Polymorphism

RESEARCH

qRT-PCR based quantitative analysis of gene expression in *C. annuum* L. in response to *P. capsici* infection

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ABSTRACT

Despite the economic importance of chili pepper (*C. annuum* L.), its productivity is highly affected by oomycete pathogens, *P. capsici*. It is a soil-borne pathogen and has a broad host range in the families of Solanaceae. Several investigations have been made to detect and analyze the gene expression associated with pathogen infection in *C. annuum*. However , understanding the disease response in different pepper landraces against *P. capsici* infection is limited. Hence, the present study aimed to analyze gene expression associated with *P. capsici* infection using qRT-PCR from our transcriptome sequencing data of previously established resistant (GojamMecha_9086) and susceptible (Dabat_80045) chili pepper. Based on the qRT-PCR results, a high expression level (~ 2.70 fold) was exhibited by glycine-rich cell wall structural protein-like (*GRCW*) gene followed by non-specific lipid transfer protein GPI-anchored 2-like (*nsLTP*) gene (~1.99 fold) in RI leaf. In contrast, *GRCW* gene expression was significantly down-regulated in SI leaf (with ~ -2.86-fold change), showing their role during *P. capsici* infection. However, the squamosa promoter-binding protein 1(*SPBP*) gene was down-regulated in both RI (~ -1.64 fold) and SI (~ -1.95) leaves. In conclusion, the level of resistance and susceptibility in the two contrasting landraces is possibly due to the difference in the level of genes expression and molecular variations in the defense response. The current findings could be exploited by molecular breeders to help improve chili peppers by selecting landraces with defense-related genes. Further functional validation using reverse genetics methods such as VIGS, RNAi, and CRISPR/Cas9 system is needed.

KEYWORDS: *C. annuum*, *P. capsici*, qRT-PCR, Gene expression, Defense response

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INTRODUCTION

Chili pepper (*Capsicum annuum* L.) is grouped under the Solanaceae family and genus Capsicum. It can be used as a spice, consumed fresh, or in powder form when red (Dias et al, 2013; Wahyuni et al, 2013; El-Ghoraba et al, 2013). Moreover, chili pepper (Capsicum spp.) has medicinal values as a drug, condiment, ointment, and relief of pain (Pawar et al, 2011; Chamikara et al 2016). Despite its economic importance, many pathogens cause disease in chili pepper and affect its yields worldwide. Among these pathogens, oomycete pathogens, *Phytophthora capsici* is considered as the most economically important pepper pathogen causing diseases like downy mildew, Phytophthora late blight, collar rot, purple blotch, fruit and root rot in chili pepper (Yin et al, 2012). It is a soil-borne oomycete pathogen (Barksdale et al, 1984; Ristaino and Johnston 1999; Walker and Bosland, 1999) and has a broad host range in the families of Solanaceae such as tomato, eggplant, pepper, squash, pumpkin, zucchini, cucumber and watermelon in the family Cucurbitaceae (Ristaino and Johnston, 1999).

Several studies have been conducted to detect and analyze the genes in *C. annuum* associated with pathogens. Differential regulation of numerous genes related to defense response, including PR genes, hormone homeostasis in peppers against *P. capsici* infection, has been reported (Wang et al, 2015; Kim et al, 2019; Zhen-Hui Gong et al, 2013). For instance, the *CanPOD* gene was significantly induced in pepper leaves under *P. capsici* infection (Wang et al, 2013a). The *CaLTPI* and *CaLTPIII* genes were predominantly expressed in various pepper tissues infected by *P. capsici* (Jung et al, 2003). Lipid transfer protein (*LTP*) isolated from the seeds of *C. annuum* is known as *Ca-LTP1* involved in morphological deformation and changes in the cells of pathogens (Majid et al, 2016). However, understanding the disease response in different pepper landraces against *P. capsici* infection is limited (Wang et al, 2015). The advancement of NGS technology coupled with high-precision bioinformatics technologies has enhanced understanding of the molecular mechanism associated with foot-rot (Huasong et al, 2016). Wang et al. (2015) employed RNA-sequencing technology to uncover differentially expressed genes associated with *P. capsici* infection in resistant line PI201234 and susceptible line "Qiemen" of chili pepper. Sequence comparison of the candidate resistance gene analogs (*RGAs*) was studied between two resistant and susceptible capsicum landraces under *P. capsici* infection (Jundae et al, 2019). The transcriptome sequencing using Illumina sequencing methods provided valuable sequencing information from Aleo vera root and leaf tissue (Choudhri et al, 2018). Despite the numerous NGS-based studies, understanding of the disease responses and resistance mechanisms in pepper against *P. capsici* infection remains limited (Wang et al., 2015). Therefore, the present study's objective was to analyze the expression levels of genes in resistant (GojamMecha_9086) and susceptible (Dabat_80045) chili pepper associated with *P. capsici* infection using qRT-PCR from our Illumina transcriptome sequencing data. The current finding provided invaluable information about the expression of genes in C. capsici associated with *P. capsici* infection, which could help molecular breeders select landrace containing resistance genes for improving disease resistance in *C. annuum*.

MATERIALS & METHODS

Plant growth, inoculation and RNA isolation

The chili pepper seeds of previously established resistant (GojamMecha_9086) and susceptible (Dabat_80045) (Rabuma et al, 2020) varities were obtained from the Ethiopian Biodiversity Institute (https://www.ebi.gov.et/). The seeds were grown in pots containing sterilized soil, sand, and compost with a ratio of 1:1:1 (Andrés Ares et al, 2005) in the Department of Bio and Nanotechnology laboratory

greenhouse, Guru Jambheshwar University of Science and Technology, Hisar, India. The seedlings of both resistant (GojamMecha_9086; control & infected) and susceptible (Dabat_80045; control & infected) genotypes were grown in three biological replicates with three seedlings/ replication/pot for qRT-PCR analysis. Moreover, *P. capsici* was isolated from infected pepper tissue from farmer's fields around Hisar and inoculation was performed as per the methods used in Andres Ares et al, (2005) and Rabuma et al, (2020).

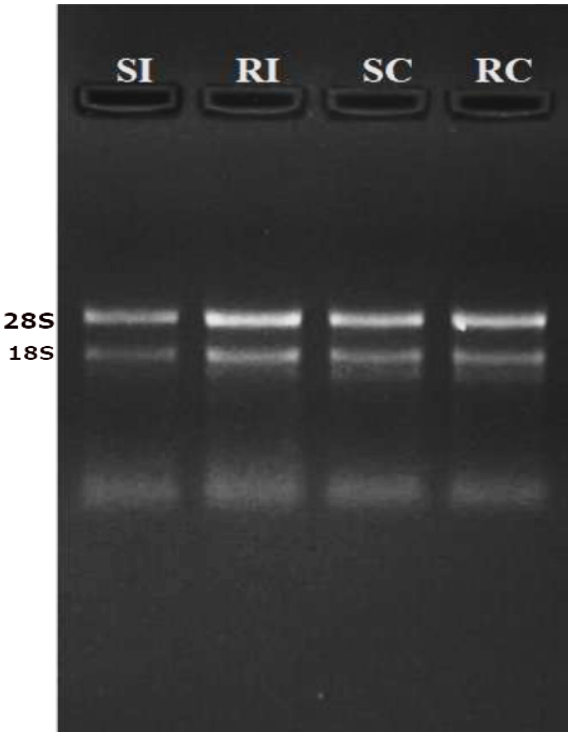

Figure 1. Quality analysis of RNA samples on 1% denatured Agarose gel.

Total RNA isolation and cDNA preparation

After five days post-inoculation (dpi), the 4th and 5th leaves of control and inoculated leaf samples were collected for RNA isolation. The total RNA was isolated from the four samples, i.e. resistance control (RC), resistance infected (RI), susceptible control (SC) and susceptible infected (SI) leaf sample using Quick-RNA Plant Miniprep Kit (ZYMO Research) as per the manufacturer's protocol. The quantity and quality of isolated total RNA were checked using Nanodrop spectrophotometer and 1% agarose gel electrophoresis for 45 min at 90 V, respectively (Figure 1). Furthermore, the quality of the RNA samples was thoroughly evaluated by Bioanalyzer 2100 (Agilent, USA) to ensure >8.5 integrity number (RIN). The cDNA was synthesized using RevertAid First-strand cDNA synthesis kit (Thermo Fisher Scientific, USA) using Oligo dT(18) primers as per the manufacturer's protocol. Then, the synthesized cDNA was used for expression analysis of genes associated with *P. capsici* infection via qRT-PCR.

Expression analysis of genes associated with *P. capsici*

For expression analysis of randomly selected genes using qRT-PCR, we have utilized the transcriptome data from four-leaf samples (RC, RI, SC and SI) of chili pepper. Six most differentially expressed genes (DEGs) were randomly selected from our transcriptome sequencing data (sequencing data archived on SRA at the link https://www.ncbi.nlm.nih.gov/biosample/16251797) and primers were designed using Primer Express 3.0.1 software (Table 1). The qRT-PCR reaction was performed in three replicates for each sample using SYBR® Green Jump Start™ Taq Ready Mix™ (Sigma) on Applied Biosystems' Step One™ Real-Time PCR System (Choudhri et al, 2018). qPRT-CR analysis was performed with a total reaction volume of 10µl containing 5µl of 2x SYBR Green JumpStart Taq ready mix, 1µl of each forward primer (10 µM) and reverse primer (10 µM), 2µl of 100ng/µL cDNA and 1µL of nuclease-free water. The amplification of the qRT-PCR run method was adjusted as per the following conditions: the reaction system was heated to 94°C and denatured for 2 min, the primary reaction (denaturation at 94°C for 15 seconds, annealing 58-60°C for 60 seconds, and template extensions at 72°C for 15 seconds) for 40 cycles. The qRT-PCR generated data was analyzed using StepOne™ Software v.2.2.2. The *C. annuum actin-7 like* gene (GenBank: GQ339766.1) was used as an internal control, and expression levels between control and treated leaf samples were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and

Schmittgen, 2001). The standard error in expression levels of three replicates was denoted by error bars in the figures, and the results were expressed as mean value ± SD. The qRT-PCR amplified product was run and visualized on 1% agarose gel as per the method used by Gupta et al. (2017).

PCR based analysis of expressed sequence tag (EST) genes associated with *P. capsici* infection

From the selected two contrasting landraces, GojamMecha_9086 (resistant) and Dabat_80045 (susceptible), DNA extraction was carried out using the CTAB protocol adapted from the Kalisz lab (Doyle and Doyle, 1987) with minor modifications. For PCR analysis, the three most differentially expressed EST genes were randomly selected and three sets of primers were designed using web-based tools Primers 3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) from our transcriptome sequence data (archived on SRA at the link https://www.ncbi.nlm.nih.gov/biosample/16251797) (Table 2). The thermal cycler reaction was performed using GoTaq® Green master mix reagent kit (Promega, USA) as per the manufacturer's instructions with little modifications as a total reaction volume of 10µl containing 5µl of GoTaq master mix, 1µl of each forward primer (10 µM) and reverse primer (10 µM), 2µl DNA and 1µL of nuclease-free water of molecular biology grade. The annealing temperature for each primer was optimized using a gradient PCR Peqlab machine by mediating the calculated annealing temperature of each primer. The amplification conditions for the PCR run method was adjusted as follows: the reaction system was heated to 94°C and denatured for two min, then the reaction was performed as (denaturation at 94°C for 2min, annealing 58-60°C for 60 seconds, and template extensions at 72°C for 15 seconds) for 35cycles. The PCR amplified products were visualized using 1% agarose gel electrophoresis as described by Gupta et al, 2017.

Table 1. The list of primers used for qRT-PCR based expression analysis of genes *C. annuum* L. associated with *P. capsici* infection.

Sr. No.	Gene ID	Primer Name	F Primer (5′ to 3′)	R Primer (5′ to 3′)	Amplicon size (bp)	Tm (^{0}C) (PrimerBlast)
1.	XP_016540871.1	GRCW	TCAGACCACAGCAGCAAGAG	ACTCCACCAACGTTGCTACC	153	60.3
2	XP_016545510.1	SPBP	GCCTTCCTGTCAAGTTGAGG	TTTCTGAACCTGCTGCATTG	156	60.0
3	XP_016542753.1	PAL	TTGCACAAGTTGCATCCATT	GCACCACCATTCTTGGTTCT	156	60.0
4	XP_016577409.1	nsLTP	AATGCCACAAAACCGTCATC	GAAGGAGACCCTGGAGAACC	146	60.8
5	XP_016560122.1	PODs	TCATTCTTTCGGTTGCCGGA	ACTACCTAGCCTCAAGGGCA	100	59.0
6	XP_016550553.1	UDP-GcT	CCAGTCTCGACGAACAACAA	TCGAATTCCATCCACAGTGA	140	60.0
Internal control genes						
Sr. No	Gene		F Primer (5′ to 3′)	R Primer (5′ to 3′)	Amplicon size (bp)	Tm (^{0}C)
1	actin-7-like		TCCTCGCATCACTTAGCACC	GCCCATCTCAAAGACTTGCC	138	58.0

RESULTS

Quality and quantity analysis of RNA

The Nano-drop spectrophotometer total RNA quantitative analysis revealed a total of 1653 ng/l in RC, 1239 ng/l in RI, 462 ng/l in SC, and 528 ng/l in SI leaf sample (Table 3).The quality of isolated total RNA from four-leaf samples (RC, RI, SC,SI) checked on 1% denaturing RNA agarose gel exhibited two intact bands, i.e. 28S (the upper more intensified band) and 18S (the lower band),

confirming the intactness of RNA isolated from the four-leaf samples (Figure 1). The mean peak sizes from the Agilent Tape Station profile result are shown in Figure 2. Hence, this QC passed total RNA samples were used for gene expression analysis via the qRT-PCR based method.

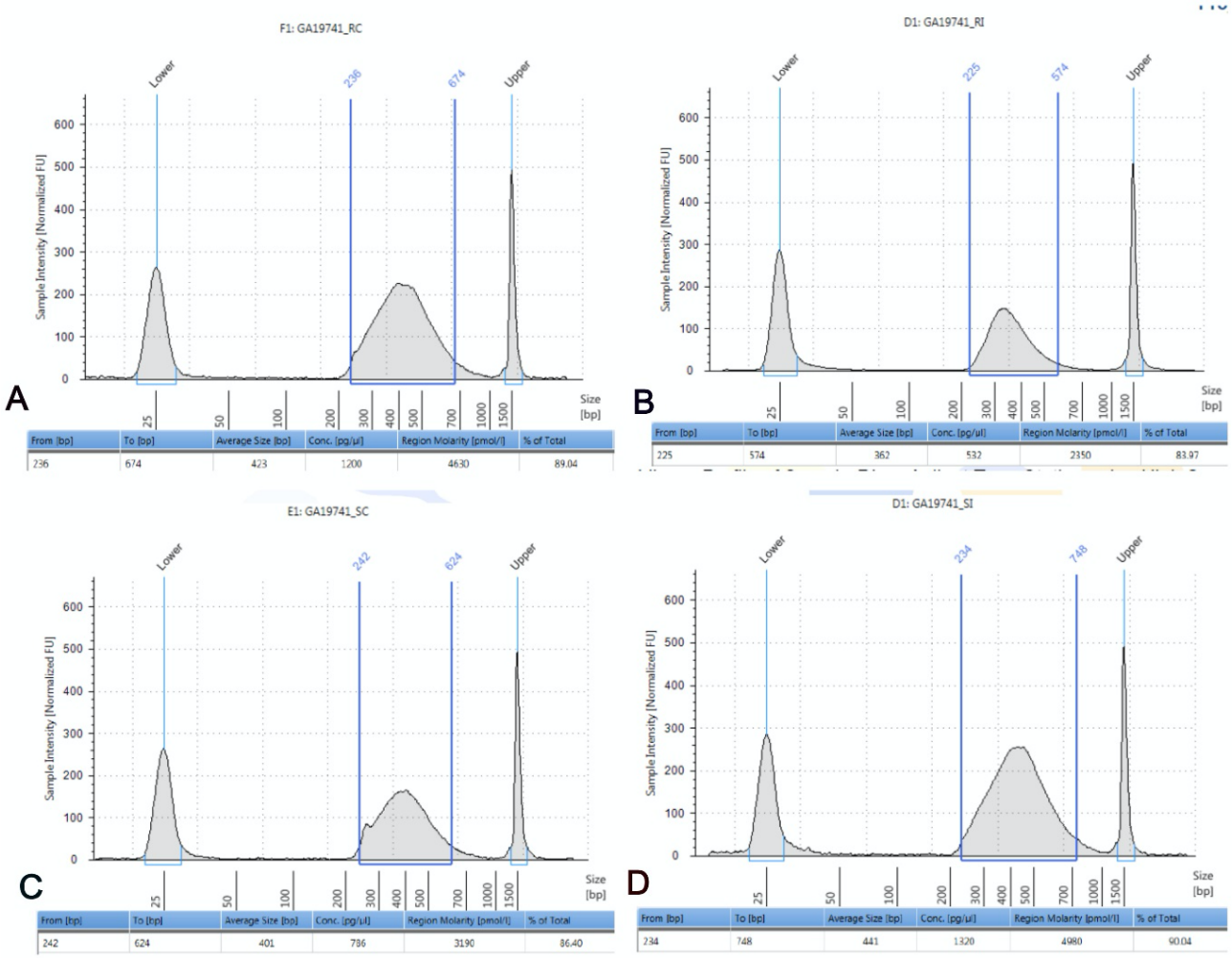

Figure 2. The profile of RNA from leaf Sample-A) RC, B) RI, C) SC, and D) SI on Agilent Tape Station using High Sensitivity D1000 Screen Tape.

Expression analysis of genes associated with *P. capsici* infection

The qRT-PCR expression analysis revealed all the six analyzed genes, except the squamosa promoter-binding protein 1(*SPBP*) gene, shown to be up-regulated between RC and RI leaf samples (Figure 3). Moreover, all genes, except the phenylalanine ammonia-lyase(*PAL*) gene, were down-regulated in between SC and SI leaf samples (Figure 3). The highest expression level (~2.70 fold) was exhibited by the glycine-rich cell wall structural protein-like (*GRCW*) gene followed by non-specific lipid transfer protein GPI-anchored 2-like gene (~1.99 fold) in RI leaf, showing their involvement in defense response against *P. capsici* infection. However, the peroxidase 42 gene exhibited lower expression (~1.07 fold) under *P. capsici* infection in the RI leaf. The glycine-rich cell wall structural protein-like (*GRCW*) gene expression was significantly down-regulated in the SI landrace leaf (~ -2.86-fold change). The squamosa promoter-binding protein 1(SPBP) gene was down-regulated in both RI (~ -1.64 fold) and SI (~ -1.95) leaves. Comparatively, motst of the genes analyzed were up-regulated in

resistance landrace(RI) leaf but down-regulated in susceptible (SI) landrace under *P. capsici* infection.

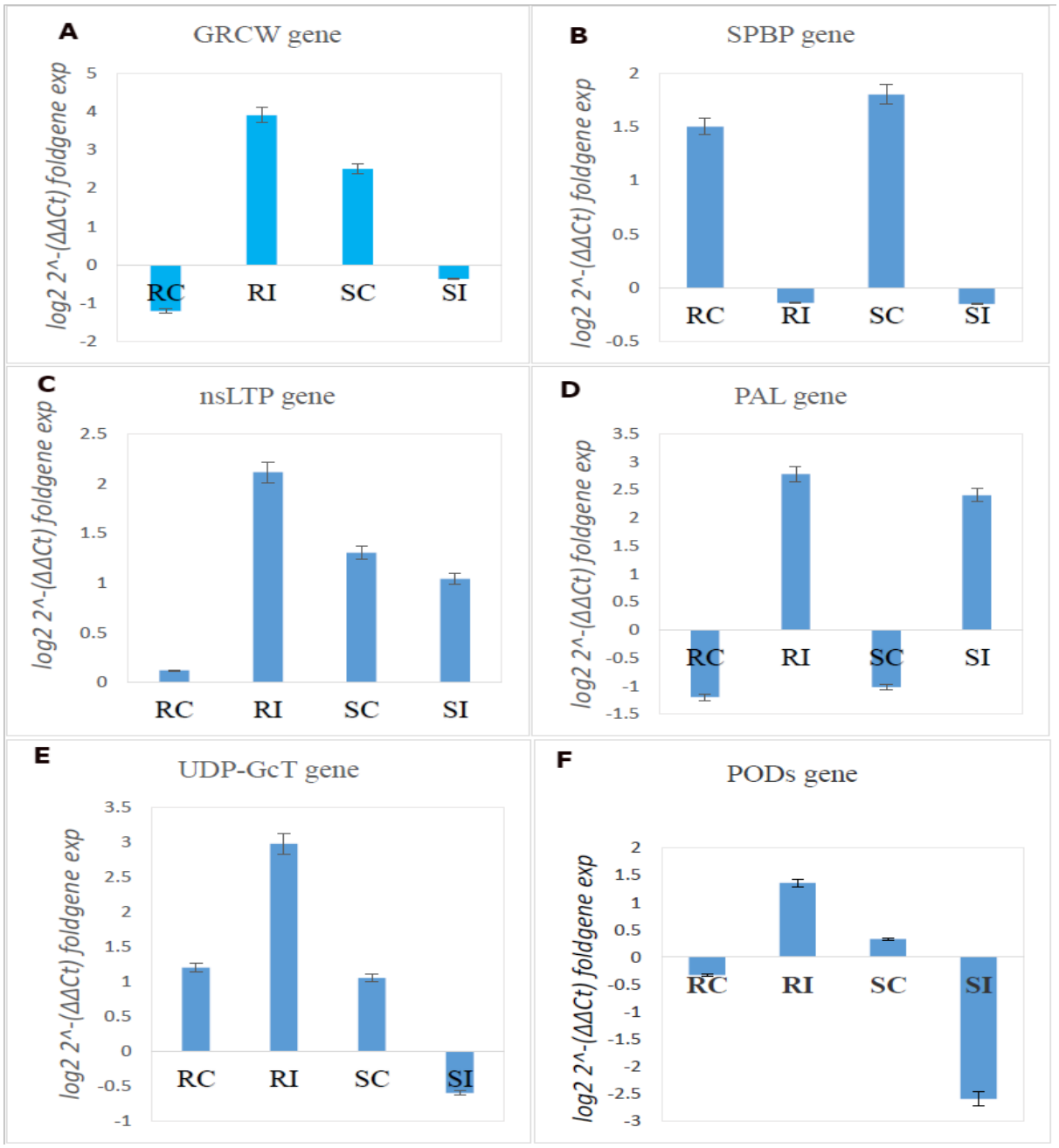

Figure 3. Expression analysis of differentially expressed selected genes in *C.annuum* L. associated with *P.capsici* infection. A) glycine-rich cell wall structural protein-like (*GRCW*) gene, B) squamosa promoter-binding protein 1(*SPBP*) gene, C) non-specific lipid transfer protein GPI-anchored 2-like (*nsLTP*), D) phenylalanine ammonia-lyase(PAL) gene, E) UDP-glycosyltransferase 74E2-like (*UDP-GcT*) gene, F) peroxidase 42 (*PODs*) gene.

Table 2. Three pairs of primers for genes related to *P. capsici* infection were designed for PCR analysis.

CDS/Unigenes ID	Genes associated *P.capsici* infection	Primer Sequence		Length (bp)	Tm(° C)	GC %
CDS_8957_Unigene_25357_Transcript_38867	XP_016581374.1 putative lipid-transfer protein DIR1	Forward	GCTGCAGGAACATCAACAAC	20	59.3	50.00
		Reverse	CAACTTGCTTGGGATCGAG	19	59.4	52.60
CDS_18554_Unigene_4480_Transcript_6844	XP_016555530.1 endochitinase EP3-like	Forward	ACAAAACTGTGGGTGTGCAG	20	59.6	50.00
		Reverse	CGTGAGATTATCCCCAGCAT	20	59.9	50.00
CDS_19877_Unigene_47749_Transcript_71400	XP_016563728.1 defensin J1-2-like	Forward	CCCTCCCCCACTAAAGAAAA	20	60.3	50.00
		Reverse	GAATTAACAACCCTCGAGGAAC	22	59.0	45.50

Table 3: Quantification of RNA samples using nanodrop spectrophotometry.

Sample ID	NanoDrop reading(ng/µl)	NanoDrop OD A260/280	Nano Drop OD A260/230	Remark
RC	1653	2.05	2.01	QC Pass
RI	528	2.10	2.30	QC Pass
SC	462	2.01	2.20	QC Pass
SI	1239	1.99	1.79	QC Pass

Gene expression associated with *P. capsici* infection

The PCR analysis revealed that more intensive PCR product bands of the three genes were observed in the treated sample leaf of chili pepper. The PCR product band for the putative lipid transfer protein gene was highly intensified in the RI leaf as compared to the corresponding control leaf (RC), while the PCR product band intensity was the same for both SC and SI leaves. Moreover, the band intensity for NP_001311656.1endochitinase EP3-like precursor gene was high in the RI leaf and low in RC, while the intensity of the band was similar for both SC and SI leaf samples. The expression intensity for XP_016563728.1 defensin J1-2-like is more visible in resistance (RI) than in susceptible (SI) leaf landrace, showing high expression in resistance landrace under *P. capsici* infection. Each of the three EST genes has the PCR product band size across all four leaf samples, i.e., *PLTP* gene=500bp, *Ep3* gene=745bp and defensin(*dfsn*) gene=700bp (Figure 4).

DISCUSSION

qRT-PCR is a generally acknowledged method for gene expression analysis due to its precision and reproducibility (Demidenko et al, 2012). The qRT-PCR expression analysis of selected genes showed that the differential expression of glycine-rich cell wall structural protein-like (*GRCW*), squamosa promoter-binding protein 1 (*SPBP*), non-specific lipid transfer protein GPI-anchored 2-like (*nsLTP*), phenylalanine ammonia-lyase (*PAL*), UDP-glycosyltransferase 74E2-like (*UDP*-GcT), and peroxidase 42 (*POD*s) genes were associated with defense response against *P. capsici* infection. The gene for phenylalanine ammonia-lyase (*PAL*) was

up-regulated (~1.58 fold) between RC and RI leaf samples. The pepper (*C. annuum* L.) *PAL* (*CaPAL1*) gene induced in pepper leaves by avirulent Xanthomonas campestris pv. vesicatoria (Xcv) infection and overexpression (OX) of *CaPAL1* gene in Arabidopsis conferred increased resistance to Pseudomonas syringae pv. tomato (Pst) (Kim and Hwang, 2014).

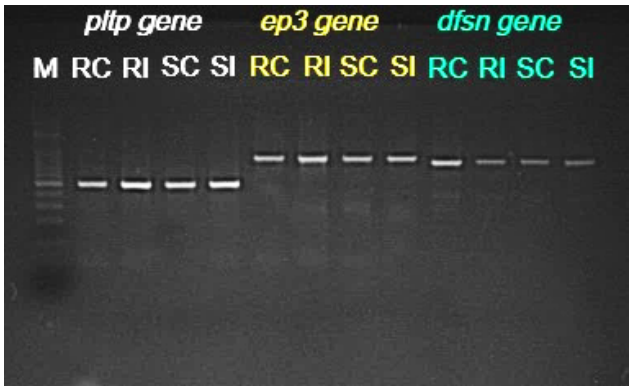

Figure 4. The PCR amplified product band using the three primers of EST genes associated to *P.capsici* defense genes (1ca-PLTP, 2ca_EP3, and 3ca_dfsn J1-2 for three differentially expressed genes i.e. putative lipid transfer protein gene, endochitinase EP3-like gene, and defensin J1-2 like gene, respectively) associated with defense response against *P.capsici* infection. The band size for the *pltp gene* is 500bp, *ep3* gene is 745bp and the defensin (*dfsn) gene* is 700bp.

In the present study, the gene for squamosa promoter-binding protein 1(*SPBP*) was down-regulated (~ -1.64 fold) between RC and RI leaf samples. These gene families were involved in different pathways like plant morphogenesis, floral transition, male sterility, biosynthesis of gibberellic acid (GA), the transition from the vegetative to reproductive stage, endoplasmic reticulum (ER) stress signalling, and environmental stress responses (Cardon et al, 1997; Zhang et al, 2007; Shikata et al, 2012; Ning et al, 2017; Zhang J. et al, 2017; Gong et al, 2016). Gong and co-workers identified a total of 15 members of the SBP-box gene under both compatible and incompatible strains of *P. capsici* infection and showed that the SBP-box genes of pepper could be classified into six groups (Gong et al, 2016). Silencing the squamosa promoter binding protein (SBP)-box (*CaSBP08*) and *CaSBP12* genes enhanced pepper resistance to *P. capsici* infection, indicating that *CaSBP12* negatively regulates defense response against *P. capsici* (Gong et al, 2019; Gong et al., 2020). Moreover, CaSBP08 overexpression in *Nicotiana benthamiana* enhanced susceptibility to *P. capsici* infection (Gong et al, 2020). Therefore, our result is supporting the result of Gong et al. (2019, 2020), depicting that the down-regulation of the squamosa promoter-binding protein 1(SPBP) (-1.64 fold) gene enhanced resistance in resistance (RI) landrace against *P. capsici* infection.

Plant non-specific lipid-transfer proteins (*nsLTPs*) are small basic proteins, abundantly present in the higher plants, and play essential roles against biotic and abiotic stresses (Gang et al, 2015; Ji et al, 2018). Non-specific lipid transfer proteins (nsLTPs) belong to the pathogenesis-related protein family, and several of them act as positive regulators during plant disease resistance (Wang et al, 2021). The *CaLTPI*, *CaLTPII*, and *CaLTPIII* are corresponding to pepper lipid transfer protein (LTP) genes isolated from leaf lesions of pepper (*C. annuum*) infected with Xanthomonas campestris pv. Vesicatoria (Jung et al, 2003). Recently, Wang and co-workers reported the expression of the *StLTP10* gene in potato leaves induced by *P. infestans*, indicating the *StLTP10* gene positively regulates plant resistance to *P. infestans* (Wang et al, 2021). The *CaLTPI* and *CaLTPIII* genes were predominantly expressed in various pepper tissues infected with *P. capsici* (Jung et al, 2003). Sarowar and co-workers reported the up-regulation of *nsLTPs*, *CaLTP1* and *CaLTP2* genes in pepper enhanced resistance to oomycete and bacterial pathogens (Sarowar et al, 2009). We also recorded up-regulation of non-specific lipid-transfer proteins (nsLTPs) (~1.99 fold) between RC and RI leaf samples, showing its role in enhancing resistance against *P. capsici* infection. However, qRT-PCR expression analysis revealed that non-specific lipid-

transfer proteins (nsLTPs) gene was down-regulated (~ -0.26 fold) in between SC and SI leaves, possibly resulting in enhancement of susceptibility of the landrace to *P. capsici* infection. Furthermore, *C. annuum* lipid transfer protein (*CaLTP1*) gene expression was induced in the incompatible interaction with TMV-P0 (Park et al, 2002).

Neutelings and co-workers investigated the potential role of the *UGT72E* gene family in regulating lignification in Arabidopsis (Neutelings et al, 2020). Moreover, Pastor and co-workers investigated the role of glycosyltransferases *UGT74F1* and *UGT74F2* gene in establishing basal resistance of Arabidopsis against *Pseudomonas syringae* pv tomato DC3000 (Pst) (Pastor et al, 2014). Campos et al. (2019) reported that the *Twi1* glycosyltransferase gene regulates quercetin and kaempferol levels in tomato plants, affecting plant resistance to viral infection. Zhang et al. (2014) reported the UDP-glycosyltransferase gene modulating the tolerance of plants against various biotic and abiotic stresses. Xie et al. (2016) reported the accumulation of the gene encoding UDP-glycosyltransferase in *C. annuum* exposed to 24-epibrassinolide (*EBR*). Glycosyltransferases (*UGT*s) gene was highly expressed in Arabidopsis following infection with *Pseudomonas syringae* pv tomato or after treatment with salicylic acid, methyl jasmonate, and hydrogen peroxide (Saindrenan et al, 2005). Tripathi and co-workers have reported the up-regulation of UDP-glycosyltransferase 73C6-like (~1.92 fold) in Musa balbisiana against *Xanthomonas campestris* pv. Musacearum (Tripathi et al, 2019). Our qRT-PCR expression analysis revealed accumulation of UDP-glycosyltransferase 74E2-like gene (~1.78 fold) in resistant landrace (RI leaf) and its down-regulation (~-1.65 fold) in susceptible landrace (SI leaf), showing its potential role in modulating tolerance against *P. capsici* infection in resistant landrace.

CONCLUSION

qRT-PCR expression analysis of genes showed the role of these genes in defense response against *P. capsici* infection in the two contrasting chili pepper landraces, i.e. GojamMecha_9086 and susceptible (Dabat_80045). It was shown that genes involved in defense response against *P. capsici* infection were differentially accumulated in RI compared to SI leaves. The level of resistance and susceptibility among the chili pepper landraces is primarily due to the difference in gene expression and molecular variations in the resistance mechanism. Furthermore, in the current study, the defense-related genes associated with *P. capsici* infection need further functional validation using reverse genetics methods such as VIGS, RNAi and CRISPR/Cas9 system. The current findings could be used as a basis by molecular breeders to select landraces containing defense-related and help to improve chili pepper.

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Author's contribution

Conceptualization, Data curation, Formal analysis, Investigation, Methodology, writing an original draft, Software, Validation, T.R, A.C and V.C., Project administration, Resource, V.C, Writing-Review and editing, Supervision, Om.P. G and V.C

Conflict of interest

The authors declare no conflict of interest relating to financial interests or personal relationships that could have influenced the work reported in this paper.

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