming any non-specific amplification which we observed in some instances with RT-PCR based amplification. Further, it could also be due to redundancy of the genes or gene sequences which needs to be ruled out with further experimentation.

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