Phytophthora capsici infection causes dynamic alterations in tRNA modifications and their associated gene candidates in black pepper

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1. Introduction

The transfer RNAs (tRNAs) are known as adaptor molecules that are implicated in the process of protein translation. A mature tRNA requires extensive post-transcriptional modifications to execute its normal functions in a cell. The modifications vary from simple addition or substitution of functional groups to complex biosynthetic reactions [34,6,19]. However, research on tRNA modifications began decades ago when Holley et al. [25] noticed the presence of unusual nucleosides like inosinic acid, l-methylinosinic acid, l-methylxanthosine, and 5,6-dihydro uridylic acid; the investigations on its significance are still growing.

The tRNA modifications confer structural stability and integrity to tRNAs and facilitate the efficient translation of proteins [15]. The tRNA modifications are prevalent at the wobble position of tRNAs to enhance the codon-anticodon pairing [29,51]. Moreover, the tRNA modifications in the anticodon loop regulate the translational rate of specific genes during adversities [12,18]. Whereas certain modifications in the variable loop, the D arm, and the T arm confer structural stability to tRNAs [15]. Interestingly, the abundance of these tRNA modifications is altered in response to various environmental stimuli [38,15]. For example, in humans, the modification levels are dynamically altered during diverse physiological conditions like cancer [14,18], neurological disorders [3,5] etc. Whereas in plants, the 2'-O-methyladenosine (Am) nucleoside was abundantly found during salt stress. In Rice, the overexpression of OsTRM13- methyltransferase implicated in the synthesis of Am showed higher resistance to salt stress [59,60].

The tRNA modifications: Am, Cm, m1A and m7G and their cognate methyltransferases (MTases), were upregulated during abiotic stresses in Rice and Arabidopsis [59,60]. Another study in Rice demonstrated that a tRNA His guanylyltransferase- AET1 aid the plant to withstand high temperatures [10]. But, most of the studies done in plants to understand the implication of tRNA modification in stress response were confined to abiotic stress. The investigations on the fate of tRNA modification profiles reprogrammed during a biotic attack are scanty. A study has demonstrated that a nonfunctional SUPPRESSOR OF CSB3 9 (SCS9) protein which codes a MTase, has enhanced disease susceptibility in plants, showing the functional importance of tRNA modifications in biotic stress regulation [45].

In plants, the pathogen attack builds up reactive oxygen species (ROS) in the cells, which ultimately causes oxidative stress or damage to the cells [53]. According to previous reports in model organ-
isms like yeast, oxidative stress alters the tRNA modification profile [8]. Although tRNA modifications and how they respond to stress are well probed in organisms like bacteria, yeast and humans, the chore of tRNA modifications during biotic stress in higher plants is least explored. The lack of such studies is mainly due to the complexity of defining tRNA modifications using conventional genetic and biochemical methods in higher systems. In such circumstances, the availability of omics data can be exploited to study more about tRNA modifications and associated enzymes [55].

In the current study, we investigated how Phytophthora capsici infection affected the tRNA modification profile of black pepper. The study identified the different tRNA nucleoside modifications in black pepper and the enzyme gene candidates involved in the modification pathways. Additionally, the expression of genes associated with tRNA modification in P. capsici-infected and uninfected black pepper was studied. The study explained the impact of stress on tRNA modifications and their cognate enzymes implicated in stress response in black pepper. An attempt to investigate the various tRNA modifications and associated genes during pathogen attack in a non-model spice crop like black pepper can also aid in annotating its obscured functions in higher plants.

2. Results

2.1. Detection of ROS generation and cell death during P. capsici infection using DAB, NBT and trypan blue staining

During pathogen infection, the plants undergo a series of chemical reactions, and the release of ROS is one among them. Generally, Hydrogen peroxide (H2O2) and Super oxide (O2-) accumulation are considered markers of ROS burst. In black pepper during P. capsici attack, the outburst of H2O2, O2- and related cell death was detected using 3,3′-diaminobenzidine (DAB), Nitro tetrazolium blue (NBT) and Trypan blue staining, respectively (Fig. 1). The trypan blue stained cell death occurred in the infected leaves. DAB staining has shown the accumulation of H2O2 in pathogen-infected leaves. Similarly, the release of superoxide radicals was also seen more in pathogen-challenged leaves when reacted with NBT.

2.2. tRNA isolation

The tRNAs extracted from the total RNA samples were run on the 7.5 % urea gel, as shown in Fig. 2. The red rectangle box indicates the 60–90 nt tRNA band. These tRNAs were excised and used for further analysis.

2.3. Identification of modified tRNA nucleosides of black pepper

The LC-MS analysis of tRNAs was done at Arraystar, USA, to identify the various tRNA modifications present in black pepper. Interestingly, from the fifty-two standard modifications used for the analysis, a maximum of fifty different tRNA nucleoside modifications were detected. These fifty modifications include (Supplementary Table 1) twenty ‘U’ derivatives, eight ‘A’ derivatives, ten ‘C’ derivatives and five ‘G’ derivatives.

2.4. Identification of methylation modifications enzymes from black pepper

Our analysis revealed that the nucleoside methylations catalyzed by MTases were predominantly found in black pepper. So, further study was done to extract the MTase candidate genes from black pepper. Fifty-nine MTase candidates were identified from black pepper based on protein sequence homology with thirteen MTase sequences from yeast, twenty-two from Arabidopsis and twenty-two from Rice, and also from the NR classification of black pepper transcriptome data using local tblastn in BioEdit (Table 1).
Table 1

Table showing the identified fifty-nine MFase candidates from black pepper.

| Black Pepper MFase Protein | Yeast Candidate Gene | E-value | Arabidopsis Candidate Gene | E-value | Rice Candidate Gene | E-value |
|-----------------------------|----------------------|---------|---------------------------|---------|----------------------|---------|
| Unigene10018_All Trm1p      |                      | 1.00E–56| At3g23220                  | 2.00E–163| LOC_Os03g57280      | 7.00E–158|
|                            |                      |         | At5g15810                  | 2.00E–170| LOC_Os01g21360      | 6.00E–149|
| Unigene10022_All Trm1p      |                      | 8.00E–26| At3g03230                  | 1.00E–118| LOC_Os03g57280      | 2.00E–118|
|                            |                      |         | At5g15810                  | 2.00E–120| LOC_Os01g21360      | 1.00E–83 |
| Unigene10021_All Trm1p      |                      | 3.00E–33| At3g03230                  | 4.00E–107| LOC_Os03g57280      | 5.00E–105|
| CL1469.Contig5_All Trm1p    |                      | 3.00E–33| At3g03230                  | 8.00E–113| LOC_Os01g21360      | 1.00E–100|
| CL1469.Contig4_All Trm1p    |                      | 1.00E–29| At3g03230                  | 7.00E–107| LOC_Os01g21360      | 7.00E–105|
| Unigene6901_All Trm2        |                      | 8.00E–27| At3g13300                  | 7.00E–105| LOC_Os01g09750      | 2.00E–98 |
|                            |                      |         | At2g28450                  | 7.00E–100| LOC_Os04g04140      | 4.00E–95 |
|                            |                      |         | LOC_Os02g39370             | 1.00E–18 | LOC_Os01g29409      | 1.00E–10 |
| Unigene285_All Trm2         |                      | 9.00E–13| At2g28450                  | 7.00E–21 | LOC_Os04g04140      | 4.00E–09 |
|                            |                      |         | LOC_Os01g09750             | 4.00E–09 | LOC_Os02g39370      | 7.00E–98 |
| Unigene3795_All Trm5p       |                      | 2.00E–51| At3g56120                  | 9.00E–116| LOC_Os01g29409      | 2.00E–56 |
|                            |                      |         | At3g21300                  | 3.00E–09 | LOC_Os02g39370      | 4.00E–09 |
| CL8815.Contig2_All Trm5p    |                      | 2.00E–53| At4g27340                  | 7.00E–134| LOC_Os01g29409      | 1.00E–135|
| CL839.Contig3_All Trm5p     |                      | 1.00E–07| At4g04670                  | 9.00E–134| LOC_Os01g29409      | 7.00E–09 |
| CL1131.Contig3_All Trm7p    |                      | 3.00E–36| At4g27340                  | 3.00E–175| LOC_Os05g49230      | 0        |
|                            |                      |         | At5g01230                  | 2.00E–34 | LOC_Os06g94140      | 4.00E–35 |
|                            |                      |         | At5g13830                  | 2.00E–17 | LOC_Os09g27270      | 4.00E–52 |
| Unigene10222_All Trm8p      |                      | 5.00E–69| At5g24840                  | 2.00E–106| LOC_Os03g60750      | 4.00E–17 |
|                            |                      |         | At5g17660                  | 3.00E–11  | LOC_Os01g29409      | 1.00E–109|
| Unigene10020_All Trm1p      |                      | 3.00E–33| At3g32320                  | 7.00E–94 | LOC_Os03g57280      | 2.00E–90 |
| Unigene16681_All Trm1p      |                      | 1.00E–20| At3g32320                  | 1.00E–97 | LOC_Os01g21360      | 1.00E–77 |
| Unigene7198_All Trm1p       |                      | 9.00E–09| At3g32320                  | 2.00E–72 | LOC_Os01g21360      | 1.00E–69 |
| CL10116.Contig2_All Trm1p   |                      | 2.00E–08| At3g32320                  | 8.00E–76 | LOC_Os01g21360      | 2.00E–58 |
| CL10116.Contig1_All Trm1p   |                      | 2.00E–08| At3g32320                  | 6.00E–11 | LOC_Os03g57280      | 3.00E–10 |
| Unigene16680_All Trm1p      |                      | 9.00E–06| At3g32320                  | 7.00E–11 | LOC_Os01g21360      | 1.00E–12 |
| Unigene10017_All –          |                      | 9.00E–06| At3g32320                  | 7.00E–08 | LOC_Os01g21360      | 2.00E–09 |
| Unigene10019_All –          |                      | 9.00E–06| At3g32320                  | 4.00E–27 | LOC_Os03g57280      | 5.00E–26 |
| CL1469.Contig3_All –        |                      | 3.00E–42| At5g15810                  | 3.00E–38 | LOC_Os01g21360      | 3.00E–32 |
| Unigene22464_All –          |                      | 4.00E–15| At3g32320                  | 6.00E–34 | LOC_Os01g21360      | 5.00E–31 |
| CL1469.Contig6_All          |                      | 7.00E–15| At3g32320                  | 6.00E–34 | LOC_Os01g21360      | 5.00E–31 |
| Unigene22463_All            |                      | 1.00E–14| At3g32320                  | 5.00E–16 | LOC_Os03g57280      | 9E–17    |
| CL1469.Contig1_All          |                      | 8.00E–07| At3g32320                  | 1.00E–06 | LOC_Os03g57280      | 3.00E–08 |
| CL2435.Contig5_All          |                      | 1.00E–08| At3g32320                  | 5.00E–08 | LOC_Os03g57280      | 2.00E–08 |
| CL839.Contig1_All           |                      | 8.00E–11| At3g32320                  | 5.00E–10 | LOC_Os03g57280      | 2.00E–16 |
| Unigene22693_All Trm7p      |                      | 4.00E–37| At5g510230                 | 5.00E–15 | LOC_Os04g04140      | 5.00E–65 |
|                            |                      |         | At4g27340                  | 3.00E–06 | LOC_Os01g29409      | 8.00E–17 |
|                            |                      |         | At5g13830                  | 4.00E–07 | LOC_Os09g27270      | 6.00E–14 |
|                            |                      |         | LOC_Os03g60750             | 4.00E–06 | LOC_Os06g94140      | 4.00E–06 |

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2.5. Phylogenetic and conserved motif analysis of MTase in Black pepper

A phylogenetic tree was constructed to study the evolutionary significance of the fifty-nine MTase candidates. The tree has shown that the candidates have clustered into closely related groups. The phylogenetic tree has been clustered into six groups (Fig. 3). Group I include the candidate genes of black pepper needed for m7G and mc5U methylation in Arabidopsis, Yeast and Rice, the group II consist of m3C and m5U candidates. The group III consists of the MTases of m5C, m2G and Cm. Group IV and V include Am, m1G, m5U and m1G, m2G, respectively. Group VI possess the candidates for Am and Gm modifications.

Further, the obtained sequences were aligned in MEME for visualizing the conserved domain in the MTase candidates in black pepper. Eleven candidates from black pepper shared conserved domains with the MTases from Arabidopsis, Rice and Yeast. Based on the conserved domain analysis of black pepper, Yeast, Arabidopsis and Rice datasets, the MEME analysis yielded three groups (Fig. 4).

Table 1 (continued)

| Black Pepper MTase Protein | Yeast Candidate Gene | E-value | Arabidopsis Candidate Gene | E-value | Rice Candidate Gene | E-value |
|----------------------------|----------------------|---------|-----------------------------|---------|---------------------|---------|
| CL166.Contig5_All          | Trm7p                | 6.00E−12| At5g01230                   | 8.00E−11| LOC_Os06g49140      | 1.00E−11|
|                            |                      |         | At4g25730                   | 5.00E−15| LOC_Os05g49230      | 1.00E−14|
|                            |                      |         | At5g33830                   | 3.00E−16| LOC_Os09g27270      | 3.00E−14|
| CL8431.Contig2_All         | Trm140p              | 9.00E−07| At5g24840                   | 1.00E−12| LOC_Os05g49230      | 8.00E−08 |
| Unigene15091_All           |                      |         | At5g13830                   | 3.00E−16| LOC_Os09g49230      | 8.00E−06 |
| CL7021.Contig3_All         | Trm8p                | 4.00E−10| At5g13830                   | 3.00E−16| LOC_Os09g49230      | 8.00E−06 |
|                            |                      |         | LOC_Os06g49140              | 1.00E−12| LOC_Os09g27270      | 3.00E−14|
| CL6988.Contig2_All         |                      |         | LOC_Os02g51490              | 1.00E−06| LOC_Os05g49230      | 8.00E−06 |

Fig. 3. The phylogenetic tree of MTase candidates from Black pepper. Unrooted phylogenetic tree of MTase candidates from Black pepper, Oryza sativa (Rice), Arabidopsis thaliana and Saccharomyces cerevisiae (Yeast) [Phylogenetic relationship among various MTases candidates from Black pepper, O. sativa, A. thaliana and S. cerevisiae] by employing the maximum-likelihood method based on Jones-Taylor-Thornton (JTT) Protein substitution model. The six groups of Black pepper genes clustered together were annotated with green lines for group I, Opera Mauve (light pinkish purple) lines for group II, light orange lines for group III, medium purple lines for group IV, red lines for group V and steel blue lines for group VI respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
The first group has shown aspartic acid (D) conservation in the 9th position. Moreover, a motif of APG from the 13th position was also seen in group I. The contigs in group I were candidates of tRNA (cytidine32/guanosine34-2-‘)-methyltransferase and Ado Met-dependent rRNA MTase SPB1. In group II, amino acid residues were conserved throughout all the sequences. This group’s contigs and unigenes of black pepper represented tRNA (guanine26-N2/guanine27-N2)-dimethyltransferase. Conserved motifs like DFY at the 14th to 16th position and FV at the 20th position were present in group II. The other amino acid residues which have shown conservation in all enzyme candidates were K, P, S, K, Y, C, S and H. Moreover, the most significant number of conserved amino acid residues were distributed between the Lysine (K) at the 2nd position and 31st position.

Furthermore, in group III, the longest motif PDPHK, among all three groups, was found along with a GG- double glycine motif. Additionally, this group found the longest motif between two Leucine residues. Moreover, a prominence of D, P and G were found in group II. The other amino acid residues which have shown conservation in all enzyme candidates were K, P, S, K, Y, C, S and H. Moreover, the most significant number of conserved amino acid residues were distributed between the Lysine (K) at the 2nd position and 31st position.

Fig. 4. The Conserved motif analysis of group I, group II and group III MTase candidate genes from black pepper. The X-axis indicates the position of each residue within the motifs identified from black pepper; Y-axis indicates bit score values. The degree of conservation within the group of proteins studied was denoted by the size of the residue letter. The table below each illustration is the translated protein sequence from black pepper, protein sequence from Yeast, Arabidopsis and Rice for each member, with the name of the protein, starting position, the p-value of the conserved motif and the whole motif sequence.
the three groups. Thus, the conserved motif analysis by MEME has confirmed the presence of the conserved catalytic domain of MTases.

2.6. Comparison of MTase gene expression during *P. capsici* infection in black pepper

The gene expression of MTase candidate genes during *P. capsici* challenge was done to understand the impact of biotic stress on tRNA nucleoside methylations. The FPKM of fifty-nine genes were compared with the control to understand the fate of these genes during the pathogen challenge (Fig. 5). The study revealed the MTase gene expression during *P. capsici* infection and uninfected black pepper samples showed differential expression, among which eleven genes were significantly expressed (p < 0.05) (Fig. 6).

The significantly regulated contigs include the one which represents MTases like tRNA (guanine-N(7))-methyltransferase subunit WDR4-like protein, which is needed for the formation of N7-methylguaninate at position 46 (m7G46) in tRNA [39], tRNA (cytidine(34):2-O)-methyltransferase (TrmL) which is involved in the methylation reaction of 34th position of C to Cm, Cm to Cmm5U and also involved in the methylation of Cmnm5U to Cmm5Um [4]; the tRNA (guanine(37)-N1)-methyltransferase (TRM5)1-like protein which methylates the N1 position of guanosine-37 near to the anticodon loop. Moreover, it catalyzes the initial step in the biosynthesis of wybutosine (yw), a modified base that is essential for the proper decoding of tRNA anticodons [7] and tRNA (guanine(26)-N(2))-dimethyltransferase execute two successive methylations of guanosine (G) to N2-, N2-methylguanosine (m2G), and N2-methylguanosine (m2G). These gene candidates were significantly upregulated in infected samples. Altogether the enzymes involved in the formation of Guanosine and Cytosine derivatives like Cmnm5U and also involved in the methylation of Cmnm5U to Cmm5Um [4]; the tRNA (guanine(37)-N1)-methyltransferase (TRM5)1-like protein which methylates the N1 position of guanosine-37 near to the anticodon loop. Moreover, it catalyzes the initial step in the biosynthesis of wybutosine (yw), a modified base that is essential for the proper decoding of tRNA anticodons [7] and tRNA (guanine(26)-N(2))-dimethyltransferase execute two successive methylations of guanosine (G) to N2-, N2-methylguanosine (m2G), and N2-methylguanosine (m2G). These gene candidates were significantly upregulated in infected samples. Moreover, the enzymes involved in the formation of N1-methyladenine at position 58 (m1A58) in tRNA [39], tRNA (guanine(26)-N(2))-dimethyltransferase execute two successive methylations of guanosine (G) to N2-, N2-methylguanosine (m2G), and N2-methylguanosine (m2G). These gene candidates were significantly upregulated in infected samples. Moreover, the enzymes involved in the formation of N1-methyladenine at position 58 (m1A58) in tRNA [39], tRNA (guanine(26)-N(2))-dimethyltransferase execute two successive methylations of guanosine (G) to N2-, N2-methylguanosine (m2G), and N2-methylguanosine (m2G). These gene candidates were significantly upregulated in infected samples.

2.7. Comparison of tRNA modification profile of *P. capsici* infected vs uninfected black pepper

The present study investigated how the composition and abundance of tRNA-modified nucleosides change in response to *P. capsici* infection in black pepper. LC-MS analysis can detect and quantify tRNA nucleoside modification and was used to identify and quantify the tRNA modification during stress response (Fig. 8).

The LC-MS analysis has shown that the modifications, namely, N4-acetylcytidine (ac4C), 5-formyl-2'-O-methylcytidine (f5Cm), 5-methyluridine (m5U), 5-methoxycytidine (mo5U) and 5-methoxy carbonylmethyl-2'-O-methyluridine (cm5U) have shown consistent abundance in the 24 hpi and 48 hpi samples. Moreover, the increase in fold change of these modifications during pathogen attack in black pepper, we attempted to uncover the status of tRNA modifications during *P. capsici* infection. The study has explored the various tRNA nucleoside modifications, corresponding enzymes in black pepper and their response to pathogen attacks.

2.8. Real-time analysis of enzymes involved in ac4C modification

The abundance of ac4C in the LC-MS analysis during *P. capsici* infection has led us to investigate the crucial enzyme, N-acetyltransferase 10 (NAT10). NAT10 is involved in the acylation of tRNA nucleoside cytidine (C) to N4-acetylcytidine (ac4C) [16]. The qRT-PCR analysis has shown that the NAT10 expression was increased at 24 hpi but later decreased at 48 hpi (Fig. 9).

3. Discussion

Black pepper is a well-known spice crop, fostered with various health benefits and has been used for ages. The globally used spice crop is exposed to various biotic and abiotic threats. Among them, the infection caused by *P. capsici* is the most devastating threat to black pepper cultivation. Plants respond to infections by reprogramming their molecular and cellular mechanisms. The outburst of ROS is one of the primary levels of defence opted by the plants during pathogen invasion [21]. The accumulation of ROS and associated cell death in black pepper during *P. capsici* challenge were detected using histochemical methods like Trypan blue, DAB and NBT, as these are the standards to detect ROS accumulation. Even in other plants like strawberries, pumpkins, etc., the same procedure was used to visualize ROS outbursts after pathogen challenges [46]. Interestingly, studies in yeast demonstrated that under oxidative stress, the tRNA modifications are dynamically altered [11,42]. The present study is the first attempt to unveil the impact of stress in tRNA modifications in a non-model spice crop like black pepper. Despite several studies that have revealed the molecular level changes during pathogen attack in black pepper, we attempted to uncover the status of tRNA modifications during *P. capsici* infection. The study has explored the various tRNA nucleoside modifications, corresponding enzymes in black pepper and their response to pathogen attacks.

The technical intricacies in tRNA isolation and purification hinder thorough research on tRNA modification in non-model plants.
Fig. 5. The Bubble plot showing the differential expression of MTase candidates in Black pepper during *P. capsici* infection (Pn_IL) in comparison with control (Pn_CL). FDR (False Discovery Rate) control is the statistical method used here for the normalization of FPKM values.
The size-specific separation of tRNAs using Urea polyacrylamide gel electrophoresis is a facile and widely accepted method for tRNA studies in higher organisms [35]. Further, an LC-MS approach was used to scrutinize the alterations in the tRNA modification profile of black pepper during *P. capsici* infection. Though LC-MS cannot pre-

**Fig. 6.** The eleven MTase candidates in Black pepper show significant differential expression (p < 0.05) during *P. capsici* infection. The blue colour represents low-expressed MTase candidate genes, and the red colour represents highly expressed MTase candidate genes. Pn_CL control; Pn_IL *P. capsici* infected samples. The FPKM values were used to plot the heatmap. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Fig. 7.** The qRT-PCR analysis of gene candidates shown significant differential expression during 24 hpi. The x-axis is the hours post-infection, MSL is the mock leaf, and 24 hpi is 24 h post-infection with *P. capsici*. While performing the unpaired test, p values less than 0.05 are summarised with one asterisk 0.001 are summarised with three asterisks, and p values less than 0.0001 are summarised with four asterisks, ns means non-significant p-value summarised greater than 0.05.

**Fig. 8.** The heatmap shows the tRNA modification profile comparison in uninfected, 24 hpi and 48 hpi black pepper leaf samples. MRM (Multi reaction monitoring) peaks of modified nucleoside were extracted and normalized to the quantity of the tRNA purified. The 24SL and 48SL are the 24, and 48 hpi *P. capsici* infected samples, and groups 24MSL and 48MSL are the 24 h and 48 h mock-treated samples.
dict the exact positions of modifications, it is mainly used for the qualitative and quantitative analysis of nucleoside modifications under different cellular conditions [56]. For example, the LC-MS accurately quantified the abundance of different tRNA nucleoside modifications like Cm, m5C and m2G modifications in S. cerevisiae during oxidative stress induced by H2O2 [8,49]. Our study identified fifty different modifications in black pepper, whereas similar studies in Arabidopsis and Rice have predicted twenty-one and twenty-two different nucleosides, respectively [59,60].

Furthermore, our analysis showed that most of the modifications in black pepper tRNA were methylations catalyzed by enzymes called MTases. Methylation are the products of complex pathways requiring various enzymes and protein complexes. For instance, the mcm5U modification reaction in yeast needs nearly 25 gene products [11,57]. So, later, we investigated the various tRNA MTase gene candidates in black pepper. But the biochemical and molecular extraction of the entire set of MTases gene candidates in a non-model spice crop like black pepper using conventional methods brings many challenges. So, we relied on the omics datasets of black pepper to predict the MTases gene candidates in black pepper [41,31,32]. Previous studies in yeast, Arabidopsis, Rice etc., have used the protein sequence homology-based extraction of tRNA modification gene candidates from the omics datasets [11]. Similarly, we extracted the MTase gene candidates from the publicly available black pepper transcriptome based on sequence homology with yeast, Rice and Arabidopsis. Further, the bioinformatic characterization of the black pepper MTases shown the presence of multiple conserved motifs compared to yeast, Arabidopsis and Rice, which may be crucial in rendering catalytic activity to the enzymes [22].

Similar to modifications, corresponding enzymes that catalyze these reactions also have a crucial role in stress response [43,44]. In line with this during P. capsici infection, the MTase candidate genes involved in the methylation of Guanine, Cytosine and their derivatives were upregulated in black pepper. Predominantly, the candidate genes associated with the methylation of m7G, Cm, Cmnm5U, Cmnm5Um, m1G, Wybutosine, m1G, m2G and m3G were significantly upregulated, which suggests the importance of tRNA methylations during the stress response. Intriguingly, we noted the increase of corresponding nucleoside modifications and some of their intermediates in the LC-MS profile of pathogen-challenged black pepper. Our results were in partial agreement with previous reports in Rice and Arabidopsis where the tRNA modifications and their related MTases gene expression of tRNA nucleosides namely Cm (2′-O-methylcytidine), m1A (1′-methyladenosine), and m7G (7-methylguanosine) were implicated in plant stress regulation [59,60]. Surprisingly in black pepper, the differential analysis of MTases during P. capsici infection didn’t signify the enzymes responsible for Am modification which was increased in Arabidopsis during biotic stress.

Furthermore, the LC-MS profile demonstrated that N4-acetylcytidine (ac4C) was the most abundant modification present in black pepper during P. capsici infection at 24 hpi. The ac4C modification is known to present at the wobble position of tRNA Met in E.coli [33], tRNAGln, tRNAGlu, tRNALys, tRNAPro, and tRNAser from halobacteria species [37]. So, the absence of ac4C disturbs the correct codon-anticodon pairing. Additionally, in humans, the significance of ac4C during stress response was noted during several diseases [27]. However, in plants, the ac4C modification was previously known for enhancing translation efficiency [2]; for the first time, the current study reports the abundance of ac4C modification in P. capsici infection hints its implication in stress regulation. The synthesis of ac4C modification is catalyzed by the enzyme- N-acetyltransferase 10 (NAT10) or its homologous enzyme [28]. The qRT analysis showed an increase in gene expression during 24hpi. This observation support the increase of ac4C abundance at 24 hpi. As mentioned earlier, the expression of enzymes associated with modification is very critical in the determine the presence of corresponding modification in the cell. For example, the down regulation of TRMT61B gene which catalyses m1A58 in three mt-tRNAs Leu, Ser, and Lys has resulted in the decrease of corresponding modifications in Alzheimer’s disease [13].

The f5Cm modification that is present at the 34th position of tRNA Leu [26] was the second most abundant modification at 24 hpi. Further, during 48hr post infection, the tm5U modification was significantly elevated. The presence of tm(5)U modification was reported at the 34th wobble position of tRNA Leu, tRNA Trp and tRNA Lys, tRNA Gln, tRNA Glu [50]. In humans, the lack of tm5U modification in 34th wobble position leads to MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes) disease [29,30]. However, the enzymes involved in the formation of tm5U modification are not reported yet [20,47]. Nevertheless, there exists a temporal alteration in the modification profile, the most abundant modifications ac4C, tand m5U, f5Cm were found in the wobble position of multiple tRNAs. The modifications present at the wobble position of the anticodon loop are crucial in the codon-anticodon pairing, thereby facilitating the translation. The significant increase of such modifications during pathogen infection can be an adaptive response chosen by the plant to enhance the translation during stress. Similarly, in yeast the modifications of tRNALysUUU, tRNAGlnGUU and tRNAGluUUC at the wobble positions were increased during high temperatures and restored at normal conditions [1].

Nevertheless, we have identified and quantified an extensive set of tRNA nucleotides, and we never intend to claim that the tRNA modifications, which were not detected in the study, are not present in black pepper; also the possibility of missing their detection in the LC-MS approach cannot be ignored. In an LC-MS study in yeast could not detect 2′-O-ribosyladenosine phosphate (Ar(p))
moreover, the 5-Carbamoylmethyl-2'-O-methyluridine (nmc5Um) was  
uncertainly detected by collision-induced dissociation (CID)  
due to weak signal strength [48]. Furthermore, the LC-MS data  
cannot map the exact position of modification in tRNAs. But mapping  
these abundant modifications in black pepper can highly assist in  
confirming the functional relevance of these modification in stress  
response. Here we assume that the modifications which have a  
regulatory role in defence regulation may lie in the anticodon  
region to enhance the translation during the stress response. Stud- 
ies have shown that the modifications in the anticodon regions  
are crucial during the translation of stress-responsive proteins [9,27].

In conclusion, we identified a wide range of tRNA modifications  
in black pepper, a non-model spice crop which is extensively used  
for its taste and other medicinal benifits. Among the various pre-  
dicted tRNA modifications, methylations were predominant in  
black pepper. Since MTases catalyze the methylations, we further  
annotated the tRNA MTase gene candidates from the publicly avail-  
able black pepper transcriptome. Furthermore, comparison of  
MTase candidate gene expression during  
P. capsici- black pepper interaction  
showed their significant upregulation. Our study revealed  
the differential expression of tRNA modifications and cognate  
enzymes during  
P. capsici infection in black pepper. Moreover, the LC/MS profile of black pepper tRNA modification during  
P. cap- 
sici infection demonstrated a time-dependent variation in the pro- 
file. Since, to date, the pathways of tRNA nucleoside modification  
are not fully depicted even in model organisms, our study is the  
first attempt to outline the story of tRNA modifications and their  
role in stress response in a non-model spice crop like black pepper.  
So, our future efforts will be to overcome the present limitations of  
the study by isolating the individual tRNAs and mapping the modifi- 
cations associated with them in black pepper. Additionally, to  
validate the direct role of tRNA modification in stress response.  
Similar studies can assist researchers in predicting the hidden  
functions of tRNA modifications in other higher plants.

4. Materials and methods

4.1. Plant inoculation by the pathogen

Black pepper young, healthy plantlets were inoculated with a  
pure culture of  
P. capsici collected from the Department of Plant  
Pathology, College of Agriculture, Vellayani. Kerala, India. The  
P. capsici culture was maintained in Potato Dextrose Agar at 28 °C.  
The 48 hr old  
P. capsici culture plug was used to inoculate the abaxial  
side of black pepper leaves. The inoculation procedure was done  
according to Kattupalli et al. [31,32]. The uninfected plants were  
mock-inoculated with PDA plugs with sterile water. Both the  
infected and the uninfected plants were maintained under the  
same environmental conditions. The leaf above the infected leaf  
(systemic leaves) from both uninfected and infected plants in trip- 
licates were harvested at different time intervals like 24 hpi and 48  
hpi for further studies.

4.2. DAB, NBT and trypan blue staining

The pathogen infection in plants leads to the release of several  
reactive oxygen species. DAB staining [17,52], NBT Staining [36,23]  
were done for detecting the release of H2O2 and O2 respectively  
whereas Trypan blue staining was carried out to evaluate cell  
death as previously described with slight modifications [24].  
Briefly, the  
P. capsici inoculated leaf samples were immersed in  
DAB solution and kept for shaking for about 4–5 h. Later the sam- 
ple was treated with a bleaching solution (ethanol: acetic acid:  
glycerol = 3:1:1) in a water bath at 95 °C for 15 min. The step  
was repeated by immersing the leaf samples in fresh bleaching  
solution until the chlorophyll was fully bleached out and then visu- 
alized under a light microscope. The detection of O2涟 which is gen- 
erated by the activity of NADH dehydrogenase was done by NBT  
staining. NBT was dissolved in Sodium Phosphate Buffer (pH 7.5),  
them the leaves were immersed in the solution and incubated in a  
shaker for 3–4 h at 27 °C. Later, NBT was removed by adding  
100 % ethanol and kept in the water bath for 10–15 min at 90 °C  
and then visualized under the light microscope. Trypan blue stain-  
ing solution was prepared as previously mentioned by Heese and  
team (6 vol of ethanol, 1 vol of water, 1 vol of lactic acid, 1 vol of  
glycerol, 1 vol of phenol and 0.067 % wt/vol trypan blue) [24].  
The stain is then added to the leaf sample and heated in a boiling  
water bath for 10 min or till the leaves were completely devoid of  
chlorophyll. Later, the samples were washed with 1X Phosphate-  
buffered saline (PBS). A set of three young plants were used as bio- 
logical replicates, and leaves were sampled from each plant for all  
microscopic analysis.

4.3. Total RNA isolation

The leaf samples were collected from the uninfected (mock-  
infected at 24 h and 48 h) and infected samples at 24 and 48 hpi.  
Total RNA was isolated from all the samples using the miRvana  
Total RNA isolation kit (Thermo Fisher) according to the manufac- 
turer’s protocol. Briefly, 1 gm of freeze-dried leaf samples were  
homogenized with lysis buffer and RNA isolation aid. The mixture  
was centrifuged at 12,000 rpm for 5 min, 4 °C. The supernatant was  
collected then 1/10th volume of miRNA homogenate additive and  
an equal amount of acid phenol: chloroform (Thermo Fisher) were  
added. The upper aqueous phase was retrieved, and 100 % ethanol  
was added and allowed to pass through a filter cartridge provided  
by the manufacturer (Thermo Fisher). The flow-through was dis- 
carded, and the filter cartridges were washed with miRNA wash  
solution I and then with miRNA wash solution II. The filter car- 
tridge was transferred to a fresh collection tube, and RNA was  
recovered with 100 μL of preheated (95 °C) elution buffer or  
nuclease-free water. The quality and quantity of thus obtained  
total RNA samples were assessed by absorbance measurements  
at 230, 260, and 280 nm in a NanoDrop™1000 spectrophotometer  
(Colibri, Germany). The integrity of total RNA was checked by  
1.2 % (w/v) Agarose Gel Electrophoresis. The isolated total RNA  
with quality and integrity was submitted to Arraystar, USA for  
LC-MS analysis.

4.4. tRNA isolation

tRNA was isolated from total RNA samples by Urea-PAGE elec- 
trophoresis. The total RNA for each sample was separated by 7.5 %  
PAGE (29:1 acrylamide: bisacrylamide) containing 7 M urea. The  
60–90 nt tRNA band was excised from the gel and then extracted  
0.3 M NH4Ac and precipitated with glycogen and ethanol. Purified  
tRNA was quantified using Qubit RNA HS Assay kit (ThermoFisher,  
Q32855) and proceeded to further downstream analysis.

4.5. tRNA digestion and LC-MS analysis

Purified tRNA was hydrolyzed to single nucleosides and  
derephosphorylated by a 50 μL enzyme mix (10 U Benzonase  
(Sigma), 0.1 U Phosphodiesterase 1 (US Biological), 1U Alkaline  
Phosphatase (NEB)]. Pre-treated nucleosides solution was depro- 
etinized using Satorius 10,000-Da MWCO spin filter. Then the reac- 
tion was incubated at 37 °C for 3 h. Parallely, a 10,000-Da MWCO  
spin filter (Satorius) was rinsed by adding 300 μL of deionized  
water and centrifuging for 5 min at 16,000 g at 4 °C. The hydro- 
lyzed RNA sample was transferred to the rinsed spin filter and cen- 
trifuged for 10 min at 16,000 g at 4 °C. The filtrate was collected for
downstream LC-MS analysis. The single nucleosides mixtures from black pepper tRNA were injected into the LC-MS system and set up the HPLC condition according to solvent gradient (Solution A, HPLC-grade water with the relevant amount of formic acid to obtain a final formic acid concentration of 0.1 % (vol/vol); Solution B, 100 % acetonitrile with the appropriate amount of formic acid to achieve a final formic acid concentration of 0.1 % (vol/vol).

4.6. Transcriptome-wide extraction of tRNA methyltransferase candidates from black pepper

The protein sequences of known tRNA nucleoside MTases from S. cerevisiae, Arabidopsis (https://www.arabidopsis.org/) and Rice (https://rice.plantbiology.msu.edu/index.shtml) were considered as query sequences. These query sequences were used to retrieve black pepper MTase gene homologs with tblastn search using bioedit software (https://bioedit.software.informer.com/). Along with these, using NR and Swissport annotations, we have curated all the MTase genes from the black pepper transcriptome. All obtained sequences were translated into all six frames using Geneious software (https://www.geneious.com/) for further analysis. A cut-off e-value was set as 1.0E-6 for the initial identification of candidate genes. A phylogenetic tree of MTases was constructed with the Maximum likelihood method in MEGA X software, bootstrap analysis was performed with 1000 iterations. Protein sequences were manually verified by protein domain analysis on NCBI-CDD (Conserved Domain Database) website (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The logos and conserved motifs were identified by MEME online searching engine (https://meme-suite.org/tools/meme) with default parameters setup.

4.7. Gene expression analysis of MTase candidate genes

The spatial expression pattern of MTases were analyzed by the comparative analysis between control and P. capiscit challenged transcriptomes of black pepper plants (SRX853636- PnIL; SRA050094- PnCl). The expression levels of each MTase candidate unigenes were taken by their corresponding FPKM (fragments per kilobase per million mapped reads). The raw reads from the mRNA transcriptomes were submitted in the NCBI Sequence Read Archive [NCBI: SRX853636 (PnIL) and SRA050094 (PnCL)] treated and control transcriptome which was previously done by our lab were used for the analysis. The calculation of Unigene expression uses FPKM (RPKM) method (Fragments Per kb Million reads), the formula is shown below.

$$\text{FPKM} = \frac{(106 \times C)}{(NL/103)}$$

Set FPKM(A) to be the expression of Unigene A, and C to be number of fragments that uniquely aligned to Unigene A, N to be total number of fragments that uniquely aligned to all Unigenes, and L to be the base number in the CDS of Unigene A. The FPKM method is able to eliminate the influence of different gene length and sequencing level on the calculation of gene expression. Therefore, the calculated gene expression can be directly used for comparing the difference of gene expression between samples. A heatmap representing all the MTases genes in uninfected and infected samples was generated using the edge R package. 1 µg of total RNA was used for cDNA synthesis using high-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time quantitative reverse-transcription (qRT-PCR) was performed using ABI Quastntudio using POWER SYBR Green qPCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. Each qRT-PCR reaction was conducted in 10 µL volumes containing 1 µL of cDNA (5 ng), 5 µL of SYBR green and 5 pmol of forward and reverse primers with the following conditions: 40 cycles at 95 °C for 15 s and 65 °C for 15 s. Negative PCR controls (Non template control- NTC) were prepared to detect possible contamination. The primers used were shown in Supplementary Table 2. The expression of these genes was compared with that of endogenous control gene 5.8 s rRNA. Relative mRNA ratios were calculated by $2^{-\Delta\DeltaCT}$ [40]. The experiments were done in triplicates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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List of author contributions

A.U and E.V.S conceived the research plans and designed the experiments. A.U, K.D performed the wet-lab experiments, and S. B assisted the same. A.U, K.D and P.V did in silico experiments and did data analysis. A.U wrote the article with the contributions of all the authors; S.B, P.V and K.D made critical revisions. E.V.S supervised and complemented the writing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcsbj.2022.11.002.

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