Expression analysis of fungal disease resistant genes in tomato (*Solanum lycopersicum L.)*

BY

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CERTIFICATE

The Thesis submitted by **Sehrish Bashir** to National institute of Genomics and Biotechnology, National Agricultural Research Center, Islamabad, Pakistan, is accepted in its current form. This thesis fulfills all the requirements for facilitating her with a Degree of Master of Philosophy in **Plant Genomics and Biotechnology.**

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AUTHOR'S DECLARATION

I would like to declare that the data presented in this thesis "**Expression analysis of fungal disease resistant genes in tomato (*Solanum lycopersicum L.*)**" is generated by me from original research work during the scheduled period of study under the supervision of **Dr. Nazia Rehman,** at National Institute of Genomics and Advanced Biotechnology, NARC, Islamabad Pakistan. The Results and materials used in this thesis never presented anywhere else before.

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DEDICATION

I sincerely dedicate this piece of work to my loving and caring parents and cooperative siblings whose continuous prayers and immense support made my path smooth and comfortable to my goals. They are my mentor and I could not ask for more to be proud on them.

Thank you so much to all for being there.

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LIST OF ABBREVIATIONS

Word	Abbreviation
A.solani	*Alternaria solani*
Avr	Avirulence effectors recognition
BCI	Bio Resources Conservation Institute
BLAST	Basic Local Alignment Search Tool
CDD	Conserved Domain search Database
cDNA	Complementary DNA
CDRI	Crop Disease Research Institute
CNL	Coiled-coiled domain
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
CT	Threshold cycles
EB	Early blight
FAO	Food and Agricultural Organization
GOP	Government of Pakistan
GWAS	Genome wide association studies
H/D	Healthy and Diseased
HRI	Horticulture Research Institute
LB	Late blight
LRR	Leucine rich repeat
NBS	Nucleotide binding site
NCBI	National Center for Biotechnology Information
NLR	Nucleotide binding -leucine-rich repeat
NPR1	Non-expressor pathogenesis related protein
PAL	Phenyl ammonium lyase
PCD	Programmed cell death
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar

Pi	*Phytopthora infestans*
qRT-PCR	Quantitative Real time Polymerase Chain Reaction
RB	Rpi-blb1, Rpi-blb2
RCBD	Randomized Complete Block Design
R-gene	Resistance gene
Rpi	Resistance to *Phytopthora infestans*
SA	Salicylic Acid
SAR	System acquired resistance
S-gene	Susceptible gene
TNL	Toll like interleukin intracellular signaling
WGS	Whole genome sequencing

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ABSTRACT

Early and late blight diseases caused by *Alternaria solani* and *Phytopthora infestans* respectively, adversely affects the tomato production collectively responsible for 49-91% yield loss estimated in Pakistan. Resistance against fungal pathogens is conferred by various resistant R-genes mainly NLR in diverse species. Resistance mechanism of NLR genes is effective method to avoid the fungicide applications and culture practices. In present study, we determined six reported blights resistant genes (NPR1, chitinase, PR1, PAL, Rpi and NRC1) and identified the expression pattern of R-genes in five *Solanum lycopersicum* accession (38046, 38037, 38039, ROMA and 19890) and one wild specie *Solanum Chilense* (19906). Structural analysis of these fungal resistant genes was carried out by different Bioinformatics tools. Evolutionary relationship was studied by constructing phylogenetic trees. Expression analysis revealed differential expression pattern of resistant genes in five tomato genotypes and up-regulated in resistant genotype 19890. *S. lycopersicum* NLR genes containing NBS-LRR domain protein randomly distributed on 12 chromosomes. Conserved motif pattern for NLR genes clustered in one clade and further classified in CNL, NL, N and TN groups within genome. Phylogenetic analysis of resistance genes in tomato with other species showed close relatedness with other members of *Solanaceae* family. Current findings conclude the screened resistant genotype has expressed strong elevated response against fungal pathogens which proposes the possible involvement of R-genes in defense mechanism and their signaling pathway. It will facilitate the functional validation of resistant genes and developing modern resistant cultivars through gene pyramiding or speed breeding.

Keywords: Tomato (*Solanum lycopersicum*), *Alternaria solani*, *Phytophthora infestans*, fungal resistance genes, phylogenetic relationship, Expression pattern analysis.

INTRODUCTION

1.1 Tomato: Biological description

Tomato (*Solanum lycopersicum)* belongs to *Solanacea* family contains 12 chromosomes (2n=2x=24) with 950Mb genome size. Tomato is a dicotyledonous plant which shows di or tetraploidy number (Ali *et al*., 2013). It is one of the most popular home gardening edible fruit and second most consumable vegetable after potato (*Solanum tuberosum*) and marked economically as the 4th horticulture crop (Noonari *et al.,* 2015*).* In terms of area, tomato has been placed next to potato but it holds first as a processing crop that being grown throughout the year, globally (Mehdizadeh *et al.,* 2013). Recent advances have facilitated the whole genome sequencing (WGS) of "Heinz 1706" tomato cultivar by Tomato genome consortium which can further provide insight to GWAS. It provides functional analysis for morphological diversity and agronomic trait linked genes characterization (Feng *et al*, 2020). Tomato has been considered a well characterized species for genetic studies second to model plant *Arabidopsis thaliana*, than other *Solanum* family members including potato (*Solanum tuberosum*), tobacco (*Nicotiana attenuata*), *Capsicum annum* and Bringil (*Solanum melogena*). It is been known as industrially important cash fruit vegetable, in many countries especially in Pakistan due to its extravagant materials (Li *et al*., 2018). Tomato is a seasonal plant but now available round the year as efforts had been made to get it for various beneficial products. Genetic studies of qualitative and quantitative traits are made applicable through breeding which offer highest productivity, fruit quality improvement and resistance to biotic and abiotic stresses (Costa *et al.,*).

1.2 Center of origin

Tomato's center of origin is South America particularly Peru. It is known as center of diversity for wild tomato species and Mexico is known for cultivated species (Alajrami *et al.,* 2020). Most dominant features of tomato which famed it world's no.1 processed fruit are relatively short growth period give fruits in about 65–85 days with high yield fruit per plant (Ejaz *et al.,* 2011).

1.3 Morphological characteristics, growth pattern and consumption

Phenotypic characteristics of the tomato plant are sympodial shoot, compound leaves and plump fruit. Diversity among leaf shape and fruits of different tomato varieties can be exhibited from cherry size to full ripen round tomatoes (*Solanum pimpenifollium*) or elongated fruit with broad or narrow leaves to fully spread shaped potato leaves (Kimura *et al.,* 2008). Depending upon growth pattern tomato matures in two ways, determinate and indeterminate habit. These patterns involve maximum plant height and fruit production simultaneously and complete growth and development followed by fruit ripening throughout the season, respectively (Alajrami *et al*., 2020).

As a fresh vegetable, tomato is being widely used in salad, as food item its routinely consumption and complementary ingredient in Asian kitchen. It is being co-cooked with other vegetables and meat which made it mandatory throughout the year (Adenuga *et al.,* 2013). Highly inelastic demand for tomato has made it attractive for the producers. Though, it is being widely cultivated in 140 countries of the world as a cash crop compared with cereal crops, which has the ability for generating opportunities for serving rural areas especially in Asian countries (Gondal *et al.,* 2012). Dried and canned tomatoes as processed products have set better economy with multi taste outcomes like sauces, soups, ketchup, purees, pulp, juices, etc. (Adenuga *et al.,* 2013).

1.4 Nutritional composition

Tomato comprised of many nutrients present naturally in abundance with nutritional composition providing vitamin A, C and E, calcium (Ca), potassium (K), iron (Fe), minerals and phenolic compounds. It is famous for deliberated source of carotenoids especially β- carotene and antioxidant Lycopene. These are involved in human health regulation by playing significant role in our diet to avoid the vascular diseases and various forms of tumor growth (Tahir *et al.,* 2012). Tomatoes are 95% water, 4% carbohydrates, and contain less than 1% of proteins and fats (World Atlas 2019). Particularly, tomato fruit color is the accumulation of carotenoid that differs from green (chlorophyll), yellow to red orange. Fruit color variation is observed during growth stages

at the start of ripening, maturation and fully ripen stage which determines the quantity of these compounds from more to less (Kimura *et al.,* 2008).

1.5 Tomato diverse species

There are 13 wild species known up to date which interestingly show the diversity for desired traits. *Solanum chilense* and *pimpenifollium* are wild species that can promote other tomato cultivars by introducing desirable traits. Variety of tomato species for resistance against diseases or environmental stresses and traits like enhanced yield, growth and quality are being improved in *Solanum lycopersicum* (Quinet *et al.,* 2019). Being a subtropical plant, 16 - 29°C is the required growth temperature at day with 11 °C during night. Fruit setting occurs at 19-24 °C and its fruit color changes at 20- 29°C (Adams *et al.,* 2001).

1.6 Tomato Production and consumption rate in Pakistan and globally

According to Food and Agricultural Organization (FAO), tomato has been grown over 16% of vegetable area globally, a reason for its ranking 6th out of 15 fruit vegetables in annual world production (FAOSTAT 2018). In 2017, 170.8 million tons tomato production was computed worldwide (World Altas 2019). Though in Pakistan, tomato production was decreased in past 5 years (2014-2019), by 0.30% average, yearly. It was calculated from 268,900 to 599,588 tons in 2001 to 2014. In 2019, production was 561,293 tons lesser than in 2014 (Knoema, World Data Atlas 2019). However, rise in population has increased the demand for tomato and its consumption rate due to its high nutritional value in comparison to other vegetables at low price (Akbar *et al.,* 2018).

Area wise tomato production was also decreasing by 0.75% on average in past 5 years from 62,930 ha in 2014 to 55,258 ha area calculated in 2019 (World Data Atlas 2019). Production rate and area have determined that china is a top most country followed by U.S as major producer in the field of fruit vegetables. In case of tomato India has replaced it with great production which inclined to export a huge amount of tomatoes, round the globe (Qasim *et al.,* 2018). Pakistan has been ranked 33rd based on area

availability and usage after growing in both during and off season to maintain national food security and making economical contribution (Wahid *et al.,* 2017).

Out of worldwide tomato production about 23% of tomatoes are processed into various products for daily uses and remaining is being consumed as fresh as harvested (Hlihor *et al.,* 2019). Tomato yield with quality varies in countries or regions like Asia is accountable for bigger share than Europe, America and Africa (FAOSTAT 2019).

Tomato consumption is concentrated in China, India, North Africa, the Middle East, the US, and Brazil and these countries accountable for 74% of world's annual production (Quinet *et al*., 2019). Although, consumption rate in Pakistan was 668 tons in 2018 which has placed it at 128th position in the world (FAOSTAT 2019). It is valuable that Pakistan has been growing over three time higher rate for tomato consumption than international and making only 1/4th of the world average consumption (Khokhar *et al.,* 2013). Pakistan had shared export only 0.1% and import value of tomato is 502286 thousand rupees and the quantity is 35860265 kg (GoP 2015).

1.7 Limiting factors affecting tomato production

Tomato production hinders by most of the ins and outs of limiting factors which are accountable worldwide. Low knowledge about cultivation, pre-treatment and during progress care from biotic and abiotic stresses, pesticide applications, climate issues, farmer's negligence, post-harvest losses and overtime market destination are major constraints (Li *et al*., 2018). Abiotic stresses including extreme temperature, drought and high salinity affect almost every stage of tomato life cycle (Krishna *et al*., 2019). Moreover, fungicide or pesticides spray against disease attack are unfortunately become necessary during development period. It is being executed to avoid numerous diseases which can pose serious health problems in consumers in result of any reaction. Tomato has been counted in top 10 contaminated fruit list instead of high production rate and growth (Hlihor *et al*., 2019).

Apart from stresses, even better quality tomato production need optimization so that they would be available in the dry season for national market and take advantages by share

exchange (Karuku *et al.,* 2017). Depending upon developmental stages, temperature may vary that show negative linear relation with fruit number and use of fertilizers (Asci, *et al.,* 2014). Whereas, socioeconomic and institutional factors are the consequential of reduction in the quantity, quality and market value of agricultural commodities (Emana *et al.,* 2017).

1.8 Tomato yield loss

Tomato yield loss by sway of various biotic elements involves bacteria, fungus, virus and other deadly elements is becoming a challenging approach for the farmers (small landholders), producers and consumers which deserves an elucidation (Krishna *et al.,* 2019). Conventional methods have not been proven effective against disease control because there are no such great cultivars produced through breeding. Horizontal gene transfer or Marker assisted selection are still in the process for making complete resistant cultivars (Bitew 2019). Pathogens become more virulent with constant change of genetic material and excessive use of biocides is a threat to national food security so on broader level world food availability. This serious issue urges for functional genomics to overcome the yield problem as alternative method to confer genetic resistance (Maeda *et al.,* 2020).

Climatic condition may favor the pathogenic attack by temperature, humidity, Salt and air exchange. If these conditions are not controlled by any preventive measures it may spread into epidemic. Variable conditions provide ideal environment for foliar, stem and soil borne plant diseases which exhibit in either root or aerial parts and cause deterioration (Saleem *et al.,* 2011). Casual pathogens must be controlled by making strategies of Integrated Pest Management by chemical, physical and biological parameters or approach therapeutically (Khokhar & HRI, 2013). During cultivation or post-harvest storage, tomato crop is susceptible to more than 200 diseases caused by an array of pathogenic fungi, nematodes, bacteria, and viruses (Sing *et al.,* 2017).

1.9 Major tomato diseases

Fungal diseases include late and early blight, powdery mildew, Grey mold, *Alternaria* stem canker, Anthracnose, Septorial leaf spot, Grey leaf spot, *Phytopthora* root rot, leaf

mold, *Vetriculum* wilt, *Fusarium* crown and root rot, *Fusarium* wilt, white old, corky root rot, *Pythium* damping off and *rhizoctonia* damping off. Bacterial diseases involve bacterial speck, bacterial spot, bacterial canker, bacterial wilt, tomato pith necrosis, tomato big bud and Bacterial stem rot. Viral attacks are known as tomato mosaic virus and tomato spotted wilt virus. All of these are concerning issue in the way of tomato crop management otherwise devastation (Tsitsigiannis *et al*. 2008).

1.10 Fungal blight diseases

Tomato is susceptible to biological and non-biological factors (Akhtar *et al.,* 2012). *Alternaria solani* and *Phytopthora infestans* are the causing agent for early blight (EB) and late blight (LB) which in prevalence accounted for 49 to 91% yield losses in Pakistan (Saleem *et al.,* 2011). LB can infect and devastate tomato plants at any developmental stages including stem and fruit lesions while EB infection is usually associated with plant physiological maturity and fruit load (Foolad *et al.,* 2008). Fresh plants are at lower risk for *A.solani* infection than older plants with fruit load that exhibit greater susceptibility (Akhtar *et al.,* 2019). Cold or low temperature with high humidity (Nov-Jan), low humidity with increased temperature (Mar-Jun) and rainy conditions provokes LB incidence. Even foliage and fruit infection also contribute to tomato yield losses (Saleem *et al.,* 2016).

1.11 Late blight

Late blight is notorious for ‘’Irish famine’’ resulted in more than 20% decree of population due to hunger and potato starvation (Foolad *et al.,* 2008). Tomato varieties usually perform poorly in the field with favorable conditions for diseases incidence. No resistant cultivar showed prominent results till, which shows huge success for the prevention of fungal diseases (Kamoun *et al.,* 2005). Late blight pathogen dwells the soil and distributes through moving spores (Vianna *et al.,* 2017).

Phytopthora infestans not a true fungus rather a fungus-like organism which is classified as an Oomycete, member of the Chromista kingdom (Nelson, 2008). It is a specialized pathogen that causes disease on various tissues of potato and tomato crops. It is

recognized by the presence of greyish green spots on the infected leaves mainly on lower side that may evolve into irregular necrotic brown lesions also on stems and fruits. Ultimately it causes leaf loss and then plant death (Akhtar *et al.,* 2016). Late blight pathogen has a wider host range including *Solanum. L. esculentum*, *S. tuberosum, S. sarrachoides, S. triflorum, S. dulcamara, S. sisymbriifolium, Nicotiana benthamiana* and plants from the genus *Calibrachoa* (Akhtar *et al.,* 2012). *P. infestans* adopts two-step infection cycle initially a hemibiotroph where the pathogen requires living host cells called asymptomatic phase. It is followed by necrotrophic phase of host tissue degradation by suppressing the plant immunity causing programmed cell death (PCD) (Fan *et al.,* 2021).

Chronic use of chemicals for late blight control management has reduced the profit margins to farmers. It is not a successful way to eradicate the disease and reduce the worldwide economic loss which is reported more than one trillion US dollars, annually. Despite of the fact, if crop left unprotected then whole farm can be destroyed in 7-10 days (Majeed *et al.,* 2017).

1.12 Early blight

Early blight or target spot disease caused by mitosporic fungus *Alternaria solani*, a destructive disease of aging plant tissues appeared with stem blight, leaf blight and fruit rot or lesions resulted in damaging symptoms during all stages of plant development (Pandey *et al.,* 2003). The yield loss of tomato fruit was 79% at a disease intensity of 72%. It has reportedly reduced the tomato yield by 1.36% and fungicide control expenditure is counted $32 million in tomato (EL-Tanany *et al.,* 2018). Symptoms mainly involve regular spots presenting “bull eye” which enlarge in diameter make concentric rings or yellowish crown. Lesions are on the lower older leaves that may dwell into seedling, stem, blossom blight and fruit drop (Raza *et al.*, 2016). Management of early blight with chemicals or fungicidal sprays is not effective and feasible. Growing resistant varieties are the only operative way and eco-friendly because *Alternaria* species always damage fruits (Haggag and Saber 2007).

Fungicides limitation and their effects urge the need for identification of high yielding varieties through breeding program. It can be done by making hybrid using resistant cultivars if available for blight infection. A genetic level solution is due to health and safety concerns that may cause mutations by reprogramming normal genes or permanently silencing them, last for several generations (Chaerani *et al.,* 2006). Though, resistant varieties are either not available or not durable besides of the fact, that process is costly to develop, requires huge investment and technical knowledge (Ravikumar, *et al.,* 2013). Breeding for early blight resistance has been practiced but there is a lack of strong incidence in making cultivated lines by the quantitative expression and polygenic inheritance of resistance against *Alternaria.* Some accessions of wild species, may show high levels of early blight resistance, but no favorable results (Chaerani *et al.,* 2007). Recently, the first linkage map has been developed on the basis of interspecific crosses, with loci controlling EB resistance (Upadhyay *et al.,* 2016).

1.13 Genetic basis of disease resistance

Resistant (R) genes are widely present in plants against pathogenic diseases but variation in pathogen strains and their reaction to those R-genes make them ineffective which caused hurdle and demand for updated method of protection. Defense mechanisms like pyramiding of genes (NLR genes which are being identified till date and working against LB), QTL mapping, marker assisted selection, cultivars with desired trait, CRISPR and transgenic lines are involved in researches to protect plants from pathogens (Wang *et al.,* 2019).

1.14 R-genes against biotic factors

Genes encoding the resistant proteins (R-proteins) are classified in 5 groups containing NB-LRR, receptor-like trans-membrane proteins, serine–threonine kinases (STKc), receptor-like kinases (RLKs) and atypical R genes (Ma *et al,* 2021). Major class is NLR that encodes nucleotide-binding (NB) site along with leucine-rich repeats (LRR) domain (Bakker *et al.,* 2011). The predominant function of these genes with NB-ARC, LRR and NB-LRR domain, is in disease resistance (Guo *et al.,* 2011). Structural feature of N-terminus has subdivided the NLR family in TNL Toll/interleukin 1 receptor (TIR)-NBS-

LRR based on resemblance with *Drosophila* Toll and *mammalian* IL-1 receptors, intracellular signaling domains while and CNL (coiled-coil) a putative coiled-coil domain, respectively (Lozano *et al.,* 2012).

1.15 Late blight resistance

Several varieties of *Solanum* species have been screened for resistance to late blight, but have not reported yet at the farmer's level (Fontem 2003). It has been stated earlier that genetic control of the resistance was conferred by single dominant allele means vertical gene transfer with the hybrid breeding (Labate *et al.,* 2007). In tomato, few R-genes and immunity specified genes are reported, yet understudy. Some quantitative, race specific and broad spectrum LB-resistance genes have been identified as *Ph-1* and *Ph-3* on chr-7 and 9 respectively, in previous studies. These resistant genes were introduced in potato as a single dominant allele and *Ph-2*, a partially dominant allele on chr-10 (Irzhansky and Cohen 2006). *Ph-3* was effective against specific *Pi* isolate (*Pi*-16 from Taiwan) which had overcome the earlier reported genes *Ph-1* and *Ph-2* combination (Chunwongse *et al.,* 2002).

An advance breeding line conferring strong resistance, containing *Ph-5* gene on chr-10 has been recognized through selective genotyping after crossing the resistant line with susceptible one. Molecular markers are being experimented on F2 and F3 populations to isolate the trait linked resistance in different varieties with desired genes (Foolad *et al.,* 2008). Eleven different Resistance (R) genes have been identified in *Solanum demissum* and several have been introduced into modern potato varieties against late blight (Visker *et al.,* 2003). Late blight disease has an adverse effect on tomato and potato production equally. So far as more than 20 (Resistant to *phytopthora infestans*) R*pi*-proteins have been identified which are applicable in breeding program for making cultivars. These proteins are named after wild species and categorized based on their respective NBS-LRR domains (Yang *et al.,* 2017). NBS-LRR or NLR genes are located inside the plant cell for *P. infestans* avirulence effectors (Avr) recognition (Sharma *et al.,* 2019). *Solanum demissum* and *pimpenifollium* show resistance toward late blight with genes present like Rpi-vnt1 from *S. ventturii* (Yang *et al*., 2017).

Recently, R- genes have been identified for cloning against *Pi* in sexually incompatible specie named *S. bulbocastanum* and *S. stoloniferum*. Pyramiding or stacking of these broad-spectrum genes into a cultivar can be effective including *Rpi-blb1* (RB), *Rpi-blb*2, *Rpi-blb*3 and *Rpi-bt1* (Rakosy-Tican *et al.,* 2020). Resistance is based on pathogen recognition and initiation of defense response has provided a dominance in plants but at some stages changes required (Ballvora *et al.,* 2002). Susceptible gene-based resistance is another way to avoid the pathogen attack but it is recessive, where S-gene product is assumed to be impaired which is being misused by pathogen. It was reported earlier that silencing of five out of six S-genes has shown complete resistance and remaining one had provided reduced susceptibility against *Pi* isolate Pic99189 in potato cultivars (Zhu *et, al.* 2012). S-genes of tomato and potato are considered to be orthologoues of *A. thaliana* susceptible genes (Sun *et al*., 2016). Resistance protein analogue having LRR domain called helper NLR is also functionally required to main R and NLR genes (Wu, *et al*., 2017).

1.16 Early blight resistance

EB resistance is a quantitative trait where only wild type tomato accessions like *S. arcanum, S. peruvianum*, *S. neorickii* and *S. chilense* have shown competency against *Alternaria* species till date. Though, *S. habrochaites* was found to have both, susceptibility and resistance for EB. Even, hybrid lines or cultivars resulting from crosses of these wild species do not possess satisfying crop qualities which hindered *A.solani* attack (Upadhyay *et al.,* 2016). Pathogenesis-related (PR) proteins in EB resistance and their use as biochemical markers for genotype selection with involvement of transcription factors are important as their elevated response against *A. alternata* was observed, effectively (Moghaddam, *et al,* 2019).

Overexpression of pathogenesis related genes is classified in 17 major families based on their biochemical and biological properties (chitinase, glucanase, thaumatin, defensin and thionin). They may individually or in combination exhibit significant level of defense response in plants against a wide range of pathogens through SAR mechanism and initiation (Sajad *et al.,* 2018). Hydrolytic enzymes are accumulated after pathogen

induction which produces salicylic acid responsible for pathogenesis related (PR) proteins expression (Salim *et al.,* 2011).

NPR1-like protein family, another R-gene family which interacts with defense related proteins and provide shield against disease attacks. It is also being considered as SA receptors provide new insight for *Arabidopsis originated NPR1 gene against biological and A-stresses in plants (*Backer *et al.,* 2019*).* Modifications of the SA pathway (receptor against fungal infection) has made it to be reviewed by CRISPR–Cas9, just to increase the defensive response through mutations (Sharma *et al*., 2019).

Expression analysis is an efficient approach to understand the pattern of genes transcription during any developmental stage and also to study the plant responses under biotic and abiotic stresses. The aim of current study is to identify the expression behavior of genes related to blight resistance in wild and cultivated tomato genotypes under pathogenic attack. Moreover, to structurally analyze the genes conferring resistance against early and late blights.

Objectives of study

The main objectives of the study are as under;

1. Structural analysis of fungal resistant genes in tomato
2. To determine the phylogenetic relationship of fungal resistant genes in tomato with *Arabidopsis thaliana*, a model plant
3. To identify the expression pattern of fungal resistant genes in tomato.

MATERIAL AND METHODS

2.1 Identification of fungal resistance genes for early and late blights and sequence retrieval

To identify the fungal resistance genes against blights in tomato (*Solanum lycopersicum*), *EnsemblPlants* database was used. Late blight *Rpi* and NRC1 belonging to NLR genes in potato retrieved from the database also chitinase and PAL gene sequences for tomato blight diseases. Other NLR genes included Pikp-2 from Rice, ZAR1, RPP13, RPP11, RPS2 and R-genes like PR1 and NPR1 showing broad spectrum resistance against pathogens were taken from Arabidopsis. These genes sequences were used as query to find out their orthologous through Basic local alignment search tool (BLASTP) in tomato (*Solanum lycopersicum*). Protein, genomic, CDS, and promoter sequences were retrieved from Ensembl database after blastP. Threshold values were subjected for identity that ranged from 100-70%, E-value adjusted to be not less than E-05 and overlapping genes duplicates for NLR and R- genes were removed also. 61 sequences were finalized on the basis of selected domain in tomato resistant genes (Cheng *et al.,* 2021; Li *et al.,* 2018; Wei *et al.,* 2021).

2.2 Analysis of conserved domains and motifs

SMART tool was used to identify the conserved domain in the orthologous. Conserved domains were further validated by NCBI batch CD search database. MEME software (Multiple Em for Motif Elicitation) was used to identify the motifs for conserved domains protein sequences. Maximum 20 number of motif with width limit between 6 and 50 were set as parameters to analyze the conserved amino acid regions in all the sequences (Yang *et al.,* 2021).

2.3 Gene structure and chromosomal localization

Gene structure display server (GSDS) tool was used to line up the 61 resistance genes for structural diversity. CDS and genomic sequences were given as input to visualize the gene features involved introns, exons/CDS and upstream regions in output. To further

synchronize them with their chromosomal location Tb tool was used which represented the 61 gene Ids on the 12 tomato (*S. lycopersicum*) chromosomes (Wei *et al.,* 2021).

2.4 Phylogenetic analysis of fungal resistance genes

To determine the evolutionary relationship of selected R and NLR genes of tomato and with other five species (potato, capsicum Arabidopsis, , their protein sequences were subjected for the alignment in to MEGAX (Molecular Evolutionary Genetics Analysis, V.10). By default setting, protein alignment was further supported through pairwise and multiple sequence alignment (MSA) by CLUSTALW. Pairwise deletion strategy was made practical for the removal of unwanted not aligned sequences. Neighborhood-Joining algorithm was applied with 1000 bootstrap replications. Default parameters were set for phylogeny study after alignment and (Cheng *et al.,* 2021; Yang *et al.,* 2021).

2.4.1 Phylogenetic tree visualization

Phylogenetic tree was edited by using an online tool TB tool (V.1.0971) (An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data), Bio sequence Structure Illustrator program (Chen *et al.,* 2018). Finalized R-gene sequences in tomato based on the conserved domain were represented with tree displayed with conserved motifs, their gene structure and a legend aside for both and arranged all together.

2.5 Cis-acting regulatory elements study in the promoter of resistant genes

PlantCARE database was used for promoter analysis of the fungal resistant genes to examine the cis acting regulatory elements. Promoter sequences for selected genes were present in the transcription start site of genomic sequences (present on either forward or reverse strand). Targeted 1.5kb upstream regions (5'UTR and 3'UTR) were downloaded from *Ensembl plants* database (Li *et al.,* 2019). Resultant file from PlantCare was extracted in WinRAR setup to find out the number of Cis-regulatory elements present in particularly each sequence's promoter region. These numbers were summed up for each sequence and Heatmap visualized for these elements in TBtool (Zhang *et al.,* 2020).

2.6 Protein-protein interaction

String, a bioinformatics tool for functional protein association network was used to understand the protein-protein interaction of the selected genes. Interaction of these proteins could be either possible with related protein or not on the basis of function or the structure, went for expression pattern.

2.7 Primer designing of fungal resistance genes

Primers were designed by using Primer3 software validated by primer stat. UCS PCR at UCSC-Insilco PCR genome browser was used for primer binding specificity. It was important for expression analysis of fungal resistance genes mentioned before in bioinformatics analysis. There were 6 genes selected on the basis of functionality reported effectiveness against biotic factors named NRC1, Rpi-protein for late blight. Chitinase, PAL, NPR1 and PR1 were shoeing broad spectrum against early blight understudying fungal diseases. Apart from target genes, an elongation factor EF1 was designed as endogenous control with which comparison would be made.

Table 2.1: Fungal resistant genes (early and late blight) expression primers list

Sr.No	**Expression Primers**	**Primer's (F/R) sequences**
1.	Rpi-protein-F	5'-CATCGACATGGAGAAGACAT-3'
	Rpi-protein-R	3'-CACGACTTGGTTCAGCATAG-5'
2.	NRC1-F	5'-CGCTACTTCTGAGGTTC-3'
	NRC1-R	3'-CCTGTTGCTGTTACGGATGT-3'
3.	PR1-F	5'-AGTTGGAGTCGGTCCTAT-3'
	PR1-R	3'-CCCACATCTTCACAGCAC-5'
4.	NPR1-F	5'-AATCGGAAACTTCACTGGCAG-3'
	NPR1-R	3'-CTTACCGCCTGGAGCCAGAAGC-5'

5.	Chitinase-F	5'-GACCCATCCCACATTCAA-3'
	Chitinase-R	3'-GCCATCCCCTAAAGATACAG-5'
6.	PAL-F	5'-TCAACAGCTCAATCTTCCAA-3'
	PAL-R	3'-TTCAAGGACAACTCTTGCAG-5'
7.	EF1-F	5'-GGTATTGACAAGCGTGTTAT-3'
	EF1-R	3'-TCAATGGTGATACCACGCTCA-5'

Primer being synthesized then resuspended in RNAase free H_2O to make 100µM stock solution. Stock solution further diluted with Nuclease free water and made a working solution of 10pM concentration for further use.

2.8 Plants and sample preparation

Tomato germplasm including 50 accessions of *S. lycopersicum* and 1 accession of wild *S. chilense* were collected from BCI (Bio Resources Conservation Institute), NARC (National Agriculture Research Center), Islamabad. Seeds were first planted in trays and after 15 days their seedling were shifted in pots. Two weeks growth at there was further transferred in beds made in glasshouse and open field so nursery was raised. Whole experiment was designed in RCBD (Randomized Complete Block Design) with 3 replications in glass house (contained environment) and open environment (field).

2.9 Fungal inoculum preparation and its maintenance

2.9.1 Isolation of *Alternaria solani*

Pathogenic strain of *Alternaria solani* from leaves of infected tomato plant was taken. *A.* Infected leaves were surface sterilized in 1% chlorox and distilled water. Then infected area (lesions) was cut off then placed on potato dextrose agar (PDA) plates, a well-supported media for fungal spores' cultivation. Plates with affected leaf part (disk) were incubated at 25°C ± 1°C with 12h photoperiod until sporulation, maximum for 7 days

(Saleem, *et al.,* 2016).). Identified *Alternaria solani* by morphology of colony such as black brownish color, circular ring formation and through microscope identified the spores. After confirmation culture was maintained at 4°C until for further use.

2.9.2 Isolation of *Phytopthora infestans*

Late blight culture was prepared from the infected leaves showing symptoms of late blight disease. Infected leaf samples were surface sterilized with 1% Clorox and distilled water. Then cut the affected area of the leaves and placed on the PDA media in laminar flow and kept the PDA plates under incubation. Incubation conditions were 12h photoperiod followed by first 24h in dark at temperature 18°C for 2 weeks (Duran, *et al.,* 2020). That morphological pattern of the colonies attained in white color and stellate shape, had confirmed the pathogen, *Phytopthora infestans*. Multiplication was carried out through 5th day colony growth at PDA media and kept for incubation at 18°C for 12-14 days in petri plates and preserved them at 4°C until infection was made ensured.

2.10 Fungal culture revival

Identification of *Alternaria* specie and *phytopthora infestans* was done in CDRI (Crop Disease Research Institute) and also multiplied there. Cultured plates were stored at 4°C initially but fungal growth activity would stop effectively at this temperature so prior to infection revival was necessary. It was done by selecting one sporangial colony from two of the pathogenic cultures. Colonies from the last ring were picked up in sterilized environment (laminar flow) and placed on PDA media then incubated at 22°C for 7 days in case of *A. solani* and 18°C for 12-14 days for *Phytopthora infestans* (*Pi*). Spores were harvested in distilled water for suspension and made them ready for infection. Screening of the tolerant and sensitive lines was then performed through detached leaflet assay (Zhi *et al.,* 2021).

2.11 Screening through mechanical inoculation

An agar plug from fungal cultures through sterile cork borer was picked as inoculum. Tomato leaves of different accessions were injured by using a syringe and kept the agar

plug on damaged site. Samples were tagged and dated on the day of infection in open field and observed them until symptoms appeared (Patel and Subramanian).

2.12 Lab based detached leaflet assay

Lab based detached leaflet assay was performed in sterilized contained environment in CDRI, NARC Islamabad. Spores were harvested from the cultures in 2ml sterilized distilled water to make their suspension up to 10ml and ready for inoculation. Leaves were surface sterilized wit 1% Clorox and distilled water. Detached leaves were kept on 1% water agar in such a way that control samples with water were parallel to fungal inoculum treated leaves in the same petri plate (Jabeen *et al.,* 2015). These plates were kept under incubation for the days determined before for both of the cultures. Following 7-10 days, symptoms were observed depend on inoculum effectiveness as brown concentric rings would be spotted in 10 days in open field and 7 days for lab. Then scoring was done on the basis of percentage area affected by the infection (Zhi *et al.,* 2021).

2.13 RNA Extraction

Sampling of healthy and infected tomato leaves of *S. lycopersicum* accessions (38046, 38037, ROMA, 38039 and 19890) and one *S.chilense* accession 19906 was carried out from tomato field for their RNA extraction. Before sampling leaf tissues were surface sterilized with 70% ethanol then kept in liquid nitrogen (-196°C) container immediately for intact RNA. Invitrogen kit for RNA extraction was used (Shi *et al.,* 2018).

Samples (leaves) from liquid nitrogen kept in properly labeled mortar pestle for grinding in liquid Nitrogen until leaves sample turned into powdered form. Crushed material shifted into 1.5mL labeled Eppendorf tubes already contained 1000µl/ 1mL Lysis buffer and 10µl Mercapto ethanol. Tubes were vortex for 5-7 seconds for even mixing of solution with grinded material. Centrifugation was done at 14000rpm (20,000×g) for 3 min after that supernatant from the tube was collected about 700-800µl and in the same amount 70% ethanol was added in a new Eppendorf tube. These tubes were inverted 4-5

times for thoroughly mixing of the ethanol with collected supernatant and shifted in spin cartridges.

Centrifugation was completed for 30 sec at 12,000rpm and again repeated this step until samples were finished. 700µl wash buffer I was added in spin cartridges and centrifuge for 30seconds at 12,000rpm. 500µl wash buffer II was added then in the new collection tubes and centrifuged at 12,000rpm for 30sec. This step was repeated followed by empty spin for 1-2 min at 12,000rpm. 50µl Elution buffer was added in the center of recovery tubes by discarding the collection tubes. Incubated these tubes for 1min and centrifuged them for 1-2mins at 12,000 rpm. Isolated RNA was stored at -20°C for further utilization.

2.14 Gel electrophoresis

RNA extraction of tomato leaf samples was followed by visualization of results in Gel Electrophoresis. Gel ingredients were agarose, TAE buffer and a dye, ethidium bromide. TAE buffer was prepared by taking 20ml 50X TAE buffer in 980ml of distilled water to make final volume 1000ml/ 1L. Gel was prepared by taking 1X TAE buffer in ml in graduated flask with Agarose in grams and boiled for up to 1 min and kept the flask at room temperature to cool it down. 4µl Ethidium bromide, a dye was added and mixed it thoroughly for some seconds. Poured it into gel tray (with stopper and comb) and kept for 15-20mins until gel got solidified. Prepared gel was placed in gel tank containing 1X TAE buffer while removing stoppers from gel tray. Samples were loaded by picking 3µl of loading dye to mix and dispensed it properly with 4µl of sample (RNA) in wells. 1kb Ladder (Gene Ruler^{TM} 1000bp DNA ladder Thermo Scientific) was loaded in the first well. The gel was run at voltage and current at 100volts and 300Amp, respectively.

2.14.1 Gel documentation system

Gel was visualized in Genesys, manual command giving software by placing gel under UV (Ultra Violet Trans-illuminator). It highlighted the gel images with the bands of RNA present in them.

2.15 RNA Quantification

After visualizing the bands for RNA in the samples, their concentrations were measured by instrument Biospec nano (spectrophotometer for Life sciences) in software Biospec^{TM}. Three readings of each sample were note down in ng/ul concentration with (O.D) in between 1.8-2 and absorbance at 260/280nm and 260/230nm.

2.16 First strand cDNA synthesis

Thermo Scientific Revert Aid reverse transcriptase kit (Wiame, *et al.,* 2000) was used to synthesize first strand cDNA. Adjusted volume of nuclease free water for each sample, variable concentration of RNA in ng for each, was added in PCR labeled tubes followed by 1µl oligodT and incubated for 5min at 65°C in thermo cycler (Applied Biosystem® veriti® 96 wells thermo cycler) to remove the secondary structures from RNA and ice chilled the tubes for 2-3min. 4µl RT buffer, 2µl dNTPs and 1µl of RT enzyme were added in tubes to make up to 20µl reaction volume and incubated the reaction tubes at 42°C for 1 hour. It was followed by 7-10min extension period at 72°C for the inactivation of the reaction.

2.17 Quantitative Real-Time-Polymerase Chain Reactions (qRT-PCR)

Expression pattern of early and late blight resistant genes in tomato leaf tissues was determined by using comparative ΔCT analysis in real-time PCR (Applied Biosystems) with Step-One Plus software. Gene specific primers against fungal blight diseases were used. The kit used for RT-PCR was Taq Man SYBR Green**.** Elongation factor (EF) was used as endogenous control and the target genes were Rpi-protein, NRC1, NPR1, PR1, PAL and chitinase. The total reaction volume was 10µl for single reaction in PCR tube included 5µl SYBR Green, 3.6µl water, 0.2µl primers (forward and reverse) and 1µl cDNA (template) used. The profile of RT-PCR was set as amplification was initiated from denaturation at 94ºC for 10 minutes, second stage followed by 40 cycles at 95ºC for 30 seconds (denaturation), 58ºC for 30 seconds (annealing) and added extension at 72ºC, 30 seconds. All reactions were performed in triplicate with one negative control. Specificity of the reactions was verified by melting curve analysis. Melt curve stage was

step and hold studied at 95 ºC for 15 seconds and then stage 2 at 60 ºC for 1 min. Amplification plot, melt curve plot, and standard curve data were recorded for further analysis. ΔCT and ΔΔCT values were calculated and statistical analysis was performed by standard error and T.Test for significance of the expression data.

RESULTS

3.1 Identification and chromosomal localization of fungal resistant genes

A set of 9 resistant genes with their orthologous in *Solanum lycopersicum* were identified from EnsemblPlants database. Arabidopsis, Potato and Rice sequences were subjected as queries. Among fungal resistant genes eight genes belong to NLR family including *Rpi, NRC1, Pikp-2, RPP1, RPP13, RPS2, Rpi-blb-2, ZAR1* whereas, 9th gene was pathogenesis related protein (PR1). 61orthologs were finalized for further analysis via blastP results. Selected gene sequences were mainly belong to R-gene family which proven to resist the fungal blight diseases in other related species, so as in tomato. R gene family has been classified into sub family NLR, a diverse group. It was reported (Lozano, *et al.,* 2012) that tomato contains 267 NLR genes. These were further categorized into sub-groups CNL, TNL and NB-subfamily as selected genes subfamily classification was given in Table 3.1(a).

Table 3.1: Classification of NLR genes sub family and their conserved domain

NLR sub-family	Gene Ids	Prominent domain
CNL	Solyc04g009250.1 RPP13 Solyc11g006630.1 Pikp-2 Solyc06g048910.1 RPS2	CC-NB-LRR
NL	Solyc05g009740.1 Pikp-2 Solyc11g007790.1 RPS2	NB-LRR
CN	Solyc07g039420.1 Rpi-gene Solyc05g042090.1 Rpi-blb2	CC-NB
N	Solyc07g039440.1 Rpi-gene	NB-ARC

Selective gene orthologs were widely distributed on the 12 chromosomes of tomato as shown in the Fig 3.1 below. It can facilitate the recombination process when genes were represented randomly. Scale pattern was given in Mbps with reference to tomato genome size 950Mb. Chromosomal distribution of genes in tomato which showed chromosome 4

was comprised highest number of the genes and chromosomes 3 and 8 were localized with lowest number of gene on them.

Chr-1, Chr-7 and Chr-11 were represented with 4 genes. Chr-2, Chr-10 and Chr-12 with 2 genes, Chr-5, Chr-6 and Chr-9 were shown with 11, 5 and 6 genes, respectively.

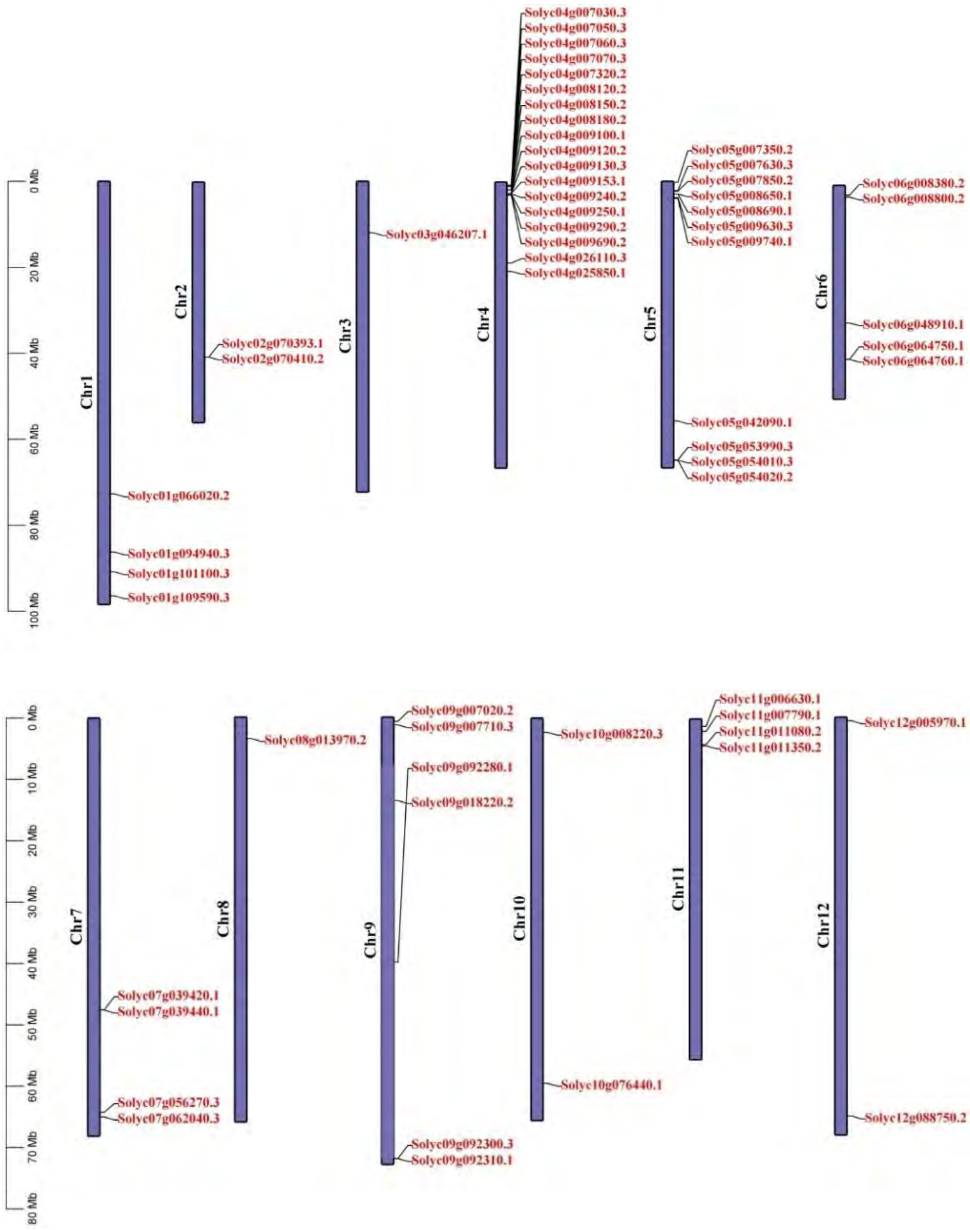

Figure 3.1: Graphical representation of chromosomal localization of NLR genes in *S. lycopersicum*.

3.2 Analysis of conserved domains

All the selected resistant were subjected to SMART tool for domain presence and validated by NCBI batch CDD search database. NB-ARC (Nucleotide-binding adaptor shared by APAF-1, Resistance genes, and CED-4) and LRR (Leucine-rich repeat) was the required domain and prominently conserved in genes.

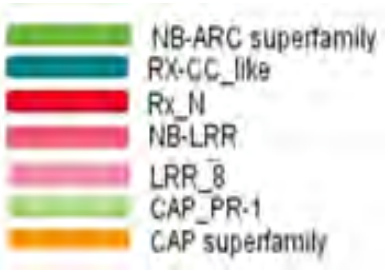

Figure 3.2: Conserved domains (NB-ARC, NB-LRR and LRR) in NLR genes and CAP domain in PR gene family of *S. lycopersicum.*

3.3. Motif recognition, gene structure and phylogenetic analysis

Gene sequences contained 3 selected domains were finalized and then subjected for motif analysis with 20 motifs per sequence. Result described the variable number of motifs present per gene sequences as shown in Table 3. Most of the sequences with NLR/NB motifs contained the specific-colored region of the conserved domain presented in Fig 3.3**C**. Gene structure analysis has determined the presence of exon, introns, 5' UTR and 3' UTR providing the insight to the structural diversity of R-gens in tomato. Presence of introns may have advantages which can increase the protein diversity through alternative splicing. It also helped in regulating the gene expression and involve in some regulatory process. Maximum and minimum number of introns and exons were also noted down through image depiction as followed in Fig 3.3. 50 genes had minimum one exon and 5 genes represented maximum number of exon. Only one gene (Solyc01g094940.3) showed maximum 7 introns, 23 genes were present with one intron and 12 genes with no introns shown in Fig 3.3**B**.

In order to comprehensively evaluate the phylogenetic relationships among the resistant genes in tomato, a phylogenetic tree was constructed. Genes comprising same motifs

pattern were clustered in the same clade. The total 5 clade were generated having 22, 17, 6, 8 and 8 genes, respectively. Conserve motif pattern was illustrated in Table 3.2.

This relationship within tomato NLR genes has shown that Rpi-blb2 gene paralogs share the same clade 1 with Pikp-2 gene and NRC1 genes. Clade 2 was clustered with Pikp-2, RPS2 and Rpi genes in same group, clade 3 with Rpp13 and RPS2 gene orthologs sharing same ancestral relationship. Clade 4 determined the pikp-2 genes sequences and clade 5 represented the PR1 and ZAR1 gene sequences shown in Fig 3.3(**A**). Fig 3.3 presented as evident of resistant genes phylogeny along with motifs and their gene structures.

Table 3.2: List of conserved domain motifs sequences of NLR genes

Conserved domain Motifs	**Gene No.**	**Absent**
Motif 2, 4, 5 and 6	52	Solyc07g062040.3 Solyc07g056270.3 Solyc01g109590.3 Solyc01g094940.3 Solyc12g088750.2
Total 20 motifs present	3	Solyc04g009250.1 Solyc04g009130.3 Solyc04g009240.2
Only 2 motifs (Motif 14 and 18) identified in tomato genes	6	Solyc07g062040.3 Solyc01g101100.3 Solyc07g056270.3 Solyc01g109590.3 Solyc01g094940.3 Solyc12g088750.2

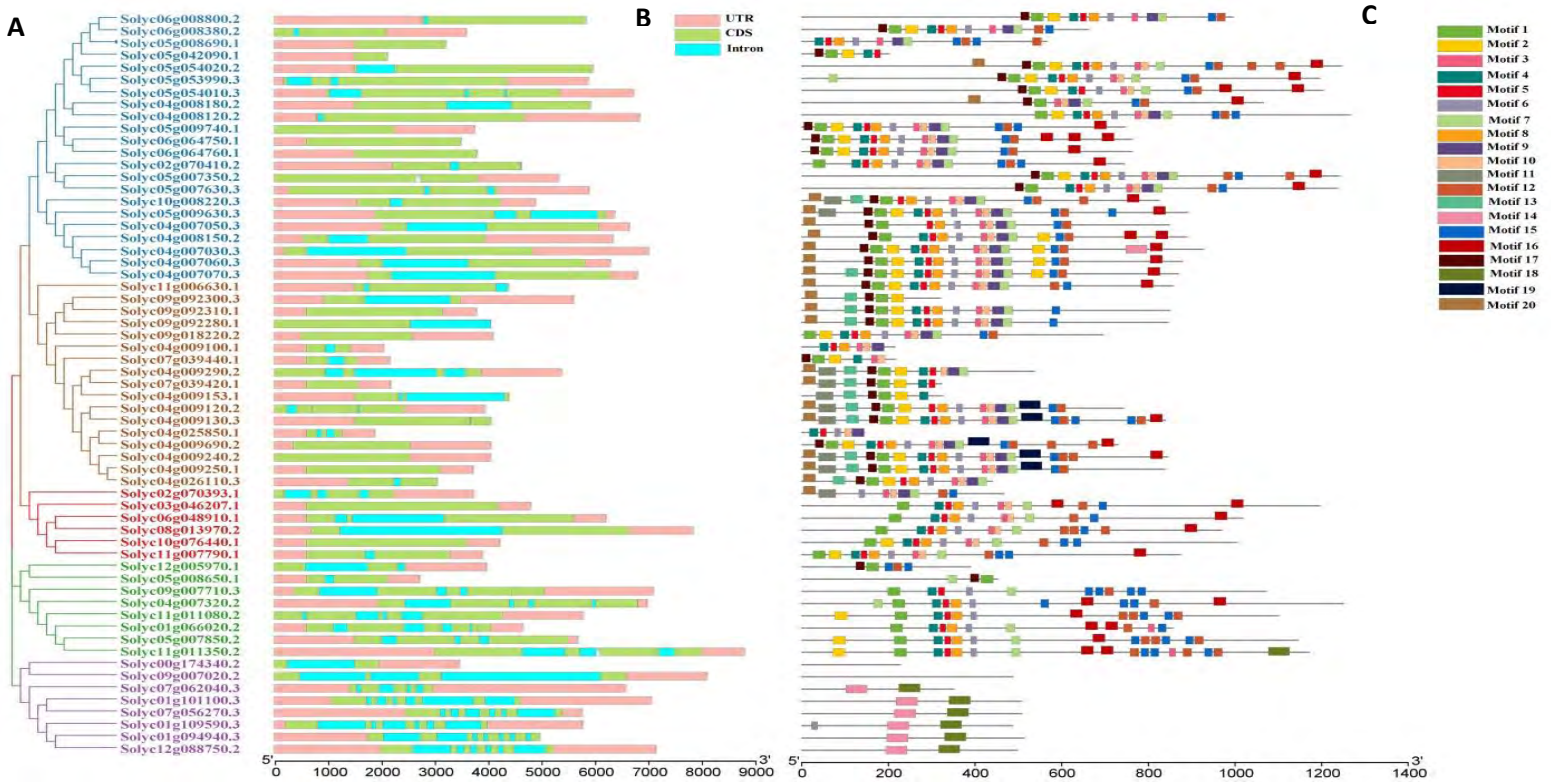

Figure 3.3: Phylogenetic tree, conserved domain motifs and gene structures of NLR genes in *S.lycopersicum*. 3.3.**(A)** The evolutionary history of the resistant genes in *S.lycopersicum* was drawn through Neighborhood-Joining method with 1000 replication Bootstrap value and conducted by using MEGA X. 3.3 (**B)** To the right of the tree, there is schematic representation of the gene structures with introns, exons and 5'UTR and 3'UTRs in sky blue, parrot and pink colors, respectively. 3.3 (**C)** whereas, next to them conserved motifs in resistant proteins are shown.

3.4.1 Phylogenetic relationship of tomato early blight resistant genes with other species

Evolutionary relationship was studied among different species for the early and late blight resistance genes. Phylogenetic tree has depicted the relatedness in tomato for R-genes with other 3 Solanum species, one model plant and a dicot plant. Their protein sequences were used as reference, retrieved from *Ensembl* plant database through BlastP with threshold to construct a phylogenetic tree after multiple sequence alignment (MSA). These included 11 genes from *Solanum tuberosum,* 12 of *Vitis vinifera* and *Nicotiana attenuata,* 13 from *Arabidopsis thaliana and* 2 from *S. lycopersicum.* Final tree sequences were categorically divided into clades according to branch length and alignment pattern. EBR tree illustrated the 5 major clades that showed R-genes in tomato present in clade 3 and 5. NPR1 gene (Solyc07g040690) was present in clade 3 showed close relation with potato, pepper and then with Arabidopsis. Chitinase (Solyc07g005100) in clade 5 showed close relation with potato. Some orthologues of R-genes Chitinase and NPR1 clustered in different clades showed divergent behavior and less relatedness.

3.4.2 Phylogenetic relationship of tomato late blight resistance genes with other species

Evolutionary relationship was studied with five different species for tomato late blight resistant genes (Rpi and NRC1). Gene orthologues were retrieved from other species came out with numbers of 49 *Solanum tuberosum,* 47 of *Vitis vinifera*, 25 of *Capsicum annum*, 10 of *Nicotiana attenuata*, 8 of *Arabidopsis thaliana* and 17 of *S.lycopersicum.* NLR genes were grouped into 5 clades which described the close relation and divergence among diverse species. Clade 1 presented the Rpi gene shared close relation with potato and then related with dicot plant *vitis vinifera*. Clade 2 expressed 7 genes closely related to potato orthologues and clade 3 with 2 orthologues while clade 4 represented 6 genes merged among *Solanacea* family members. Clade 5 showed two genes linked with *Arabidopsis* than *Solanacea* family members.

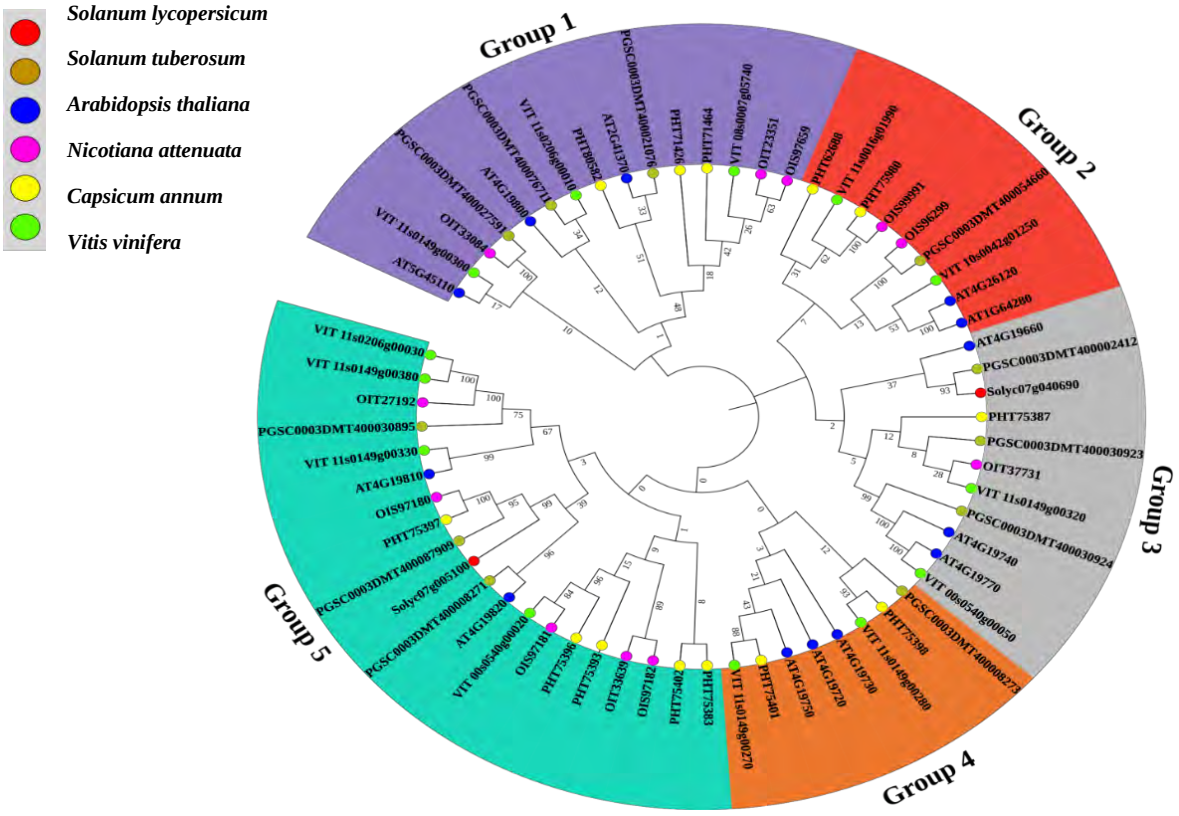

Figure 3.4.1: Phylogenetic tree of early blight resistant genes (Chitinase and NPR1) in tomato with other five species included model plant *A. thaliana*, *Solanacea* family members and dicot plant *vitis vinifera*. Branch symbols of different colors represented the different species and their relatedness with tomato orthologues.

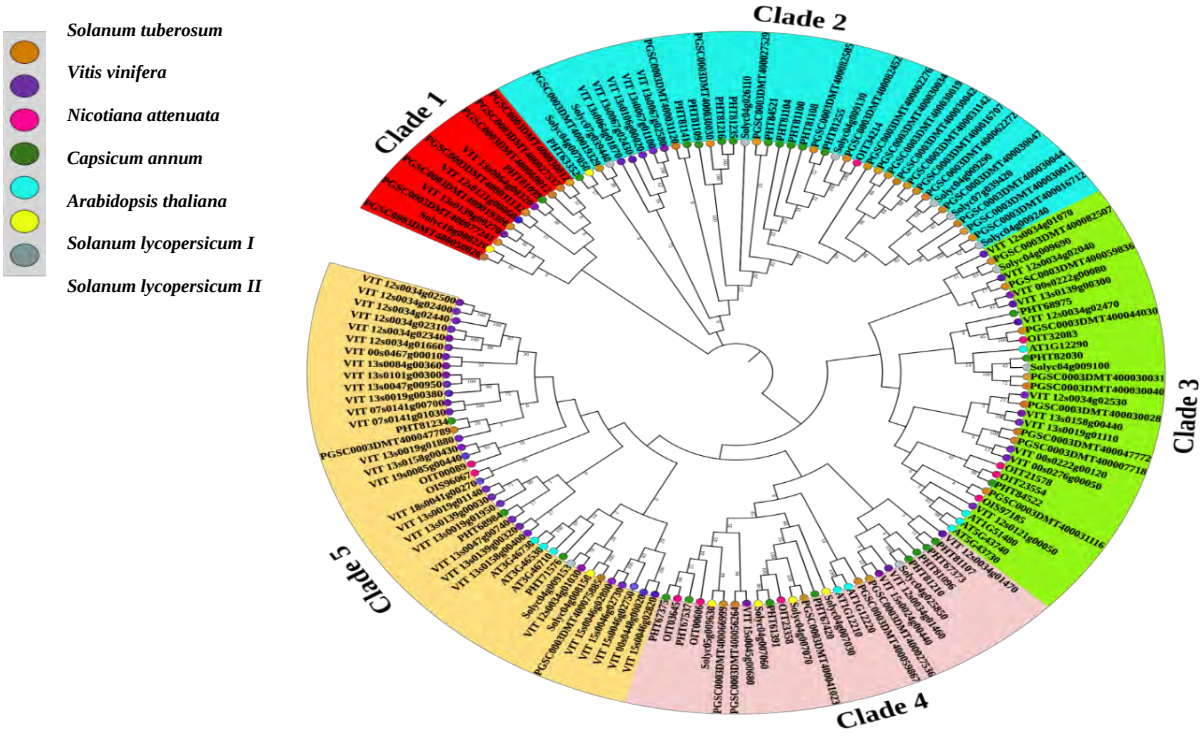

Figure 3.4.2: Phylogenetic tree of late blight resistant genes (Rpi gene and NRC1) in tomato with other five species included model plant *A. thaliana*, *Solanacea* family members and dicot plant *vitis vinifera*. Branch symbols of different colors represented the different species and their relatedness with tomato orthologues.

3.5 Analysis of Cis-acting regulatory elements in the promoters of resistance genes

Cis-acting regulatory elements specific to the resistant genes were responsible for diverse functions including plant growth and development, phytohormones, and responsive to biotic and a biotic stress in *S. lycopersicum*. 23/57 was showing response associated with growth and development, 28/57 was linked with Biotic and Abiotic stresses and 6/57 were phytohormones.

Regulatory elements named CAAT-box and TATA-box which were associated with growth and development were observed in all of the resistant genes in tomato. These 2 regulatory elements were resulted into maximum number. In comparison to maximum, there were 3 regulatory elements named RY-element, LS-7 and CTGGC-motif present with lowest value number. Highest expression to midpoint and then lower expression of these regulatory elements was also observed in TATA-box (for transcription initiation) associated with growth and development.

There were 2 elements showing high response (expression) shown in Red, 20 elements gave midpoint expression values shown in pink and 25 elements gave lower expression in response as depicted in Fig 3.5. All these regulations determined the resistance providing genes also play role in growth and development.

3.6 Protein-Protein interaction

Protein network analysis was used to determine the association on the basis of function of selective resistance genes in tomato with other related protein. Proteins were either functionally dependent or perform their function solely. Chitinase, confer resistance against early blight fungus by destroying its chitin wall, its protein had shown association with other proteins PR1 and PAL, depicted in Fig 3.6 below. Selective proteins showed zero association with each other means tend to work independently and Rpi- gene also showed association when observed alone.

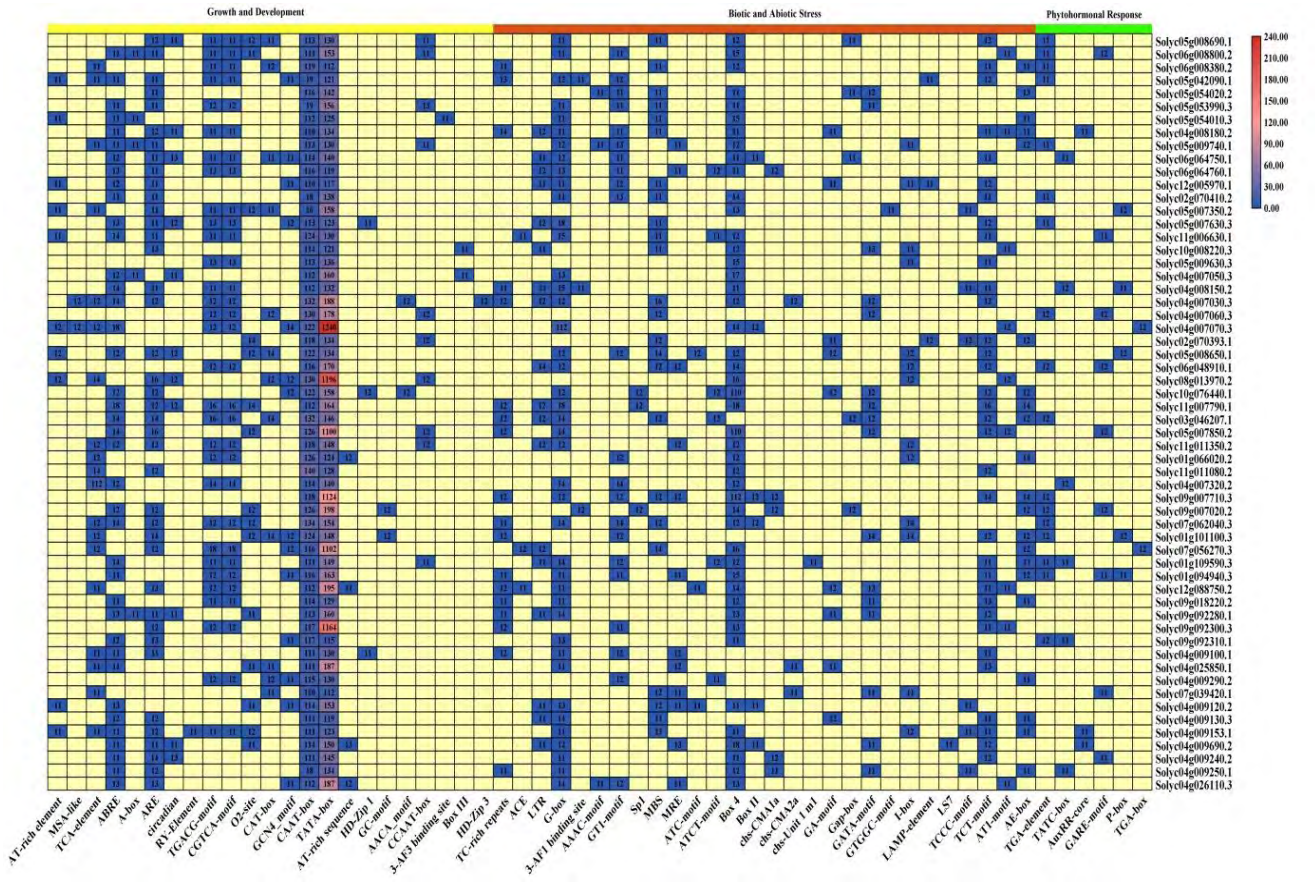

Figure 3.5: Cis-actin regulatory elements on promoters of all identified resistant genes in tomato. Scale showing the expression from highest to lowest pattern and legend representing the elements growth and development colored yellow, phytohormones response colored red and biotic and abiotic factors colored blue.

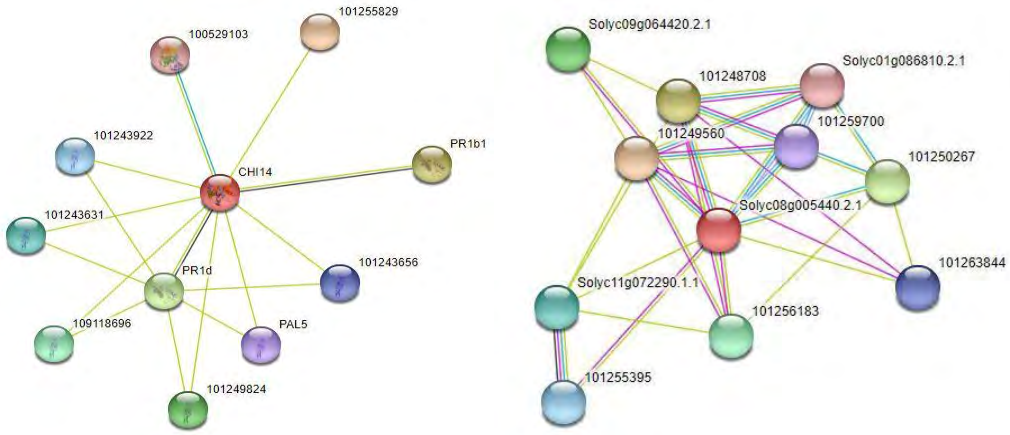

Figure 3.6: Protein-Protein interaction of early blight responsive gene Chitinase with other related proteins like PAL enzyme which acquired SA pathway and PR1 gene provide resistance against pathogenic diseases. On the right hand, association was shown for Rpi-gene against late blight, related to other *Solanum* proteins which determined their interlinked function.

3.7 Screening methods for tomato lines against blight pathogens

3.7.1 Mechanical inoculation

The screening of 36 lines against fungal resistance was performed via field experiment. It was proposed for early blight pathogen infection through mechanical way. Field experiment was shown to be very favorable for pathogen because there were relatable conditions for pathogen to attack then symptoms were appeared. Leaf samples of different tomato genotypes (cultivated and one wild accession) were first injured through injection and then inoculated by placing Agar plug on them, as this was mechanical inoculation. Symptoms started to appear at 5^{th} day and it was routinely checked till 10^{th} day post inoculation (dpi). Most of the samples were shown disease adaptive than a single variety 19890. That line was observed highly resistant in the field which shared potato leaf structure morphologically. Although a wild variety *S.chilense* (19906) showed the disease adaption earlier than resistant variety and 38039 was marked as moderately sensitive to infection through visual basis. So, results were drawn out phenotypically that symptom appearance and severe necrosis happened on different time in various

accessions. Phenotypic data was calculated on the basis of lesion size as shown in the Fig 3.7(a) There were some representative pictures of some lines (wild and cultivated) showed disease symptoms upon inoculation. Also there was an image drawn out the comparison between healthy and diseases leaf samples. It has been shown in the Fig 3.7 (b) as followed.

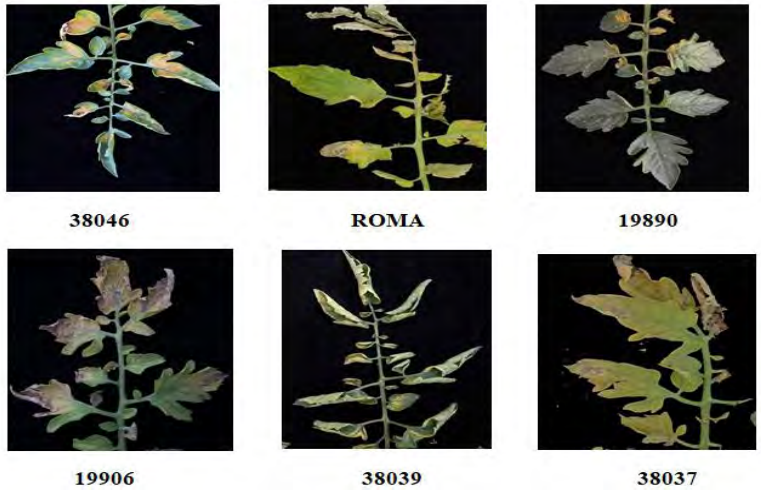

Figure 3.7.1(a): Tomato leaf tissues of six genotypes (cultivated accessions 38046, 38037, 38039, 19890 and ROMA and wild accession 19906 (*S.chilense*) at maturing stage represented early blight disease condition in open field.

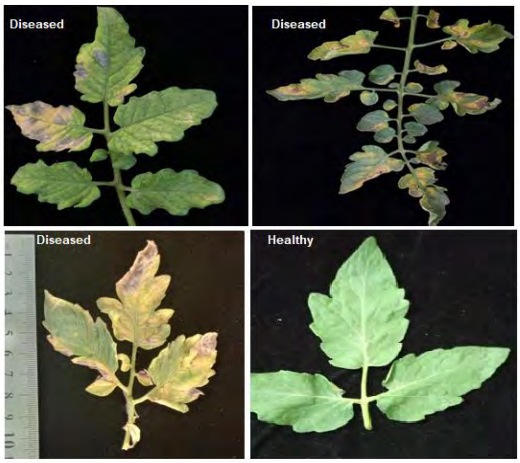

Figure 3.7.1 (b): comparison of Healthy and Diseased tomato leaf samples of a representative line from open field.

3.7.2 Lab based detached leaflet assay and disease scoring

Lab based assay was performed on the selected 36 accessions in triplicates, which were infected in the field. Outcomes of this experiment correlated with field experiment represented the early blight symptoms. Surveillance of disease on different tomato accessions was checked which determined the tolerant and susceptible lines based on affected area percentage. Fungal pathogens (*Alternaria solani* and *phytopthora infestans*) isolation was shown in Fig 3.7.2 (a). Affected area percentage based on lesion size varied in replicates of same accession for disease severity. Symptoms and % effectiveness of disease shown in six representative genotypes in Table 3.3 and Fig 3.7.2(b) illustrated below. Assay results have determined one tomato variety 19890, moderately resistant, one highly susceptible and remaining were moderately susceptible. The relative expression of accessions was shown in (Fig 3.7.3 c) based on disease severity, disease reaction and their scoring.

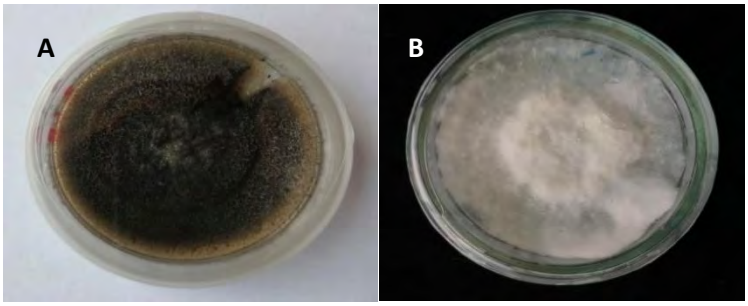

Figure 3.7.2(a): Morphological identification of *Alternaria solani* through concentric rings (A) and *Phytopthora infestans* showed white pellate (B) in culture plates.

Table 3.3: Comparison of the mean lesion size and affected area (%) in the leaves of the control (water treatment) and disease inoculated (treated with *A. solani*) tomato leaf samples.

Tomato accessions	Lesion size ± S.E(cm)	Disease Severity (%)	Affected area (%)	Disease score	Disease reaction
38046	50±0.13	9	50	3	Moderately Succeptible

38039	50±0.003	2.6	50	3	Moderately Succeptible
ROMA	83±0.075	10	83	5	Highly Succeptible
19890	25±0.005	1.53	25	2	Moderately resistant
19906	66±0.04	4	66	4	Succeptible
38037	50±0.06	8.3	50	3	Moderately Succeptible

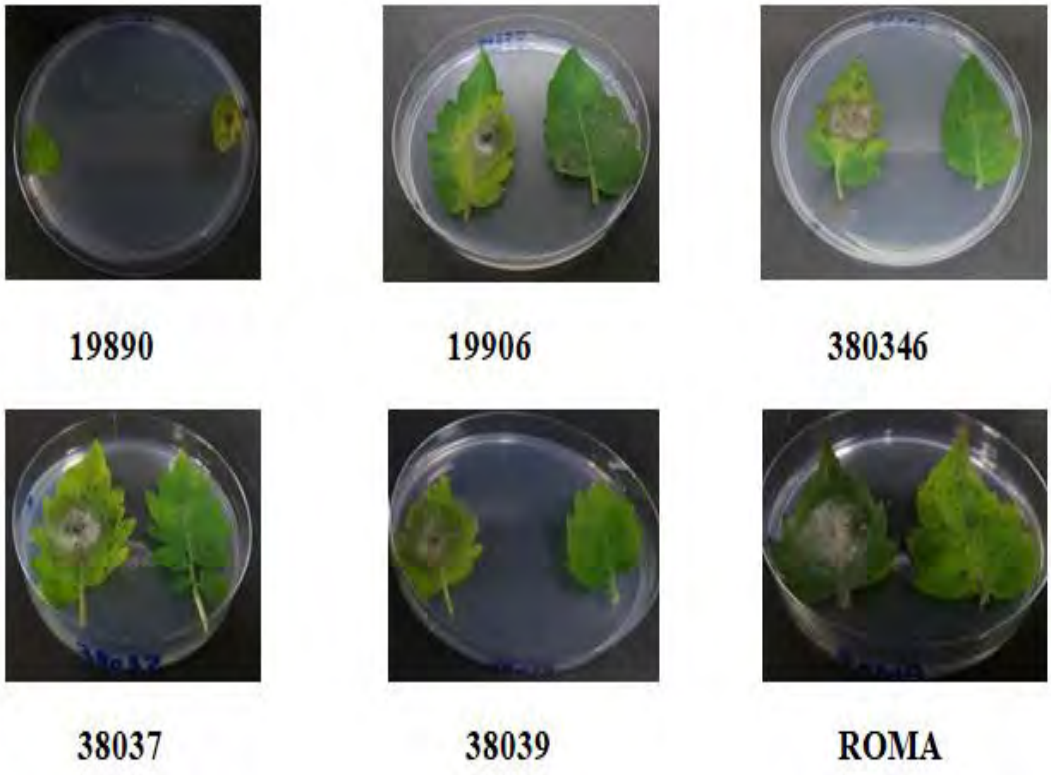

Figure 3.7.2(b): Lab based detached leaflet assay for tomato accessions against early blight inoculum *(Alternaria solani).*

Disease scoring scale

0-10% affected area HR

11-25% affected area MR

26-50% affected area MS

51-75% affected area S

76-100% affected area HS

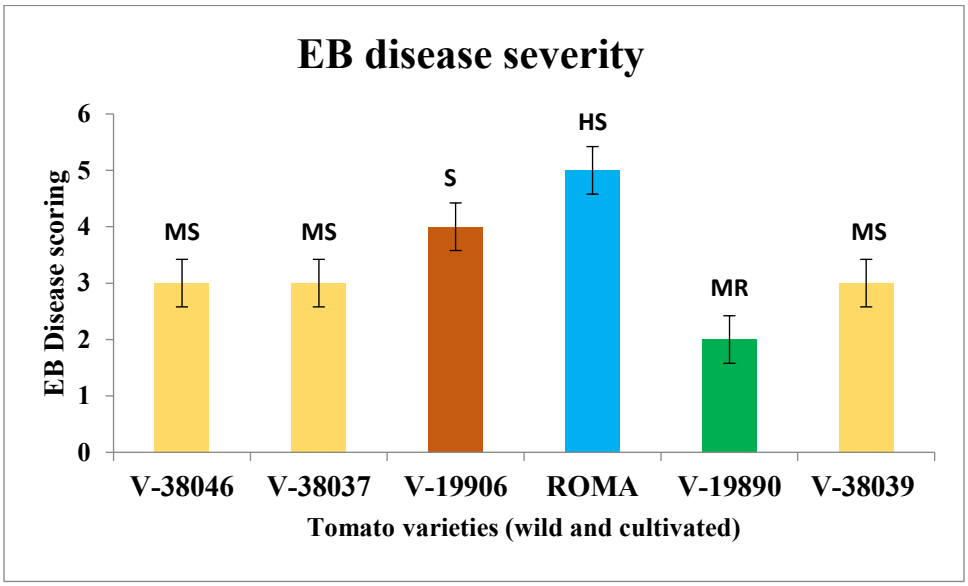

Figure 3.7.2(c): Disease scoring and disease reaction of 6 selected tomato genotypes against early blight after detached leaflet assay.

3.8 Total RNA extraction

RNA was isolated from the healthy (control) and diseased samples of six accessions (1 wild accession 19906 *(S.chilense)* and 5 cultivated *S.lycopersicum* 38046, 38037, ROMA, 38039 and 19890). Selection of these accessions as representative was supported by the associative results of phenotypic scoring data with visualized screening. RNA of healthy and disease leaf tissues (H/D) was extracted and reverse transcribed for

expression analysis of blight resistant genes. Gel image showed the isolated RNA bands represented 6 accessions in Fig 3.8.

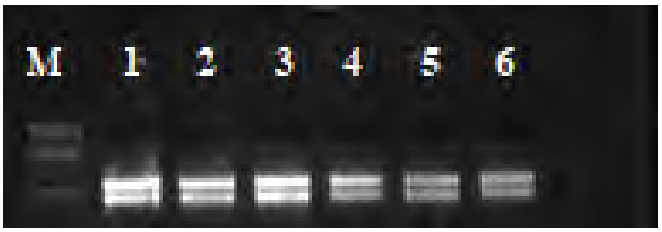

Figure 3.8: Islolted RNA of six tomato accessions (1 wild accession 19906 and 5 cultivated accessions 38037, 38046, ROMA, 19890 and 38039, their healthy and control leaf tissues).

3.9 Expression analysis of fungal resistance genes in tomato

The relative abundance of fungal resistance genes was estimated by quantitative RT-PCR. For the detection of relative expression pattern of fungal blight resistance genes, healthy (control) and diseased tomato leaves of six different accessions (mentioned above) at maturing stage were selected. It determined the relative expression of resistant genes was up-regulated on pathogen interaction in comparison to control (healthy). Threshold cycle (ΔCT) value gave the information of resistant genes at the time of amplification and ΔΔCT values inferred about relative gene expression in diseased and control (healthy) leaf samples of tomato accession at maturing stage. Expression pattern of different R- genes for early blight and NLR genes for late blight was presented in images below. Fig 3.10 (A and B) showed the expression pattern for late blight resistant genes in tomato different accession and response was observed variable with respect to pathogen in them. Resistant genotype in case of Rpi gene was showing significant expression than others while remaining accessions respond moderately but there was no significant difference in expression for ROMA against *Pi* invasion. NRC1, a helper NLR expression was strongly elevated in ROMA than resistant genotype 19890 after disease inoculation. Wild accession 19906 didn't respond well under diseased condition. Other moderately susceptible varieties were showing tolerant behavior by elevated response as compared to their control samples.

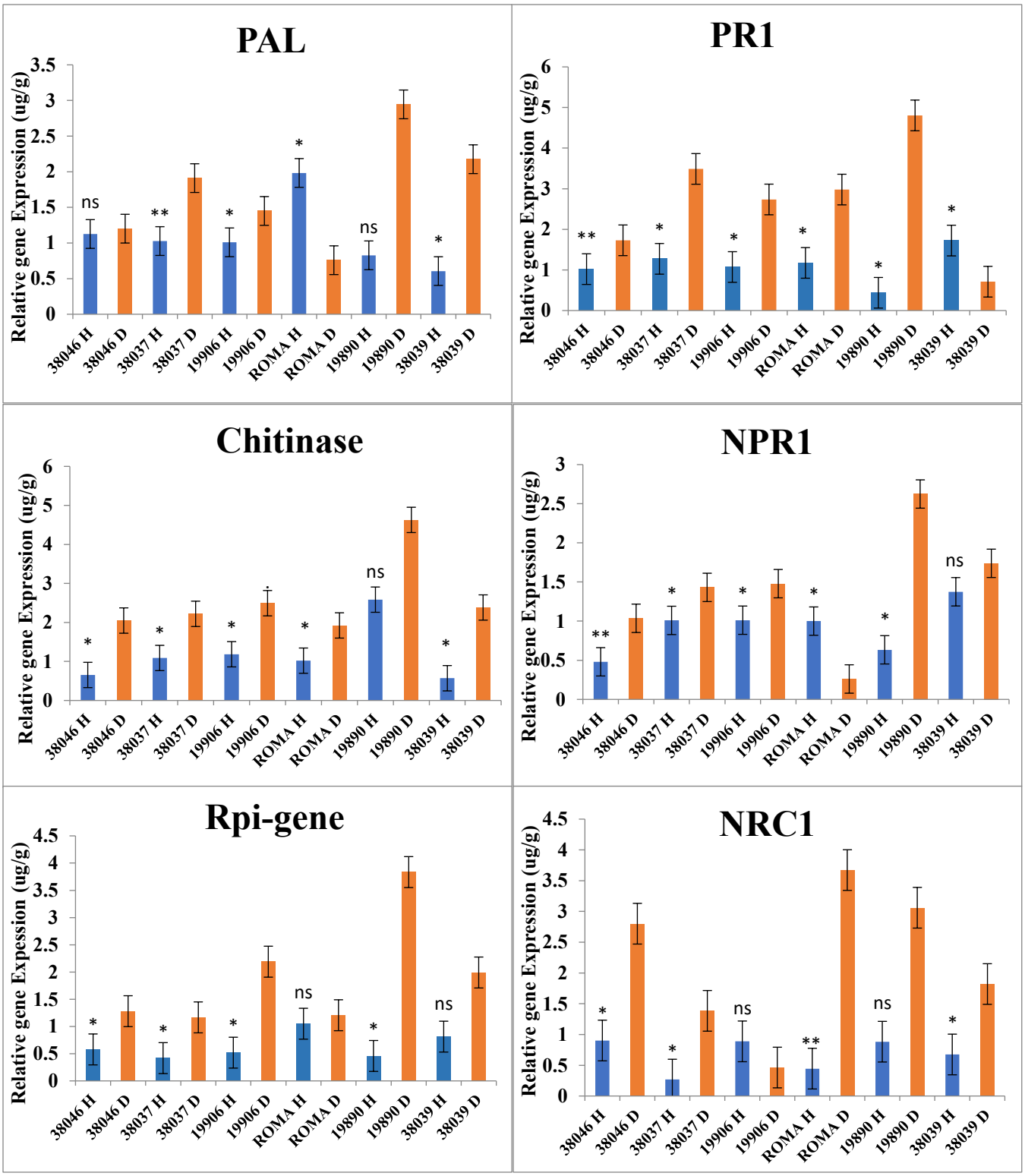

Figure 3.9: Expression pattern analysis for NLR genes (A and B) against Late blight disease and R-genes (C, D, E and F) against *A.solani* in healthy and disease plants.

Fig 3.9 (C) described the results for enzyme PAL (phenyl ammonium lyase) responsible of SAR mechanism. Up regulated response was observed in all the infected samples exception laid in resistant genotype 19890D which showed highly significant expression while ROMA gave no significant response due to susceptibility and remained unchanged with control. NPR1 gene reportedly involves in natural defense and chitinase enzyme fungal cell wall killer, both gave expression against *A.solani* presented in Fig 3.9 (D) and (E).

Expression pattern of two genes was enhanced in diseased samples and highly active in resistant genotype and low in ROMA. PR1 (Pathogenesis related protein-1) has been significantly involved in SA response initiation so expression pattern of this gene showed the elevated response upon pathogen interaction. All the inoculated samples expressed significant response except moderately susceptible line 38039 which showed no significant expression. While 19890D with up regulated response and ROMA with no significant difference in expression were represented in Fig 3.9 (F).

DISCUSSION

Tomato (*Solanacea* plant) which is economically important fruit vegetable and non-grain crop after potato, worldwide. There are many abiotic stresses which affect tomato plants in different way but its production is mainly hindered by biotic factors (Panthee and Chen 2010). Its growth is dependent of pesticides or fungicides because of many devastating fungal diseases are resulted in annual yield loss (Seo *et al.,* 2016). Fungal pathogens *Alternaria solani* and *Phytopthora infestans* are the causing agent for early and late blights that collectively responsible for 49-91% tomato yield loss estimated in Pakistan (Saleem *et al.,* 2015). Economic yield loss due to EB was reported 79% annually and critical yield loss estimated 100% in sever condition for late blight. Disease causing pathogens are capable to rapid genome changes which destroy resistance pattern of plant (Bitew 2019). Plant innate immunity is operational in defense mechanism against numerous diseases. From conventional breeding to molecular characterization, so far there are various reported R-genes actively involved in resistance against bacterial, fungal and viral diseases (de Araújo *et al.,* 2019). Determining the function of a set of resistance genes helps us in understanding the pathway of host plant defense action (Panthee and Chen 2010).

The progress in EB resistance being a quantitative trait was potentially limited which required an additive effect from various R- genes (Upadhyay *et al.,* 2014). Polygenic inheritance of genes may indicate their resistance is not long lasted (Bitew 2019). Resistant cultivars are the suggested control measures economically which can limit the use of fungicides in disease control maintenance (Cherani *et al.,* 2006). Mature tissue and ripen tomato fruit are greatly susceptible to EB disease also associated with leaf part (Grigolli *et al.,* 2011). NLR genes containing NBS-LRR or NB-ARC domain are reported that work effectively in wild species against *Pi* disease. Up to date there are 267 NLR genes are known in tomato and classified in TNL and CNL groups (Jacob *et al.,* 2015). Up till now, 20 R-genes are being characterized and 13 are being cloned in *Solanacea* species showing resistance against late blight pathogen (Bakker *et al*., 2011; Wu *et al*., 2017). Among NLR genes, Rpi- protein and Rpi-blb2 and one helper NLR gene NRC1

were studied for late blight resistance in our diverse tomato accessions. Broad spectrum NLR genes also identified in *A. thaliana* were RPP13, a resistance gene against pathogen *Hyaloperonospora Arabidopsidis*, RPP1 resistant to *Peronospora Parasitica* 1, ZAR1 gene interconnected in NLR network, RPS2 a resistance gene against the bacteria *Pseudomonas syringae* and another NLR gene pikp-2 from Rice which confer resistance to blast pathogen *Magnaporthe oryzae (*Baggs *et al.,* 2017*;* Nowicki *et al.,* 2012*;* Wu *et al.,* 2017*;* Zdrzałek *et al.,* 2020).

NLR genes understudied within tomato genome clustered linked Genome-wide (GW) identification that was suggested to work in disease resistance supported the studies on NLR genes in (Andolfo *et al.,* 2014) which showed the clustering according to their functions. Chromosomal localization of NLR genes on tomato was related to study of (Andolfo *et al.,* 2014; Wei *et al.*, 2021) by showing uneven distribution of genes on 12 chromosomes. (Nowicki *et al.*, 2012) reported about Rpi gene present on chr-07 and study result determined the Rpi gene two orthologues in tomato Solyc07g039420.1 and Solyc07g039440.1 occupied the position on chr-7. Broad spectrum resistant NLR genes were selected for phylogenetic relationship within tomato genome. These genes were clustered in groups based on sequence similarity, conserved domain and motif analysis. NLR gene carrying NB-ARC, NB-LRR and LRR_8 domains were classified in sub-family (CNL and TNL) and that result is consistent with previous study (Andolfo *et al.,* 2014; McHale *et al.,* 2006; Seo *et al.,* 2016).

Motif 2, 4, 5 and 6 out of 20 motifs identified per sequence were seen conserved present in all NLR genes. Motif analysis based on 3 conserved domain (NB-ARC, NB-LRR and LRR_8) were matched to previous study having major 3 motif and 3 minor domains in NLR genes within genome (Seo *et al.,* 2016). Phylogenetic relationship of resistance genes with other species was studied for late blight and early blight resistant genes that were separately represented in a rooted circular tree (Feng *et al.* 2020). They showed clustering of the tomato NLR genes Rpi and NRC1 with other species and grouped in clades according to study in (Wei *et al.,* 2021). Tree cluster analysis was displaying the close relation of late blight resistance genes and their orthologous in tomato with potato

from where they were originated, then related to model plant *A.thaliana* and dicot *Vitis vinifera* plant. It suggested the strong ancestral relationship of the tomato with its family *Solanacea* by revealing a high level of conservation across all NLR subgroups, comparatively (Andolfo *et al.,* 2014). Early blight resistance genes, PR1 and NPR1 (natural defense response) which conatined CAP domain while Chitinase for its enzymatic activity contained Glyco_18 or Glyco_hydro_18 (GH-18) domain has supported the findings of (Cao *et al.,* 2019). Results indicated the close relationship of these R-genes against *A.solani* with potato than other species. PAL is the key enzyme for inducing signaling pathway and elevates the expression of pathogenesis related proteins through SAR defense. It also worked against late blight pathogen (Joshi *et al.,* 2021).

Promoter analysis has categorized the cis-acting regulatory elements in 3 functions for resistance genes in tomato. These major groups were growth and development, phytohormones and abiotic and biotic stresses, supported the results of (Li *et al.,* 2019; Wei *et al.,* 2021). Protein network analysis for chitinase indicated it interaction with PR1 and PAL genes and itself belonged to PR3 gene family. It determined the diversity of chitinase binding proteins, which was helpful to understand their functional roles in defensive pathway (Cao & Tan, 2019). Likewise, Rpi gene was associated with other *S.lycopersicum* genes while other selected genes are working independently.

Field screening of tomato germplasm accessions planted in RCBD manner, was conducted through mechanical infection in accordance with (Nagesh *et al.,* 2020). Tomato accessions (wild and cultivated) were inoculated with *A.solani* pathogen and resulted in sensitive and one resistance variety. Assay was conducted for surveillance of the disease on 36 screened (field screening) tomato germplasm. Percentage disease severity was assessed for early blight in our diverse genotypes and their reaction to this disease was studied in relevance to *(*Moghaddam *et al.,* 2019). Lab assay (detached leaflet assay) has determined the susceptible or resistance genotypes based on lesion size and disease scoring was performed based on affected area in percentage, which supported the research of (Jabeen *et al.,* 2015; Pandey *et al.,* 2003). That percentage was then scaled by the study of (Grigolli *et al.,* 2011) which determined the disease severity index.

Comparison of 2 screening methods has determined adequate correlation from each other as most of the genotypes were declared moderately susceptible by showing symptoms and moderately tolerant behavior and resistant genotype which showed disease but late than sensitive ones (Moghaddam *et al.,* 2019).

In correspondence to the study of (Moghaddam *et al.,* 2019) at maturing stage, resistant genotype 19890R was showing strongest expression for PR1 in inoculated sample than control. Other genotypes which were moderately susceptible somewhat showed response except 38039 which gave down regulated response. Exception was observed in case of highly susceptible variety ROMA showed elevated response than healthy one. In case of chitinase, all the inoculated samples showed significant resistive response to disease but greater response was observed in resistant genotype. NPR1 gene expression in inoculated samples was observed up regulated with significant result and unchanged in highly susceptible variety ROMA after inoculation. Response of susceptible Wild variety 19906 was strongly increased than its control may be possible due to dominant effect of resistant genes. Resistant genotype showed the greater expression of this gene after inoculation in comparison to all other lines. Rapid induction of defensive response in case of PAL was observed in resistant genotype at maturing stage which has supported the results of (Patel *et al.,* 2011). Expression remains unchanged with no significant differences in highly susceptible variety that may happen if gene present in recessive form which did not activate the defensive pathway upon pathogen interaction. Expression of PAL was up regulated in sensitive line 38037 and 38039D.

Late blight resistance gene Rpi expression was observed in tomato cultivars and wild accession in which elevated response was detected in all inoculates samples than non-treated samples. NRC1 helper NLR gene expression was exceptionally up regulated in all inoculated moderately susceptible varieties and highly susceptible line ROMA even showed high response than resistant inoculated genotype 19890 (Wu *et al.,* 2017). Expression data results concluded that R and NLR genes respond well in disease condition. These R and NLR genes show strong increased expression in our local tomato varieties against *Phytopthora infestans* and *Alternaria .solani,* respectively.

CONCLUSION

Tomato plant is prone to fungal attack, a major reason for its yield loss. Early and late blight incidence on tomato leaves and fruits are proved to be devastated if left unprotected. Diverse tomato germplasm accessions (wild and cultivated varieties) were screened through combined field and lab assay. Different bioinformatics tools were used for structural analysis of resistant genes that recommend the functional characterization of resistant genes in future. Elevated expression in resistant genotype, moderate response in moderately susceptible lines and no significant difference observed in highly susceptible variety after pathogen interaction is determined the genetic basis of R and NLR genes might be involved in tolerance. It suggests that set of these genes collectively in tolerant cultivar can be helpful in conferring the resistance against fungal diseases.

FUTURE ASPECTS

Transcriptomic analysis of NLR genes will provide the regulatory network approaches for making resistant cultivars which will be helpful in the speed cloning and breeding program.

CRISPR Cas-9 mediated silencing of susceptible genes in tomato for EB and LB diseases will be another level of resistance. Bioinformatics studies on molecular characterization will also provide further insight to gene diverse functions.

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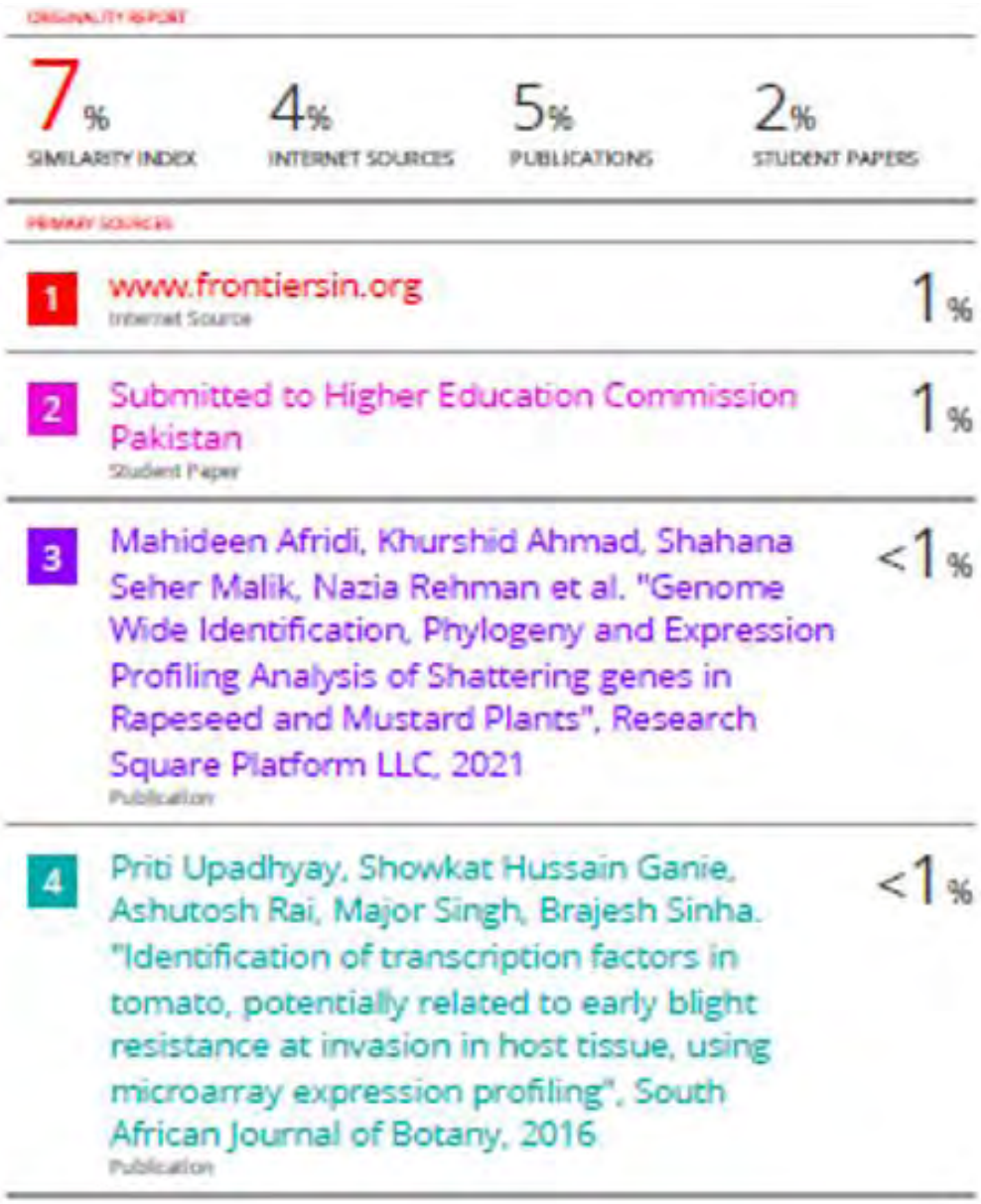
Expression analysis of fungal disease resistant genes in tomato (*Solanum lycopersicum L.*)



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