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Abstract: Circular RNAs (CircRNAs) are covalently closed non-coding RNAs that play an important role in a variety of biological processes. CircRNA profiling helps to understand biological process associated with various abiotic and biotic stresses. In tomato genotype IIHR- 2611 (resistant to ToLCBaV), a total of 193 CircRNAs were discovered, of which 72 and 121 were found in control (RC) and ToLCBaV inoculated (RI) plants respectively. Among them, 103 (53 %) were exonic CircRNA regulating the expressions of their parent genes. Relative expression of CircRNAs 2:45295638|45295796, 2:51520741|51530067 and 7:67566489|67566691 and their respective parent genes Solyc02g080530.3 (peroxidase), Solyc02g088950.2 (superoxide dismutase) and Solyc07g065840.2.1 (heat shock protein 90) response to ToLCBaV infection were analysed at different time intervals. A significantly positive correlation was observed for the expression profiles of all three circRNAs and their parent genes. Furthermore, the differential expression across samples as well as time interval indicates that CircRNA mediated gene expression is involved in viral resistance. The results of the expression assays of both superoxide dismutase and peroxidase were consistent with enzyme analysis. Overall findings demonstrated the importance of CircRNAs in ToLCBaV resistance and suggested that CircRNAs could be key regulators of gene expression during disease resistance in tomato.

Keywords: CircRNAs, RNA sequencing, ToLCBaV resistance, tomato.

INTRODUCTION

Tomato is a globally important vegetable crop and its cultivation is severely hampered by various pests and diseases including Tomato leaf curl virus (ToLCV). The losses due to infestation of ToLCV often exceeds 90 per cent (Varma and Malathi, 2003; Singh et al., 2015). Majority of genomic studies relied on molecular markers and functional analysis of genes. In order to understand the structural and functional concept of genomic regions, it is important to employ high- throughput next generation sequencing technologies (Barone et al., 2008; Wang et

al., 2018). RNA sequencing also known as transcriptomics is one such technology that allows researchers to examine both known and unknown transcripts. Non-coding RNAs (ncRNAs) are transcripts that are not part of protein-coding genes (Wang et al., 2018). Among them Circular RNAs (CircRNA) are diverse and unique family of endogenous non-coding RNAs found in plant cells (Wang et al., 2018).

CircRNAs are abundant in the eukaryotic transcriptome. Their discovery and functional involvement in biological processes has opened up a new perspective so as to know how genomic regions interact in a variety of ways. However, their specific role is yet to be understood (Zhang et al., 2020; Litholdo et al., 2018). The majority of CircRNAs are conserved across species, although their expression varies according to tissue or developmental stage, as well as during biotic and abiotic stresses. CircRNAs interact with the transcriptional complex and influence the transcriptional and post-transcriptional regulation of gene expression (Zhang et al., 2020; Shao et al., 2021). They regulate parent gene expression by acting as miRNA sponges and RNA binding proteins (RBP) sponges (Hansen et al., 2013; Ashwal-Fluss et al., 2014; Shao et al., 2021). Their biogenesis competes with linear mRNA splicing to target alternative splicing mechanism of gene regulation (Shao et al., 2021). CircRNAs also regulate the translation of parental genes through interaction with trans-acting elements (Shao et al., 2021).

CircRNA were found to be differentially expressed during pathogen interaction in Arabidopsis (Sun et al., 2016; Zhang et al., 2020), pathogen invasion in kiwi fruit (Wang et al., 2017) and interaction with leaf curl virus in tomato (Wang et al., 2018); they also have regulatory roles in response to cotton verticillium wilt and maize Iranian mosaic virus (Xiang et al., 2018; Ghorbani et al., 2018). However, there is no information on the involvement of CircRNA in ToLCV tolerance in tomatoes. Keeping this in view, the present study investigated the potential role of CircRNA in regulating ToLCV resistance in tomato. Using high-throughput sequencing technology and appropriate bioinformatic tools, we analysed transcriptome data and identified CircRNAs. Abundance of CircRNAs, chromosome distribution and their corresponding genes were analysed and further the differential expression of few selected CircRNAs and their corresponding parent genes at different interval after ToLCBaV infection were analysed through gene expression studies.

MATERIAL AND METHODS

Plant infection and RNA sequencing

Tomato genotype (Acc No. IIHR 2611) resistant to ToLCBaV was grown under control green house conditions at ICAR-IIHR, Bengaluru. Ten day old seedlings were inoculated with white fly (*Bemisia tabaci*) carrying ToLCBaV. At 0, 3, 5, 9, 15 and 21 days post inoculation (DPI) leaf samples were collected. Total RNA from all the periods

with three biological replications in each sample was isolated using RNA iso-Plus (TAKARA, BIO INC. Japan). The quality of total RNA was measured using NABI UV/vis Nano Spectrometer. Total RNA of control plants of all the intervals (0, 3, 5, 9, 15 and 21 DPI) were pooled as sample RC and total RNA of infected plants of all the intervals (3, 5, 9, 15 and 21 DPI) were pooled as sample RI and sent for RNA-sequencing at M/S Eurofins Genomics facility, Bengaluru. The libraries were made from the pooled RNA samples and sequenced on an Illumina Hiseq1500 sequencing platform with 150-bp paired-end reads following manufacturer's instructions. The raw reads were filtered to obtain the clean reads by removing reads containing adaptors and uncertain nucleotides N>10%, and also reads with low quality nucleotides (base quality <5 and Q score <20%). The RNA sequence data of both control and infected tomato (IIHR 2611) was submitted to NCBI (SRR 13493714).

Bioinformatic analysis to detect CircRNAs

CircPlant is composed of four modules. Based on total/polyA- RNA sequencing reads, CircPlant using BWA-MEM software detects plant CircRNAs and the modified CIRI2 (Gao et al, 2015; Gao et al, 2019; Zhang et al., 2020). CIRIexplore2 tool was used to identify CircRNAs with the following criteria: both ends of splice sites should be GU/AG; mismatch d" 2; Back-spliced junctions reads e" 1; The distance between two splice sites d" 100 kb (Zhang et al., 2016). The functional role of the parent genes of identified CircRNAs involved in viral resistance was taken from Sol Genomics Network ([https:// solgenomics.net](https://solgenomics.net)) and also from other publications.

Validation of CircRNAs and their parent genes using qRT-PCR assay

Following the manufacturer's instructions, cDNA for RNA samples of IIHR-2611 at both control and infected conditions (0, 3, 9 and 15 DPI) with three biological replications were synthesized using HicDNA synthesis kit (Mol Bio HIMEDIA: MBT076- 100R). qRT-PCR was performed in Quantstudio 7 Flex thermal cycler (Applied biosystems) using the intercalation dye TB Green Premix Ex Taq II (TaKaRa Cat# RR820A). PCR mixture composition and data analysis were carried out as previously described (Sorrequieta et al. 2010). PCR conditions were 30 sec at 95 °C and 40 cycles of 5 sec at 95 °C, 40 sec at 59 °C and 30 sec at 72 °C. A melting curve for every target analysed was included using the following conditions: 95 °C for 15 sec, 60 °C for 1 min, and 95 °C for 15 sec. Primer sequence for CircRNA and their parent genes is listed in Table 1. The relative expression level was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2000). Each sample contains three biological replications and three technical replications. The

housekeeping gene EF-1 α (elongation factor-1 α) was used to normalize the transcript levels in the RNA samples (Lacerda et al. 2015).

Table 1:
List primers used in the study

CircRNA_ID	CircRNA primer sequence 5'-3'	Corresponding gene primer sequence 5'-3'
2: 45295638 45295796 (Peroxide, POX)	F: TGCTTGGTCTCACACATTCA R: GCAAGATGTATAATGCGATGGAT	F: GGAATCAACACCCCTGGAGTT R: ACTTCTGGATATAAACGGTGTACCAA
2: 51520741 51530067 (Superoxide dismutase)	F: GCTTGTTCCCAAATCCTGCA R: TTACCCGAGTTCCATCCACC	F: GCGACACTTGAACCTTCCTTCCT R: AGCACTTCCCCACAGAATAATTTG
7: 67566489 67566691 (Heat shock protein 90)	F: ACATGGAGAGAATTATGAAGGCC R: TCACCTTACACAGTCCCTCA	F: TATGAAGGCACAGGCACTTAGG R: ATGATGGAGTTCTCTGGGTTGATC

Correlation was performed to mean values of log₂ (FC) in excel to analyse relationship between CircRNA and their parent gene expression.

Antioxidant enzyme analysis

In order to complement CircRNAs data, we examined SOD (superoxide dismutase) and peroxidase (POX) activity, spectrophotometrically in control and infected plants following Du and Bramlage (1994) and Chander, S. (1990) respectively. Absorption was measured at 560 nm for SOD and the increase in absorbance was measured at 450 nm up to 5 min at 1 min interval for POX. Enzyme activity was expressed in Unit/mg FW. The enzymes activity between control and ToLCBaV infected tomato samples with three replications were compared statistically by two factor analysis of variance (Anova) using online statistical software package for Agricultural Research-OPSTAT (Sheoran et al., 1998). In all analyses, P < 0.05 was taken to indicate statistical significance and Tukey's HSD Test was performed for multiple comparisons.

RESULTS

Identification of CircRNAs in ToLCBaV infected resistant tomato genotype

A total of 193 CircRNAs were identified in IIHR- 2611 genotype using CircPlant (CircRNA Identifier-CIRI2 software (Gao et al., 2015; Gao et al., 2019), of which 58 were specifically expressed in uninfected plant samples and 107 CircRNAs in ToLCBaV infected samples (Fig. 1A). While 14 CircRNAs are common to both RC and RI conditions (Fig.1A). The analysis of CircRNAs across chromosomes (Chr.) showed that all chromosomes harbour CircRNA. However, Chr. 2 has maximum CircRNAs both in control and infected conditions, accounting for 23.83

per cent of total CircRNAs identified (Fig. 1B). The distribution of identified CircRNAs differs with chromosomes where, Chr. 1, 4 and 6 had more CircRNAs in infected sample compared to control (Fig. 1B).

The results showed that CircRNAs were formed from various genomic regions. Out of 193 total CircRNAs identified from both RC and RI, 103 (53 %) CircRNAs were generated from exonic region, four (2 %) CircRNAs were from intergenic region and 86 (45 %) CircRNAs are from other regions of the genome (Fig. 1C). The CircRNAs length analysis showed that most of the exonic CircRNAs were up to 10kb and intergenic CircRNAs were <500bp (Fig. 1D). A few parent genes of identified CircRNAs involved in virus resistance includes SOD (Soly02g088950.2) and POX (Soly02g080530.3) in Chr. 2, Zinc finger transcription factor 33 (Soly04g057990) and Ariadne-like ubiquitin ligase (Soly04g079780) on Chr. 4 and Chaperonin (Soly01g028810) and Chaperonin Cpn60 (Soly01g028810) on Chr. 1 (Table 2).

Validation of tomato CircRNAs in response to ToLCBaV infection

A total of 193 novel CircRNAs were discovered from control and ToLCBaV infected seedlings of the resistant genotype (IIHR-2611). 108 of them were specific to infected samples induced due to viral infection. Among them, we listed parent genes of CircRNAs based on their functional role in defence against biotic stress (Table 2) and experimentally tested the predictions of their expressions using qRT-PCR analysis. Relative expression pattern of CircRNA and their parent genes was found to be significantly positively correlating across different interval of viral infection in all three genes with correlation coefficient of 0.89, 0.61 and 0.97 for 7:67566489|67566691 (HSP 90: Soly07g065840.2.1), 2:4529563845295796 (POX: Soly02g080530.3) and 2:51520741 51530067 (SOD: Soly02g088950.2 respectively (Fig 2). The expressions of SOD gene (up to 6.0 log₂FC).

Table 2 :
List of a few identified CircRNAs with their parental genes

CircRNA_ID	Gene_ID	Corresponding gene	Reference
1:41290623 41290915	Solyc01g028810	Chaperonin	Solgenomics
1:41290675 41291268	Solyc01g028810	Chaperonin Cpn60	Solgenomics
2:44939803 44947800	Solyc02g080050.2	Cysteine-rich receptor-like protein kinase 25	Van <i>et al.</i> , (2017)
2:45295638 45295796	Solyc02g080530.3	Peroxide, POX	Xue <i>et al.</i> , (2020)
2:51520741 51530067	Solyc02g088950.2	Superoxide dismutase	Li <i>et al.</i> , (2020)
4:55042616 55042849	Solyc04g057990	Zinc finger transcription factor 33	Solgenomics
4:64205208 64205372	Solyc04g079780	Ariadne-like ubiquitin ligase	Solgenomics
6:39320133 39320546	Solyc06g061200	Glycine-rich protein	Padmanabhan <i>et al.</i> , 2019
6:39320160 39320804	Solyc06g061200.1	Glycine-rich protein TomR2	Padmanabhan <i>et al.</i> , 2019
7:67566489 67566691	Solyc07g065840.2.1	Heat shock protein 90	TGRD
9:66931468 66931629	Solyc09g074680.2.1	Cullin 1B (ubiquitin-protein ligase activity)	Solgenomics
9:69558474 69566030	Solyc09g084465.1	Wound-induced proteinase inhibitor 1	Fan <i>et al.</i> , (2019)
11:40246227 40246508	Solyc11g040050.2	TBP-associated factor 15	TomAP

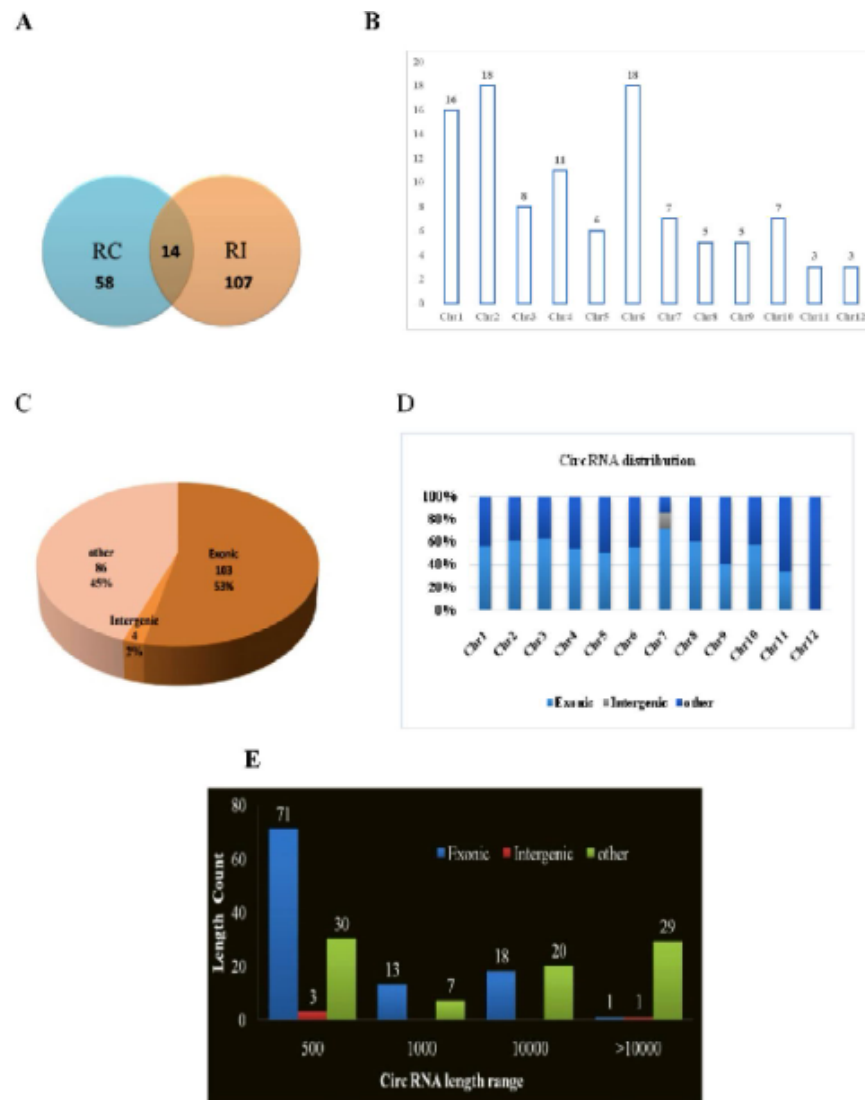


Fig. 1.

Identification and characterization of CircRNAs in response to ToLCBaV in a resistant tomato genotype. (A) Number of CircRNAs identified in RC and RI. (B) CircRNAs distribution on each chromosome. (C) The number and percentage of CircRNAs originated from exon, intergenic and other genomic regions. (D). Percent distribution of exonic, intergenic and other CircRNAs across chromosomes. (E) Classification of CircRNAs based on length range.

and its CircRNA (up to 5.63 log₂FC) was significantly higher during early stages of infection whereas, gene HSP 90 (up to 14.92 log₂FC) and its corresponding CircRNA (up to 7.17 log₂FC) expression was higher during later stages of viral infection (Nine and 21 DPI) (Fig. 2). While, the gene POX relative expression was up to 5.34 log₂FC and that of its CircRNA was up to 8.38 log₂FC (Fig. 2).

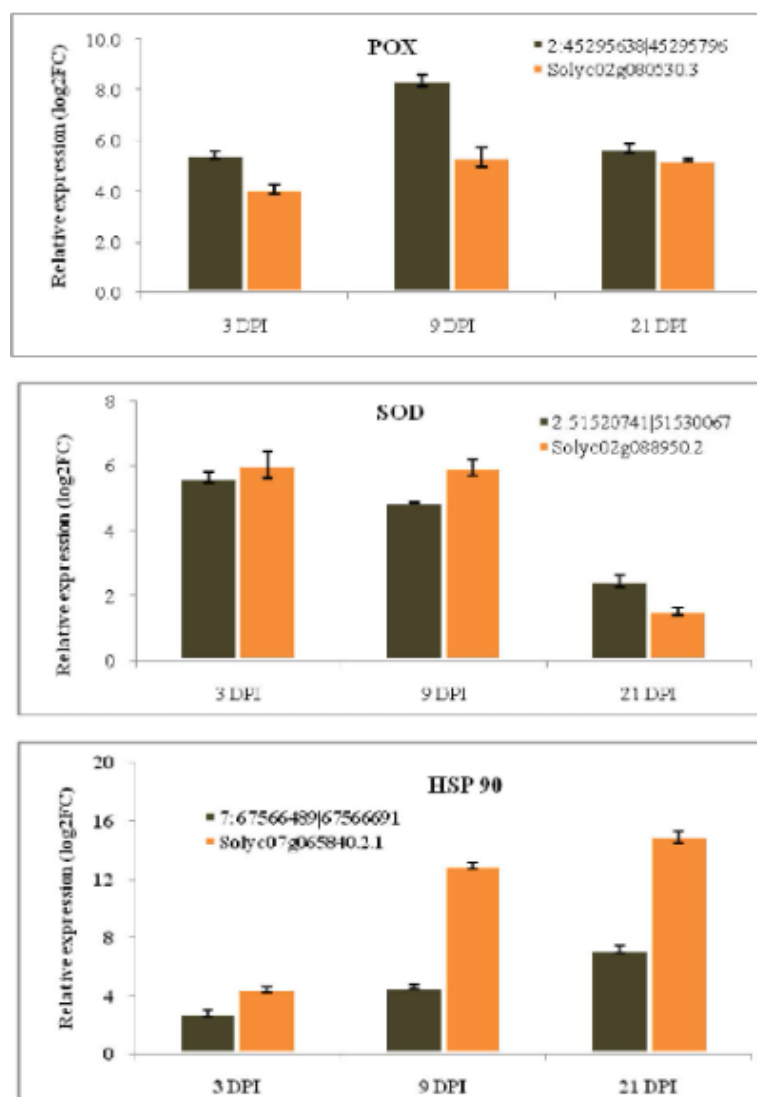


Fig. 2:

Expression profiling of ToLCBaV resistant genotype for CircRNAs and their corresponding parent genes at different days post infection. The data were normalized to Elongation factor-1, presented as the means (Standard error, $n = 3$, three biological replicates, correlation coefficient $r=0.89$, 0.61 and 0.97 for HSP 0, POX, and SOD respectively).

The enzyme analysis done to support the CircRNAs data showed that ToLCBaV infection had significantly altered the enzymatic activity of POX and SOD in resistant tomato (Fig. 3). The uninfected tomato plants had lower POX and SOD activity compared to those infected plants in all five intervals and this difference was probably due to the presence of the virus. In infected samples, the POX activity was highest at 9 and 21 DPI followed by both five and 15 DPI, while the SOD activity was significantly higher at three and five DPI followed by nine and decreased at 21 DPI. The difference in SOD and POX activity between the intervals to be more pronounced was likely due to the presence of virus. These relative expression results were in accordance with the enzyme analysis of both SOD and POX at respective day intervals and provide some insights into the important role of CircRNAs association with antioxidant enzymes in

disease response against ToLCBaV in resistant plant. These findings point out to a possible functional role for CircRNAs in plant defence against viral infection.

DISCUSSION

CircRNAs are covalently closed non-coding RNA molecules. They predominantly comprising of exonic sequences and are spliced at canonical splice sites and were first discovered in humans and mouse, although they are found in all eukaryotes (Salzman, 2016). Use of highthroughput sequencing technologies and in silico analyses, have reported the CircRNAs-mediated gene regulation in plant immune system (Litholdo et al., 2018). In tomato plants, CircRNAs identification have been performed on tomato fruit ripening (Yin et al. 2018), tomato fruit coloration (Hong et al., 2020), fruit pigment accumulation (Yang et al., 2020), responsive to *Phytophthora infestans* (Zhou et al., 2020), TYLCV infection and tomato leaves responding to multiple stresses of drought and heat (Tan et al., 2017) and also low temperature treatments (Yang et al. 2020). In this study, identification of CircRNAs in tomato genotype resistant to ToLCBaV was examined and their functions in response to virus infection process are discussed.

In our study, a total 121 CircRNAs were generated from diverse genomic regions across all chromosomes in response to viral infection. Our result showed that Chr 1, 2, 4 and 6, had a greater number of induced CircRNAs (Fig. 1B). Few of them were involved in defence response (Table 2). Similar results were found where chromosome 01 had the most CircRNAs from susceptible tomato in response to TYLCV (Wang et al. 2018) and in response to multiple stresses of drought and heat (Zhou et al., 2020). Chr. 4 and Chr. 6 harbour TYLCV resistance loci Ty-5 (Hutton et al., 2012) and Ty-1/3 respectively (Dong et al., 2016) and induced CircRNAs on Chr. 4 and 6 might be having the regulatory roles on these resistance genomic regions. CircRNAs are mainly located at exons of genes, but scarcely distributed at introns or intergenic regions (Zuo et al. 2016; Yang et al. 2020). Similar pattern was observed in our experiment that CircRNAs were generated from various genomic regions and out of 193 total CircRNAs identified from both RC and RI, 103 (53 %) were from exonic region (Fig 1C). Similar trend was observed when the CircRNAs (62 % from exonic region) were analysed in susceptible tomato (Wang et al. 2018).

The expressions of exonic CircRNAs were significantly correlated with the expressions of parent genes (Ye et al., 2015; Wang et al., 2018). In our study, we observed a significant positive correlation between relative expression of selected CircRNAs and their parent genes (correlation coefficient $r=0.89$, 0.61 and 0.97 for HSP 0, POX, and SOD respectively) (Fig. 2). HSP90 function through 26S proteasome mediated proteolytic machinery in eukaryotic cells (Sadanandom et al., 2012). Due to decrease in the degradation of the TYLCV protein V2 by the 26S proteasome, silencing of Hsp90 led to enhanced accumulation

of TYLCV CP and DNA levels as infection develops (Moshe et al., 2016). There is a significant positive correlation between 7:67566489|67566691 CircRNAs and its parent gene Solyc07g065840.2.1 (HSP90) (Fig. 2). TYLCV infection enhances defence mechanism through the activity of the antioxidant's enzymes, i.e., SOD, CAT, PPO and POX in tomato (Dieng et al., 2011; Sofy et al., 2017). The corresponding biochemical activity of POX and SOD was in similar trend with the CircRNA expression across different intervals after ToLCBaV infection (Fig. 2 and 3).

CircRNAs in plants are differentially expressed both spatially and temporally in plants, acting as important functional modulators involved in biological processes (Pan et al., 2018; Wang et al., 2016; Zhou et al., 2017). CircRNAs (Slcirc017 parent gene) regulated TYLCV infection in susceptible plant and the silencing of its parent gene (Solyc01g080200.2) resulted in decreased TYLCV virus accumulation (Wang et al., 2018). In this study also differential expression of CircRNA parent genes (Solyc02g088950.2 and Solyc02g080530.3) was observed between control and infected conditions at different intervals (Fig. 2).

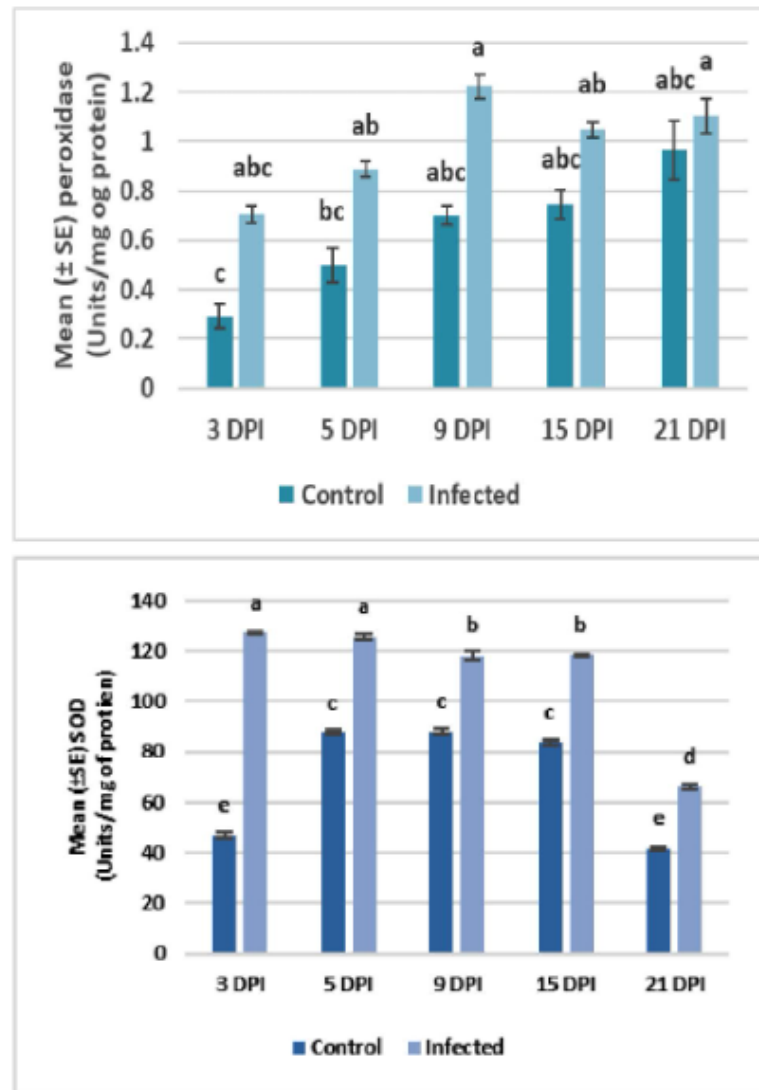


Fig. 3:

Response of SOD and POX activity in IIHR-2611 to ToLCBaV infection. Bar charts of different colour and with different letters are significantly different ($P < 0.05$, $F = 1.702$, $df = 18$, $P = 0.021$ for POX and $F = 3.75$, $df = 18$, $P = 0.021$) based on Tukey's HSD test. Data presented as means $\pm$ SE ($n = 3$)

The results indicate that the CircRNAs- 2:51520741| 51530067 and 2:45295638|45295796 positively influence the plant response to disease through genes involved in ROS scavenging enzymes (SOD and POX) providing some insights into the role of CircRNAs in association with antioxidant enzymes against ToLCBaV. The increasing activity of peroxidase after virus infection (Fig. 2 and 3) might be due to structural defence of peroxidase which was known to perform polymerization, suberization, cell wall elongation, controlling virus multiplication and wounding (Bahar et al., 2020). TYLCV infection enhances the activity SOD and POX and further these enzymes activate the plant defence mechanisms (Dieng et al., 2011; Sofy et al., 2017).

CircRNAs analysed in this study might be acting as miRNA sponges and functioning through miRNA involved regulatory pathways or any of

the other transcriptional, translational and posttranslational regulation mechanisms mentioned above. Furthermore, the precise mechanism by which CircRNAs regulate parent gene expression need to be investigated by identification of miRNA targets for the parent genes, in order to determine whether CircRNAs acting via miRNA mediated pathway or CircRNAs directly acting as parent gene regulator at transcription and translational level. The association between CircRNAs and interacting miRNAs was induced using the rice transgenic plants developed using agroinfection of rice calli with CircRNAs expression cassette (Sharma et al., 2021). There are various methods like artificial miRNA-mediated CircRNA knockdown, gain-of-function study, full-length CircRNA identification followed by CircRNA-protein interaction (Feng and Yu, 2021) to study and characterize the biological function of identified CircRNAs.

CONCLUSION

CircRNAs are emerging as a key player in RNA mediated gene regulation, having roles in several biological processes at both transcriptional and posttranscriptional stages. Many new studies on CircRNA profiling to diverse stresses found that the exonic CircRNAs positively regulates the expressions of their parent genes. This is the first report on CircRNAs for ToLCBaV resistance and we found a positive correlation between few CircRNAs and their parent genes. We hypothesised that these circRNAs must be acting as miRNA sponges and as regulators of miRNA mediated pathways in positively regulating their parental genes or acting as regulatory check points at transcription and translational level of parent gene expression. CircRNAs mediated regulation of some of their parent genes were found to be involved in host defence against viral disease. Further, research on understanding precise mechanism of these CircRNAs during viral infection and resistance against virus would help to identify target specific CircRNAs in plant viral disease resistance.

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