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Differential expression profiling of defense related genes for Leaf Curl Virus (ChiLCV) in resistant and susceptible genotypes of Chiili

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Abstract

The present investigation was undertaken to study the expression of eight defense related genes in leaf curl resistant line DLS-Sel-10 and susceptible line Phule Mukta after different days post inoculation (dpi) with viruliferous white flies to understand their role in resistance to chilli leaf curl virus (ChiLCV). The expression level of Ca PPO, Ca AsPer, Ca ATP/ADP and CaTopoll was observed to be higher in resistant genotype DLS-Sel-10 than the susceptible Phule Mukta at all the time points studied. Expression of CaNBS-LRR increased up to 12 dpi while that of Ca Thionin, and Ca SKP1 increased up to 24 dpi in the resistant line, thereafter it started declining. The CaSPI expression did not show any specific pattern in both the test plants. The heat map clustered all the genes under study into two major clusters based on their expression profiles, one comprising CaAsPer, Ca-Thionin, CaATP/ADP transporter, CaPPO and CaTopoll while other group comprised CaSKPI, Ca NBS and CaSPI. The challenge inoculation of the test genotypes also revealed that viral titre increased at a much slower rate in DLS-Sel-10 than Phule Mukta, suggesting thereby that DLS-Sel-10 is resisting the accumulation of ChiLCV and has a more active defense machinery than Phule Mukta.

Key words: Chilli, ChiLCV, defence genes, expression profiling, qPCR

Introduction

The diseases caused by ‘Geminiviruses’ are the most devastating among the viral diseases which threaten the production of crop plants. Chilli is one of the most important vegetable crops, valued for its pungency, taste, aroma and the appealing colour that it imparts to food. The crop suffers huge economic losses due to the infection by Chilli Leaf Curl begomo virus transmitted through whitefly. Occurrence of the disease has become so devastating that the farmers have abandoned cultivation of chilli in *kharif,* the main growing season. Therefore, the availability of chilli germplasm with resistance to leaf curl disease is of extreme importance to the breeders as well as farmers. ICAR- Indian Agricultural Research Institute has identified a chilli leaf curl virus (ChiLCV) resistant line DLS-Sel-10. Our group has also ascertained the nature of resistance in DLS-Sel-10 to be recessive (Maurya et al. 2019). Recessive resistance to virus has been reported in other crops like mungbean (Solanki et al. 1982; Ammavasai et al. 2004). However the mechanism of resistance as well as host factors and defense genes playing role in resistance in this line are not known.

In order to establish a successful infection, viruses control the cellular machinery of the host plants in such a way that they suppress the plant defense system and don’t allow the plant to counteract. Plants in turn have also developed a variety of resistance mechanisms to combat the pathogen. Different layers of plant defense mechanisms include production of different anti-pathogenic compounds as well as activation of specific defense signaling cascade in response to pathogen attack (Heil and Bostock 2002; Veronese et al. 2003). A resistant plant harbours specific genes which provide it resistance against particular pathogens (Singh et al. 2016). Resistant plants contain a specific kind of resistance proteins called nucleotide-binding leucine-rich repeat (NB-LRR) domain containing proteins which provide effector-triggered immunity (ETI) to the plants after recognition

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of viral effectors (Mandadi and Scholthof 2013). During host-viral interaction alteration in the regulation of transmembrane immune receptors as well as different phytohormone pathways have also been observed (Zorzatto et al. 2015; Alazem and Lin 2015). In addition plants have also been known to employ the ubiquitin-proteasomal pathway (UPS) as an antiviral defense strategy (Alcaide-Loridan and Jupin 2012).

By studying the changes in expression of certain defense related genes upon virus infection in susceptible as well as resistant plants, their role in resistance or susceptibility can be ascertained. The aim of this study was to analyze the expression levels of CaNBS-LRR, CaAsPer, CaPPO, Ca-Thio, CaTopoII, Ca ATP/ADP transporter, CaSKP1 and CaSP1 transcription factor that are known to be involved in plant biotic stress responses between Chilli varieties with varying susceptibility to *ChiLCV* infection.

Material and methods

The resistant genotype DLS-Sel-10 and susceptible Phule Mukta were maintained in insect proof glass house at the experimental farm of Division of Vegetable Science, ICAR-IARI-New Delhi for all experiments.

Challenge inoculation using white flies

Whitefly (*Bemisia tabaci*) population maintained on brinjal plants (*Solanum melongena*) under controlled temperature (28-35°C), relative humidity (30-50%) and light/dark (14/10 hr) conditions that were used for inoculation of test plants. To make the whiteflies viruliferous, healthy whiteflies were fed on pure cultures of ChiLCV) for 24 hours following standard protocols and used for challenge inoculation of the healthy seedlings of test genotypes. For challenge inoculation, the resistant and susceptible plants (grown in 4 inch pots) were covered with cylindrical plastic cages (15 x9 cm^2) having black muslin cloth at the top surface. Viruliferous white flies were released into the cages from a small hole made at the side of the cage which was kept plugged with cotton plug all the time except during release of flies into the cage.

Sampling

Plants of DLS-Sel 10 and Phule Mukta were inoculated with healthy/viruliferous white flies at 4-6 leaf stage. Thirty six plants per genotype were inoculated and five viruliferous white flies were released on each plant. The whiteflies were allowed to feed on each plant for 48 hrs after which they were killed with 2ml/ litre Rogor (Bayer crop Science). Sampling of inoculated plants was done at four time points i.e., zero (day of inoculation), 12, 24 and 36 days after inoculation. Three replicates per time point with three plants per replication were used for extraction of RNA for gene expression profiling. Only topmost four leaves from each test plant were used for RNA extraction so as to maintain the uniformity of experiment.

Symptom development of disease scoring

All the challenge inoculated plants of resistant and susceptible genotypes were observed for appearance of leaf curl disease symptoms at 0, 12, 24, and 36 days post inoculation. Scoring for chilli leaf curl disease was done according to scale developed by Banerjee and Kalloo (1987) and modified by Kumar et al. (2006) as given in Table S1.

Detection of presence of virus using PCR

Following challenge inoculation of DLS-Sel-10 and PM with viruliferous whiteflies (28 days post inoculation), plant samples from test genotypes were tested for presence of virus using virus specific primers BM861/862 (sequence information to be provided on request) with an amplification product of 453 bp. Genomic DNA was extracted from young leaf tissue (top four leaves) of each genotypes following the C-TAB method (Murray and Thompson 1980). DNA quality and quantity were assessed on a 1% (w/v) agarose gel stained with ethidium bromide (Sigma Aldrich Chemical Pvt. Ltd, Bangalore, India) and also by using a NanoDrop® ND-1000 spectrophotometer.

The PCR was carried out in 10µl volumes containing 50ng genomic DNA, Taq DNA Polymerase 2X Mastermix (Cat. # 786-449 G Biosciences) and 1.0 µM each of forward and reverse primer. Amplification conditions used for the primer pair were: one cycle of 94°C for 3 min; 35 cycles of 94°C for 0.5 min, 62°C for 1 min, and 72°C for 1 min; and a final cycle of 72°C for 5 min. Amplified products were resolved on 3.0% agarose gels with Tris/Acetate / EDTA (TAE) stained with ethidium bromide at a constant voltage of 60 V for 3 h using a horizontal gel electrophoresis system (BioRad, USA) and visualized and photographed under UV light in a gel documentation unit (Alpha imager, Cell bioscinces, Santa Clara, CA).

q-PCR for viral titre load estimation

ChiLCV being a DNA virus, viral titre in resistant and susceptible genotypes was estimated through q-PCR using the DNA from the challenged plants. Primers

for estimation of viral titre (BM942/943) were designed from the AC1/AC4 region of Chilli Leaf Curl Virus genome. The sequence of the forward primer LCV BM-942F was 5' CGGCATATGCGTCGTTGGC-AGAC 3' while that of reverse primer BM-943R was 5' TTCTTCGACCTCGTTTCCCCAACC 3'. Three biological replicates were used from each genotype. Two technical replicates were also used while running real time PCR assay to normalize any pipetting error. For qRT-PCR, 5 μl of SYBR Green mastermix (Applied Biosystem, CA,USA), 2 μl nuclease free water, 1 μl each of forward and reverse primer of desired genes and 1ìl of template DNA was used. The PCR program comprised initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 15s, annealing at 55°C for 35s, and extension at 72°C for 35 s. The ΔCt of target gene (viral gene) was normalized with internal control Ca-actin. Viral titre was quantified by relative quantification approach where in the ΔCt values in respective genotype after one day (24hrs) of challenge inoculation were used as calibrator. The ΔΔCt values were used to plot graph to obtain relative titre of virus in resistant and susceptible plants.

Expression analysis of Defense related genes

Eight different defense related genes *viz*., CaNBS-LRR gene, CaTopoII, CaPPO, CaAsPer, CaThionin, CaATP/ADP, Ca SKP1 and CaSPI were studied during the present investigation as their expression is known to be influenced by begomovirus(es) in chilli. Already published primers sequences (Kushwaha et al. 2015a; Singh etal. 2016) of these genes were used for the study.

RNA extraction

Total RNA from four upper most leaves of challenge inoculated plants of each genotype was extracted using Tri-Xtract (catalogue no-786-652, G Biosciences) following manufacturer's guidelines, quantified with a spectrophotometer, and loaded on a denaturing agarose gel to check its concentration and integrity. Two micrograms of total RNA was reverse transcribed using Verso cDNA synthesis kit (Catalog number: AB1453A, Thermo Fisher Scientific, Inc.) following manufacturer's instructions. 10 fold dilution of cDNA was made and 1 μl of diluted cDNA was used as template for each qPCR reaction. For a 10 μl qRT-PCR reaction , 5 μl of 2XSYBR Green mastermix (Applied Biosystem, CA,USA), 2 μl nuclease free water, 1 μl each of forward and reverse primer of desired gene (100nm) and 1 μl of template DNA was used. qRT-PCR was performed on *LightCycler® 96 Real-Time PCR* (Roche LifeSciences) keeping annealing temperature at 55°C for 20s. The ΔCt of each target gene was normalized with internal control Ca-actin. For construction of Heat Maps MultiExperiment Viewer Mev4.9 software was used wherein Pearson correlation was used for calculation of distance matrices and clustering was done using average linkage clustering pattern.

Results

Appearance of symptoms in challenge inoculated plants

Symptoms started appearing after 10-12 days of challenge inoculation in the susceptible genotype which progressed in severity with the passage of time while the resistant genotype DLS-Sel-10 showed only mild leaf curling after 24 days (Table 1, Fig. 1), however by 60th day post inoculation the curling symptoms recovered in DLS-Sel-10 while the symptom kept increasing with age in Phule Mukta.

Table 1. Disease score in resistant and Susceptible genotypes at different days post inoculation

Days post inoculation	Disease score	
	DLS-Sel-10	Phule Mukta
0 day	0	0
12 days	0	2
24 days	0-1	4
36 days	0-1	4

Detection of ChiLCV and Viral titre load estimation

The presence of virus (ChiLCV) was first confirmed using PCR reaction using genomic DNA from infected plants of both the genotypes under test. An amplicon of expected size (453 bp) was observed in both the resistant and susceptible genotypes, however the intensity of band was much sharper in the susceptible genotype Phule Mukta than the resistant genotype DLS-Sel-10 (Fig. 2).

Viral titre was found to increase at a faster pace in the susceptible genotype, Phule Mukta as compared to the resistant genotype DLS-Sel-10. In DLS-Sel-10, even after 36 dpi the viral titres increased by 51 folds in comparison to the load at 1dpi while in Phule Mukta it increased to 4167 and 6435 folds after 24 and 36 dpi (Fig. 3).

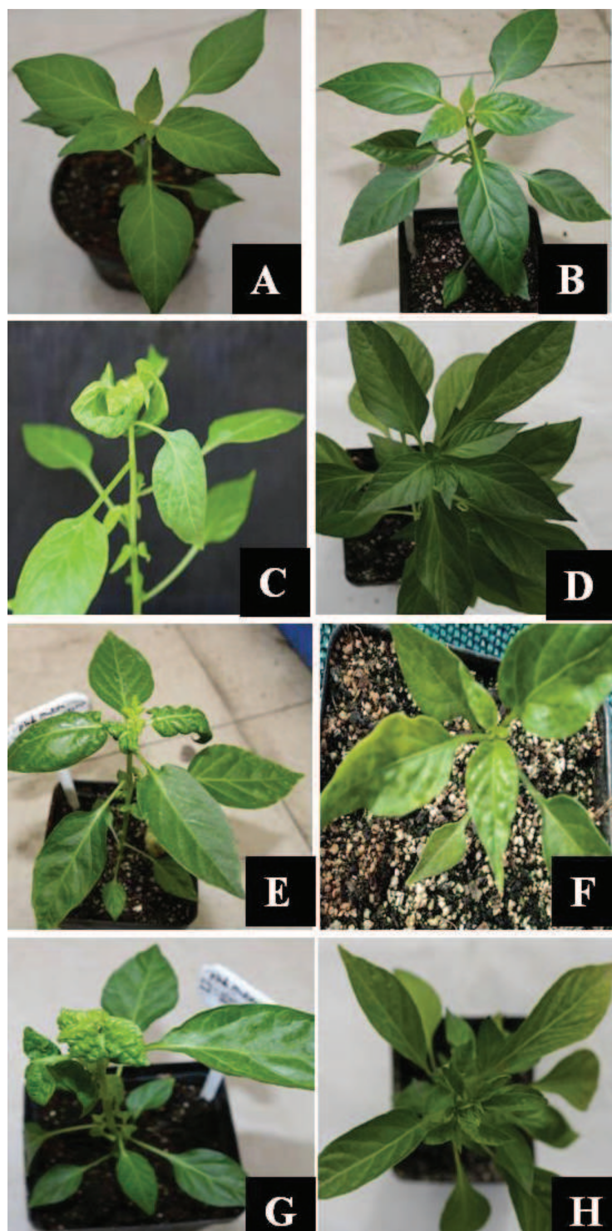

Fig. 1. Disease symptom in Phule Mukta (A, C, E, G) and DLS-Sel-10 (B, D, F, H) at 0, 12, 24 and 36 days post inoculation, respectively

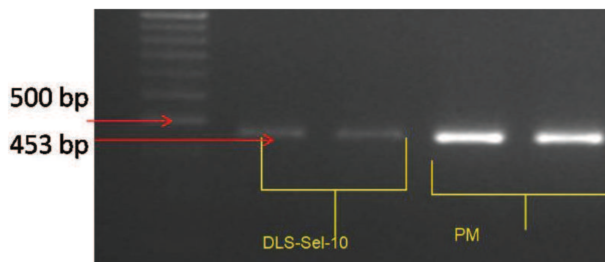

Fig. 2. Detection of presence of ChiLCV in resistant and susceptible parent

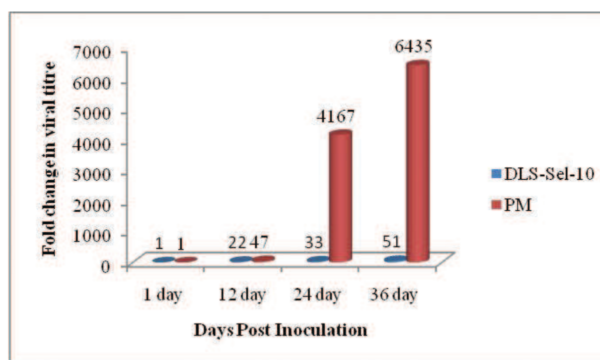

Fig. 3. Relative fold change in viral titre after different days of challenge inoculation with ChiLCV in the test genotypes

Expression of defense related host gene

Expression of eight defense related genes was studied in both resistant and susceptible genotypes after different days post inoculation with the virulent white flies (Fig. 4A-H). The expression of CaNBS LRR increased upto 12 dpi in the resistant genotype DLS-Sel-10 as well as susceptible genotype Phule Mukta (Fig. 4A). However, the expression of this gene was higher in the resistant genotype than the susceptible both at 12 (by 1.43 fold) and 24 days (by 1.22 fold) but at 36 days its expression decreased slightly (by 0.36 fold) in the resistant genotype than the susceptible genotype. The expression level of four defense related genes *viz*., Ca PPO, Ca AsPer, Ca ATP/ADP and CaTopoII was observed to be higher in resistant genotype than the susceptible Phule Mukta at all the time points studied (i.e., 12, 24 and 36 dpi). Expression of CaPPO (Fig. 4B) was highest in both the resistant (17.58 folds) as well as susceptible line (4.32 folds) at 12 dpi thereafter it kept decreasing both at 24 and 36 dpi however, the expression was much higher (17.58, 16.16 and 6.49 fold) in resistant line as compared to the susceptible line (4.32, 1.73, 3.30 fold) at 12, 24 and 36 dpi, respectively.

In case of Ca Thionin (Fig. 4C), maximum level (859.48 fold) of expression was observed at 24 dpi in the resistant genotype and till this point, expression of this gene was higher in resistant genotype than the susceptible one however, thereafter it declined in resistant genotype and increased in susceptible genotype. The expression of Ca ATP/ADP transporter gene (Fig. 4E) was higher in resistant genotype than the susceptible genotype at all the time points studied

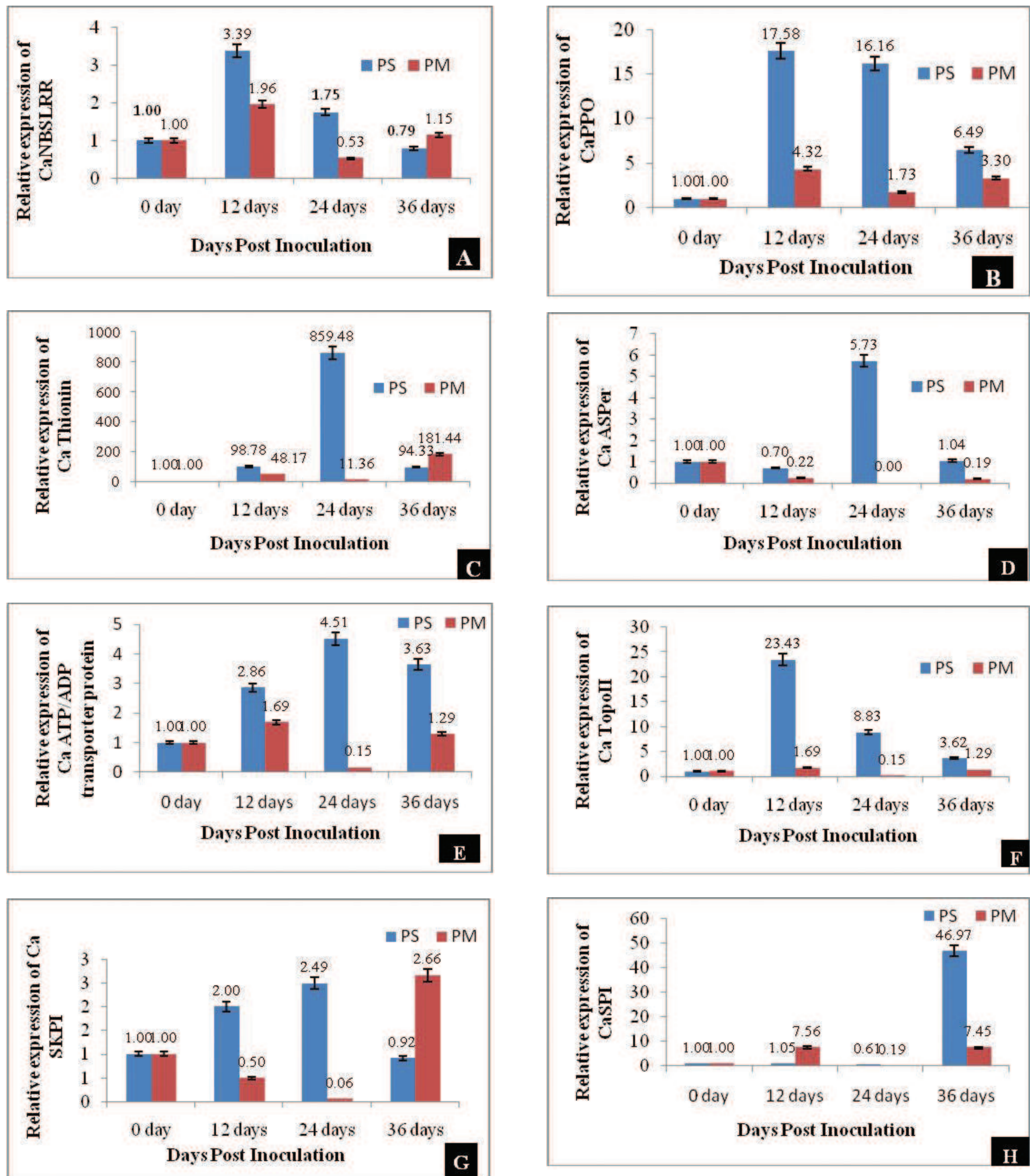

Fig. 4. Relative fold change in the expression of different genes after different days post inoculation

but expression increased in resistant genotype only upto 24 dpi. A similar trend of expression was observed in CaAsper (Fig. 4D) where also a steep rise in expression was observed at 24 dpi in resistant genotype. The expression of CaTopoII (Fig. 4F) increased up to 12 dpi (23.43 folds) in DLS-Sel-10 thereafter it decreased, however there was no specific trend in expression of this gene in Phule Mukta. The

expression profile of Ca SKP1 (Fig. 4G) revealed that its expression increased with age upto 24 dpi in the resistant genotypes DLS-Sel-10 and was lowest (0.92 fold) at 36 dpi while decreased with increase in dpi in Phule Mukta upto 24 dpi (0.06 fold) but was found to be highest (2.66 fold) at 36 dpi. The CaSPI expression (Fig. 4H) did not show much variation at different time points in resistant line upto 24 dpi thereafter it showed a sharp increase to 46.97 fold at 36 dpi while in the susceptible line Phule Mukta it did not show any specific pattern. As shown in Fig. 5 when the expression profiles of all the eight defense related genes studied under present investigation were analysed the heat map generated showed two major clusters one comprising five genes i.e., Ca AsPer, Ca Thionin, Ca ATP/ADP transporter, CaPPo, Ca TopoII and the other comprising three genes i.e., Ca SKP1, Ca NBS and CA SPI.

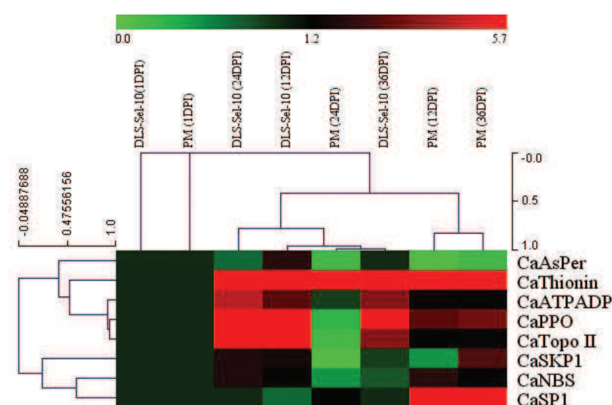

Fig. 5. Heat maps generated for the expression pattern of different defense related genes in Chilli

Discussion

Identification of resistant lines is the foremost requirement for any resistance breeding program. Srivastava et al. (2017) have earlier identified three genotypes namely, DLS-Sel-10, WBC-Sel-5 and PBC-142 resistant to ChiLCV however, the mechanism of resistance in these genotypes and the host genes responsible for resistance are still unknown. In addition, comparative studies involving conservation and dispersion of genes conferring Tomato Yellow leaf curl virus resistance was also done between tomato and chilli (Mangal et al. 2017). DLS-Sel-10 in the present study was observed to be a tolerant host as in the challenge inoculation experiment even after 36 days it did not develop severe disease symptoms whereas the disease score as well as disease symptoms kept increasing in the susceptible genotype Phule Mukta and reached to a score of 4 after 24 dpi with the virulent white flies. Further both the PCR and qPCR assays revealed the presence of virus in both the hosts *viz.*, DLS-Sel-1 as well as Phule Mukta however, the viral titre in the susceptible host increased at a very high rate as compared to ChiLCV resistant DLS-Sel-10. Viral titre was found to increase to 6435 fold after 36 days of challenge inoculation in Phule Mukta while it reached to only 51 fold in DLS-Sel-10 after the same duration. This observation is indicating that DLS-Sel-10 is allowing the virus to accumulate in it to a certain extent without any adverse effect on its fitness and without any disease symptoms. Cooper and Jones (1983) have defined tolerance as a mitigation of the impact of virus infection irrespective of the pathogen load. In a tolerant type of plant viral interaction, although a significant virus load is sustained, the plant growth, yield or reproduction attributes are only minimally affected and visible symptoms are either absent or mild. Because viruses are intracellular obligate parasites, they require host resources to complete their infection cycle (Culver and Padmanabhan 2007; Nagy and Pogany 2012; Wang 2015). Therefore, high virus fitness is at the expense of the host in symptomatic susceptible interactions. In resistant interactions, the plant fitness is preserved by preventing virus accumulation or systemic movement. According to Paudel and Sanfaçon (2018) in tolerant interactions, virus fitness is reduced by preventing over-accumulation of viral RNAs or by minimizing the concentration or activity of viral proteins that play a role in virulence which in turn limits the damage to the host and the same phenomenon was observed in DLS–Sel-10 in the present study. Further, a very mild leaf curling in the DLS-Sel-10 after 24 days post inoculation was observed but as the age advanced symptom recovery was recorded in this host while the reverse was observed in Phule Mukta. Ghoshal and Sanfacon (2014) have also reported symptom recovery phenomenon in plant-virus interactions wherein in plants initially display systemic symptoms but later recover from infection as exemplified by the emergence of young asymptomatic leaves.

Infection of the hosts by pathogens induce hypersensitive response in plants which in turn is associated with the induction of the expression of various defense related genes. A number of plant antiviral resistance genes (R genes) have been characterized (de Ronde et al. 2014; Miyashita and Takahashi 2015; Sanfacon 2015; Hashimoto et al.

2016) and many defense response genes have been reported to be activated by dominant R genes in resistant/tolerant interactions (Bengyella et al. 2015). Nucleotide-binding site leucine-rich repeat is a conserved domain protein which has been reported to have a role in specific resistance to various pathogens including several plant viral interactions involving Cucumber Mosaic Virus (CMV) (Seo et al. 2006), Tobacco Mosaic Virus (TMV) (Dinesh-Kumar et al. 2000), and Mungbean Yellow Mosaic India Virus (MYMIV) (Maiti et al. 2012). In the present study, the expression of Ca-NBS-LRR was found higher in the ChiLCV resistant genotype DLS-Sel-10 than the susceptible Phule Mukta both at 12 and 24 days after challenge inoculation, although afterwards the expression was higher in susceptible genotype. Similar observations wherein expression of NBS-LRR was found to be upregulated significantly in ChiLCV-inoculated resistant chilli cultivar Punjab Lal was observed by Kushwaha et al. (2015a). Upregulation of NBS-LRR gene (namely, CRY1) expression has also been correlated with resistance in *Vigna mungo* against MYMIV and its suppression with susceptibility in susceptible plants infected with Tomato leaf curl New Delhi virus (Kushwaha et al. 2015b).

Polyphenol oxidases (PPOs) are a group of Cu-containing enzymes that catalyse the oxidation of several phenols produced during pathogen attack to o-quinones (Taranto et al. 2017; Raj et al. 2006). They are shown to be involved in maintaining the basal defense against fungi, bacteria, and viruses (Constabel et al. 1995; Mayer 2006; Poiatti et al. 2009). They protect the cells by scavenging the reactive oxygen species and reactive phenolic molecules produced during the pathogen attack (Kushwaha et al. 2015a). In the present investigations transcripts of this gene were found to be accumulated at much higher level in DLS-Sel-10 than Phule Mukta at all the time points studied. The observations of present study appear to be in line with that of Kushwaha et al. (2015a) who also observed higher expression of Ca-PPO in ChiLCV resistant cultivar than the susceptible one following ChiLCV infection.

Thionins are antimicrobial peptides that are reported to be involved in plant defense against bacteria, fungi as well as nematodes (Epple et al. 1997; Pelegrini and Franco 2005; Ji et al. 2015; Almaghrabi et al. 2019). The expression of this gene was found to be consistently high in resistant chilli genotype than the susceptible one till 24 dpi and a highest relative fold change of 859.48 was observed in DLS-Sel-10 at 24 dpi. Kushwaha et al. (2015a) had also observed highest expression of this gene at 21 dpi followed by a decline in its expression in ChiLCV resistant line suggesting a similar mechanism exists in DLS-sel-10.

Ascorbate peroxidase constitutes one of the major antioxidant defences and hydrogen peroxide detoxifying system in plant cells. The expression of ascorbate peroxidise genes is regulated in response to biotic and abiotic stresses and is directly involved in the protection of plant cells against adverse environmental conditions (Caverzan et al. 2012). The persistent high level of expression of this gene in the resistant line in the present study is suggesting a role of this gene in imparting resistance to DLS-sel-10.

ATP/ADP transporters are responsible for ATP transport across membranes in an exchange mode with ADP (Pebay-Peyroula et al. 2003; Reiser 2004). Although no direct role of this gene in plant defence against viruses is known however, upon pathogen attack a number of vital functions of plants are compromised including energy transport across membranes and the extent of damage will be more in susceptible plants as compared to resistant ones. Consistently, higher expression of this gene in resistant chilli genotype as compared to susceptible Phule Mukta was observed till 36 dpi, which is in line with the observations made by Kushwaha et al. (2015a).

DNA Topoisomerases are the enzymes which play a role during DNA replication, transcription or recombination events. They have an ability to remove or add DNA supercoils and untangle the snarled DNA. Riffat et al. (2016) have reported that TopoII also plays a pivotal role in salt stress tolerance in plants. In the present investigations it was observed that the expression of CaTopoII was maximum (23.43 folds) after 12 dpi in resistant genotype DLS-Sel-10 thereafter it kept decreasing till 24 dpi however, the expression was much higher in resistant line as compared to the susceptible at all time points, indicating a possible role of this gene against biotic stresses also.

The Ubiquitin-proteasome system is a major proteolysis pathway for degrading unwanted and misfolded proteins in eukaryotic cells (Ciechanover et al. 2000). S-phase kinase-associated protein 1 (SKP1) is a crucial subunit of the proteasome system and is required for the maintenance of protein homeostasis (Bai et al. 1996). The expression of Ca-SKPI kept increasing in resistant line following

challenge inoculation till 24 dpi when the relative fold change in expression was 2.49 folds while it was only 0.06 folds in the susceptible line suggesting thereby the involvement of proteosomal pathway in resistance to ChiLCV. Similar expression profile has earlier been observed for this gene in ChiLCV resistant plants by Kushwaha et al. (2015a) in chilli. High levels of proteinase inhibitors are often found in many plants belonging to the *Solanaceae* family (Plate et al. 1993). Plants are reported to synthesize inhibitory polypeptides against pathogenic microorganisms (Ryan 1990). However, in the present study the Ca-SP1 did not show any direct correlation with the resistance to ChiLCV.

In general it was observed that the resistant line DLS-Sel-10 had an innate mechanism whereby it did not allow the accumulation of ChiLCV in it beyond 51 folds till 36 dpi, whereas the susceptible line Phule Mukta lacked this resistance and recovery mechanism and viral titre kept increasing with increase in dpi. Present study indicated that the genes CaNBS, CaPPO, Cathionin, Ca Asper, Ca ATP/ADP transporter, Ca TopII, and Ca-SKP1 played an important role in the resistance and recovery mechanism of DLS-Sel-10.

Authors' contribution:

Conceptualization of research (MM, AS, PK); Designing of the experiments (MM, AS); Contribution of experimental materials (AS, BM); Execution of field/ lab experiments and data collection (SJM, KS, VS); Analysis of data and interpretation (MM); Preparation of the manuscript (MM).

Declaration

The authors declare no conflict of interest.

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