Study on long non-coding RNAs expressed during *Helopeltis theivora* infestation in tea plant [*Camellia sinensis* (L.) O. Kuntze]

A THESIS SUBMITTED FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.) IN BOTANY OF GAUHATI UNIVERSITY

By

KUNTALA SARMA BORDOLOI

Ph.D. Enrolment No. Bot-16/18

GU Registration No. 045755 of 2011-12



DEPARTMENT OF BOTANY FACULTY OF SCIENCE GAUHATI UNIVERSITY ASSAM :: INDIA AUGUST, 2023 I would love to dedicate this thesis to my parents and my brother...

Declaration

I hereby declare that this thesis is the result of my own research work which has been carried out under the guidance of Dr. Niraj Agarwala, Assistant Professor, Department of Botany of Gauhati University. I further declare that this thesis as a whole or any part thereof has not been submitted to any university (or institute) for the award of any degree or diploma.

This thesis contains less than 90,000 (ninety thousand) words excluding bibliography and captions.

Kuntala Saema Bordobi

Kuntala Sarma Bordoloi

Date: 28/08/2023 Place: Guwahati



GAUHATI UNIVERSITY

Gopinath Bordoloi Nagar, Guwahati - 781014, Assam, India

Dr. Niraj Agarwala, M.Sc., Ph.D. Assistant Professor

E-mail: niru.niraj.s@gmail.com niraj_botany@gauhati.ac.in

Date: 25/08/2023

Certificate

This is to certify that the thesis titled "Study on long non-coding RNAs expressed during *Helopeltis theivora* infestation in tea plant [*Camellia sinensis* (L.) O. Kuntze]" is the result of research work of Kuntala Sarma Bordoloi, carried under my supervision, submitted to Gauhati University for the award of the degree of Doctor of Philosophy in Botany.

This thesis conforms to the standard of PhD thesis under Gauhati University including the standard related to plagiarism and has a similarity index not more than 10% (ten percent), excluding the bibliography.

Ninaj Agawala Dr. Niraj Agarwala

Nıraj Ağarwala Supervisor

Acknowledgement

First and foremost, with an immense sense of gratitude, I would like to offer my sincere thanks to my supervisor, Dr. Niraj Agarwala, for his constant guidance, motivation and constructive criticism during my research. He has been an integral source of my commitment and knowledge towards this field of interest and has intellectually contributed to my research work and its findings.

I would like to extend my deepest gratefulness to the Head of the Department of Botany, Gauhati University, for providing all the necessary facilities and for ensuring smooth conduct of my research work.

With immense pleasure I offer my sincere thanks to my RAC members and all the faculties and staff members of the Department of Botany, GU. I would also like to thank my fellow research scholars for their constant love and support.

My heartfelt thanks goes to my loving and supporting lab members Debasish B Krishnatreya, Bhaskar Dowarah and Pooja Moni Baruah for their constant help, support and criticism.

My sincere gratitude to all the authorities of Darjeeling Tea Research and Development Centre, Kurseong, West Bengal, authorities of Sungma and Turzum Tea Estate, West Bengal and Mrinmoy Purkayastha, Sonapur Tea Estate, for extending their helping hands for smooth conduct of my research. My utmost thanks to Genotypic, Bangalore, for carrying out the high-throughput Ilumina RNA-sequencing. I would like to thank Preetom Regon and Mehzabin Rehman for extending their support during setting up of qPCR experiment.

And last but not the least, I offer my sincere gratitude to the Almighty, my beloved parents and my brother, without whose love and blessings, this research work wouldn't have been possible. I express my heartfelt thanks to them for helping me through thick and thin.

Date: 28 08 2023

Kuntala Sarma Bordolai

Kuntala Sarma Bordoloi

Place: Guwahati

Abstract

Helopeltis theivora or the tea mosquito bug (TMB) is reportedly one of the most devastating pests of tea plant (Camellia sinensis) causing threat to the beverage crop. This study revealed a significant alternation of tea plant's biochemistry in terms of phenolics and flavonoids content, and increase in antioxidative enzymatic activities in response to TMB herbivory. Long non-coding RNAs (lncRNAs) constitute a group of endogenous RNAs that play gene regulatory roles in eukaryotes. In the present study, 9502 lncRNAs were identified from healthy and TMB-infested C. sinensis tissues using high-throughput RNA sequencing data, out of which 80 lncRNAs got differentially expressed in response to TMB infestation. Determination of genes that could act as potential targets of lncRNAs revealed that the identified lncRNAs could possibly target as many as 5804 genes. Differential gene expression (DGE) analysis led to the identification of 3665 differentially expressed genes (DEGs), of which, expression of 1767 genes got upregulated and 1898 genes got downregulated during tea plant's response to TMB. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs and lncRNA-target genes have shown that TMB infestation might have triggered transcriptomic reprogramming leading to altered primary and secondary metabolism in C. sinensis. Identification of TMB-responsive circRNAs (another class of non-coding RNA) in tea plant revealed differential expression of 34 circRNAs during TMB feeding. These circRNAs are found to be associated with biological pathways like "tryptophan metabolism", "biosynthesis of secondary metabolites", "porphyrin metabolism", "nucleocytoplasmic transport", "N-glycan biosynthesis" etc. LncRNAs and circRNAs can act as competing endogenous RNAs (ceRNAs) to bind with common microRNA (miRNA) response elements (MREs) involving a competition between mRNAs, lncRNAs and circRNAs. Assessment of this "sponging" activity of lncRNAs and circRNAs led to the construction of the ceRNA network consisting of 5 lncRNAs, 17 circRNAs, 33 mRNAs competing against each other to bind with 37 miRNAs. The expression of 6 DEGs and 5 differentially expressed lncRNAs (DELs) have been validated by qRT-PCR.

Table of contents

Li	ist of figures	sii
Li	ist of tables	xix
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	9
	2.1 History, taxonomy, cultivation of tea and its challenges	9
	2.2 Plant-insect interaction – an overview	11
	2.3 Transcription factors involved in plant-insect interaction	16
	2.4 Taxonomy, life cycle of <i>H. theivora</i> and its infestation on tea	17
	2.5 Non-coding RNAs – their potential in plant developmental processes	
	and stress response	20
	2.5.1 miRNA	21
	2.5.2 siRNA	23
	2.5.3 circRNA	24
	2.5.4 Long non-coding RNAs – undeniable mediators of plant	
	biotic stress response	25
3	MATERIALS AND METHODS 29	
	3.1 Sample harvesting for biochemical analysis	29
	3.2 Total phenolics estimation	29
	3.3 Totals flavonoids estimation	30
	3.4 Antioxidative enzymatic assays	30
	3.4.1 POX	30

3.4.2 APX
3.4.3 PAL
3.4.4 PPO
3.4.5 CAT
3.5 Plant materials and stress treatments
3.6 RNA isolation, library construction and Illumina sequencing
3.7 LncRNAs
3.7.1 Bioinformatics pipeline for identification of lncRNAs and
differentially expressed lncRNAs (DELs)
3.7.2 LncRNA target gene prediction
3.7.3 Functional annotation and gene set enrichment analysis
(GSEA) of lncRNA targets
3.7.4 Quantitative real-time PCR of selected DELs
3.8 Genes
3.8.1 Identification of differentially expressed genes (DEGs)
3.8.2 Functional annotation and gene set enrichment analysis
(GSEA) of DEGs
3.8.3 Quantitative real-time PCR of selected DEGs
3.9 CircRNAs
3.9.1 Pipeline for identification of circRNAs and DECs
3.9.2 Expression correlation analysis of DECs and DEGs
3.9.3 Functional annotation of DECs42
3.10 Collection of miRNA data
3.11 miRNA target prediction

	3.12 eTM prediction
	3.13 Network construction and visualization
4	RESULTS 45
	4.1 Biochemical changes in the tea plant in response to TMB-herbivory
	4.1.1 Estimation of total phenolics
	4.1.2 Estimation of total flavonoids
	4.1.3 Enzymatic antioxidants
	4.1.3.1 POX
	4.1.3.2 APX
	4.1.3.3 PAL
	4.1.3.4 PPO
	4.1.3.5 CAT
	4.2 Sequencing reads summary
	4.3 LncRNAs
	4.3.1 Identification of lncRNAs 52
	4.3.2 Characterization of lncRNAs
	4.3.3 Expression of lncRNAs 56
	4.3.4 Differential expression of lncRNAs 57
	4.3.5 Identification of lncRNA-target genes
	4.3.6 Functional annotation and enrichment analysis of lncRNAs
	4.3.7 Quantitative real-time PCR of selected DELs
	4.4 Comes
	4.4 Oches
	4.4.1 Expression of genes
	4.4.2 Differential expression of genes

	4.4.3 Functional annotation and enrichment analysis of DEGs
	4.4.4 Quantitative real-time PCR of selected DEGs
	4.5 Comparison between lncRNAs and mRNAs identified in this study
	4.6 CircRNAs
	4.6.1 Identification and characterization of circRNAs
	4.6.2 The differential expression pattern of identified circRNAs
	4.6.3 Functional characterization of DECs
	4.7 miRNA targets, eTMs
	4.8 ceRNA network
5	DISCUSSION 120
	5.1 Biochemical changes in the tea plant as a result of TMB feeding
	5.2 Change in expression pattern of genes/lncRNAs involved in primary
	metabolism
	5.2.1 DEGs related to primary metabolism of C. sinensis in response
	to TMB
	5.2.2 LncRNA mediated regulation of genes related to primary metabolism
	of C. sinensis in response to TMB 124
	5.3 Change in expression pattern of genes/lncRNAs involved in secondary
	metabolism
	5.3.1 DEGs related to secondary metabolism of C. sinensis in response
	to TMB
	5.3.2 LncRNA mediated regulation of genes related to secondary
	metabolism of C. sinensis in response to TMB
	5.4 Interaction between DEGs and their involvement in some important

defense responsive biological pathways	
5.5 Involvement of circRNAs in tea-TMB interaction	
SUMMARY AND CONCLUSION	136
REFERENCES	141
APPENDIX	177

List of Figures

Figure No.	Title	Page No.
1.1	Photographs of <i>H. theivora</i> infested tea plants	4
3.1	Healthy and <i>Helopeltis theivora</i> infested tea leaves. (a) Healthy tea leaf and bud (b) Development of symptoms after feeding (c, d) <i>H. theivora</i> feeding on leaves	33
3.2	A flowchart depicting pipeline employed for identification and characterization of lncRNAs in healthy and TMB-infested tea plants. The peach ellipses denote softwares/tools used in each corresponding step.	38
3.3	A flowchart depicting pipeline employed for identification of differentially expressed genes (DEGs) in healthy and TMB-infested tea plants. The peach ellipses denote softwares/tools used in each corresponding step.	41
3.4	A flowchart depicting pipeline employed for identification of differentially expressed circRNAs (DECs) in healthy and TMB-infested tea plants.	43
4.1	Total phenolic content in 11 clones of tea plant. (Data is represented as mean \pm SE, n = 3). Statistical significance was calculated through a one-way ANOVA followed by a post-hoc t-test with Bonferroni's correction method. P- value ≤ 0.05 was used as the significance value. Statistically significant data are represented with *.	45
4.2	Total flavonoid content in 11 clones of tea plant. (Data is represented as mean \pm SE, n = 3). Statistical significance was calculated through a one-way ANOVA followed by a post-hoc t-test with Bonferroni's correction method. P-value ≤ 0.05 was used as the	46

	significance value. Statistically significant data are	
	represented with *.	
4.3	POX activity in 11 clones of tea plant. (Data is	47
	represented as mean \pm SE, n = 3). Statistical significance	
	was calculated through a one-way ANOVA followed by	
	a post-hoc t-test with Bonferroni's correction method. P-	
	value ≤ 0.05 was used as the significance value.	
	Statistically significant data are represented with *.	
4.4	APX activity in 11 clones of tea plant. (Data is	48
	represented as mean \pm SE, n = 3). Statistical significance	
	was calculated through a one-way ANOVA followed by	
	a post-hoc t-test with Bonferroni's correction method. P-	
	value ≤ 0.05 was used as the significance value.	
	Statistically significant data are represented with *.	
4.5	PAL activity in 11 clones of tea plant. (Data is	49
	represented as mean \pm SE, n = 3). Statistical significance	
	was calculated through a one-way ANOVA followed by	
	a post-hoc t-test with Bonferroni's correction method. P-	
	value ≤ 0.05 was used as the significance value.	
	Statistically significant data are represented with *.	
4.6	PPO activity in 11 clones of tea plant. (Data is	50
	represented as mean \pm SE, n = 3). Statistical significance	
	was calculated through a one-way ANOVA followed by	
	a post-hoc t-test with Bonferroni's correction method. P-	
	value ≤ 0.05 was used as the significance value.	
	Statistically significant data are represented with *.	
4.7	CAT activity in 11 clones of tea plant. (Data is	51
	represented as mean \pm SE, n = 3). Statistical significance	
	was calculated through a one-way ANOVA followed by	
	a post-hoc t-test with Bonferroni's correction method. P-	
	value ≤ 0.05 was used as the significance value.	
	Statistically significant data are represented with *.	

4.8	Filtering of transcripts for identification of putative	53
	IncRNAs	
4.9	Coding potential analysis of the assembled transcripts	53
	through three tools	
4.10	Chromosomal distribution of identified lncRNAs	54
4.11	Classification of identified lncRNAs based on their	55
	genomic position	
4.12	Length distribution and number of exons found in the	56
	identified lncRNAs	
4.13	A venn diagram showing number and proportion of	56
	lncRNAs expressed in the healthy/control and TMB-	
	infested samples	
4.14	Boxplot showing log2 values of FPKM+1 of lncRNAs	57
	in the healthy/control and TMB-infested samples	
4.15	Volcano plot showing the differential expression of	60
	lncRNAs in control v/s TMB-infested samples	
4.16	Heatmap showing the differential expression pattern of	61
	IncRNAs in the six RNA-seq libraries	
4.17	GO enrichment of lncRNA-target genes. BP; Biological	64
	processes, CC; Cellular component, MF; Molecular	
	function. The size of the bubble represents number of	
	lncRNA-target genes assigned to the particular GO term	
	and the color of the bubble represents adjusted p-value	
	(q value).	
4.18	Gene set enrichment analysis based on GO enrichment	65
	of lncRNA-target genes. The left halve represents terms	
	upregulated in response to TMB and the right halve	
	represents terms downregulated in response to TMB.	
	The size of the bubble represents number of lncRNA-	
	target genes assigned to the particular GO term and the	
	color of the bubble represents adjusted p-value (q value).	

4.19	Gene set enrichment analysis based on KEGG pathway	67
	enrichment of lncRNA-target genes. The left halve	
	represents pathways upregulated in response to TMB	
	and the right halve represents pathways downregulated	
	in response to TMB. The size of the bubble represents	
	number of lncRNA-target genes assigned to the	
	particular KEGG pathway and the color of the bubble	
	represents adjusted p-value (q value).	
4.20	Heatmaps showing expression patterns of lncRNA-	69
	target genes in different biological pathways (a)	
	Terpenoids biosynthesis (b) Flavonoids biosynthesis (c)	
	Zeatin biosynthesis (d) Plant hormone signal	
	transduction (e) MAPK signaling pathway (f) Linoleic-	
	acid metabolism (g) Brassinosteroids biosynthesis	
4.21	Graphs showing positive/negative expression	70
	correlation between lncRNA and lncRNA-target genes.	
	Blue line signifies FPKM values (x-axis) of lncRNAs	
	and red line signifies FPKM values (x-axis) of lncRNA-	
	target genes. Figures a and b denote negative correlation	
	between lncRNA-mRNA pairs, figures c and d denote	
	positive correlation between the pairs.	
4.22	Melt curve plots of DELs TCONS_00040585 (a and b),	71
	TCONS_00083891 (c and d), TCONS_00096174 (e and	
	f), TCONS_00032903 (g and h) and TCONS_00099260	
	(i and j) in control and TMB-infested samples	
4.23	Bar diagrams showing result of qRT-PCR analysis of 5	72
	DELs in control v/s TMB-infested samples. Values in	
	the y-axis determine the relative expression of DELs	
	normalized to UBC1 gene. Error bars indicate ±SEM of	
	relative expression of triplicates.	
4.24	Boxplot showing log2 values of FPKM+1 of genes in	73
	the healthy/control and TMB-infested samples	

4.25	A venn diagram showing number and proportion of genes expressed in the healthy/control and TMB-infested samples	74
4.26	Volcano plot and expression heatmap depicting DEGs	75
4.27	Gene set enrichment analysis based on GO enrichment of DEGs. The left halve represents terms upregulated in response to TMB and the right halve represents terms downregulated in response to TMB. The size of the bubble represents number of DEGs assigned to the particular GO term and the color of the bubble represents adjusted p-value (q value).	76
4.28	Gene set enrichment analysis based on KEGG pathway enrichment of DEGs. The left halve represents pathways upregulated in response to TMB and the right halve represents pathways downregulated in response to TMB. The size of the bubble represents number of DEGs assigned to the particular KEGG pathway and the color of the bubble represents adjusted p-value (q value).	79
4.29	Melt curve plots of DEGs CSS0024393.1 (a and b), CSS0006785.2 (c and d), CSS0016212.1 (e and f), CSS0018684.1 (g and h), CSS0023703.1 (i and j) and CSS0046901.1 (k and l) in control and TMB-infested samples	81
4.30	Bar diagrams showing result of qRT-PCR analysis of 6 DEGs in control v/s TMB-infested samples. Values in the y-axis determine the relative expression of DEGs normalized to UBC1 gene. Error bars indicate ±SEM of relative expression of triplicates.	82
4.31	(a) Bar diagram showing distribution of transcript lengthof both identified lncRNAs and expressed mRNAs (b)Comparison of exon numbers between identified	83

	lncRNAs and mRNAs (c) Boxplot showing distribution	
	of FPKM values of lncRNAs and mRNAs	
4.32	Chromosomal distribution of identified circRNAs	84
4.33	Characterization of identified circRNAs in tea plant. (a)	86
	Pie chart showing classification of circRNAs based on	
	their genomic positions (b) Venn diagram showing	
	number of unique and common circRNAs identified in	
	control and TMB-infested samples (c) Bar diagram	
	showing length distribution of identified circRNAs (d)	
	Percentage of circRNAs showing homology with	
	already reported circRNAs of different plant species	
	deposited in PlantcircBase	
4.34	Differential expression pattern of identified circRNAs.	87
	(a) A heatmap showing the expression pattern of DECs	
	across all six samples (b) Volcano plot showing	
	differential expression of circRNAs	
4.35	Annotation of the DEC-target genes based on GO. The	89
	x-axis depicts the enrichment ratio between number of	
	DEC-target genes and all UniGenes enriched in a	
	particular GO term. The size of the bubble represents	
	number of DEC-target genes assigned to the particular	
	GO term and the color of the bubble represents adjusted	
	p-value (q value). BP; Biological processes, CC;	
	Cellular component, MF; Molecular function	
4.36	GSEA based on KEGG pathway of DEC-target genes.	90
	The x-axis depicts the enrichment ratio between number	
	of lncRNA-target genes and all UniGenes enriched in a	
	particular KEGG pathway. The size of the bubble	
	represents number of DEC-target genes assigned to the	
	particular KEGG pathway and the color of the bubble	
	represents adjusted p-value (q value). The left halve of	

	the figure represents pathways upregulated in response	
	to TMB and the right halve represents pathways	
	downregulated in response to TMB.	
4.37	(a) Percentage of novel and conserved C. sinensis	105
	miRNAs putatively targeting DELs (b) Percentage of	
	miRNAs targeting on DELs (c) Interactive networks of	
	C. sinensis miRNAs and DELs represented by pink and	
	purple ellipse nodes respectively. The connection	
	between miRNAs and lncRNAs are shown as blue	
	edges.	
4.38	Result of the lncRNA mimic analysis. Query signifies	106
	miRNA and Subject signifies lncRNAs. Score	
	determines the expectation threshold; Dash (-)	
	determines bulge formation.	
4.39	miRNA targeting lncRNA analysis through	107
	psRobot_tar; Score depicts the expectation threshold;	
	Query determines the miRNA and subject determines	
	the lncRNA; Asterisks (*) determine unpaired regions.	
4.40	TMB-responsive miRNA-lncRNA-mRNA network in	109
	C. sinensis. miRNAs, mRNAs and lncRNAs are	
	represented by pink prismatic shape, yellow ellipse and	
	blue round rectangles respectively. The interactions	
	between the RNA molecules are depicted by blue edges.	
4.41	miRNA targeting mRNA analysis through psRobot_tar;	110
	Score depicts the expectation threshold; Query	
	determines the miRNA and subject determines the	
	mRNA; Asterisks (*) determine unpaired regions.	
4.42	C. sinensis miRNA-DEC-DEG interaction network.	111
	miRNAs, genes and circRNAs are represented by red	
	diamonds, green octagons and blue rectangles	
	respectively. The connections between the RNA	
	molecules are depicted by black edges.	

4.43	TMB-responsive ceRNA network in C. sinensis.	119
	miRNAs, mRNAs, lncRNAs and circRNAs are	
	represented by purple prismatic shape, blue round	
	rectangles, green octagons and red diamonds	
	respectively. The interactions between the RNA	
	molecules are depicted by black edges.	
5.1	A hypothetical schematic regulatory network	131
	representing involvement of genes in defense responsive	
	biological pathways during TMB infestation in C.	
	sinensis. Peach colored ellipses represent DEGs and	
	yellow ellipses represent genes that are not differentially	
	expressed during TMB infestation. The heatmaps	
	represent expression pattern of DEGs in six RNA-seq	
	libraries (Control-1, Control-2, Control-3, Infested-1,	
	Infested-2, Infested-3). The green rectangles represent	
	plant hormones and blue rectangles represent the plant's	
	response to TMB feeding downstream of the pathway	
	involved.	

List of Tables

Table No.	Title	Page No.
3.1	List of primers designed for validation of selected DELs	36
3.2	List of primers designed for validation of selected DEGs	40
4.1	Summary of reads generated from control and TMB- infested tea samples	52
4.2	List of DELs with their log2 fold change and p-values	58
4.3	Results of lncRNA- <i>cis</i> target hybrid formation analysis using RIBlast algorithm	62
4.4	List of DECs with their log2 fold change and p-values	86
4.5	List of DECs annotated with their parental genes	88
4.6	Table showing lncRNAs acting as eTMs and the corresponding mRNAs with their available information	91
4.7	Table showing C. sinensis miRNAs targeting DELs	92
4.8	List of DECs being targeted by C. sinensis miRNAs	111