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RESEARCH

Comprehensive study of tRNA-derived fragments in plants for biotic stress responses

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Abstract

Plant growth and development are often disrupted by biological stressors as they interfere with the regulatory pathways. Among the key regulators, transfer-RNA-derived fragments (tRFs) have emerged as key players in plant defense mechanisms. While tRF-mediated responses to abiotic stress have been well studied, their role in biotic stress remains less understood, as various stressors may elicit different regulatory systems. In this study, tRF-mediated biotic responses in three species, viz. *Arabidopsis thaliana, Oryza sativa,* and *Solanum lycopersicum* are investigated using *in-silico* approaches. Analysis of predicted tRFs across various biotic stress conditions reveals specific interactions with mRNA targets, microRNAs (miRNAs), and transposable elements (TEs), highlighting their regulatory significance in plant adaptation mechanisms. These findings provide new insights into tRF-mediated stress responses and establish a computational framework for further functional studies. The study's database is publicly available at http://www.nipgr.ac.in/PbtRFdb.

Keywords TRFs · TRNA fragments · TncRNAs · Biotic stress · Stress response · Genome regulation · Plant database

Introduction

The majority of the human genome encodes for non-coding RNAs (ncRNAs) which are now extensively studied for their crucial roles in regulating gene expression and genome organization (Nemeth et al. 2023). The endonucleolytic cleavage of precursor tRNA (pre-tRNA) or mature tRNAs, which constitute 4–10% of total cellular RNA, results in the formation of a pool of tRNA-derived fragments (Lee et al. 2009; Zhao et al. 2023). It constitutes the short fragments of length 14 to 30 nucleotides (nt) called tRNA derived fragments (tRFs or tDRs), longer tRNA halves (tRHs or tiRNAs) of approximately 30 to 50 nt (Sun et al. 2020; Zhu et al. 2019a, b) and other tRFs from the internal region of tRNAs. All the classes termed as tRNA-derived non-coding RNAs (tncRNAs) are conserved across most organisms.

These genome regulatory molecules are further classified based on the tRNA from which they are derived (S. Li et al. 2018). The tRFs derived from the 5' end are tRF-5, and the 3' end generates tRF-3, resulting from cleavage in the D and T regions, respectively (Ma et al. 2021). Additionally, pre-tRNA generates 5' U-tRFs (leader tRF) and tRF-1/tsRNA (30 U-tRFs) from the 5' leader and 3' trailer regions, respectively (Fig. 1) (Zahra et al. 2021). In the case of tRNA halves, 5' and 3' tRHs are produced from cleavage in the anticodon region, containing the 5' and 3' portions of the mature tRNA (Ma et al. 2021). Therefore, the identified tRFs are classified into tRF-5, tRF-3, tRF-1, leader-tRF, 5' tRH, 3' tRH, and a miscellaneous group labeled as other tRFs (Zahra et al. 2021).

The originating tRNA type, cell type, developmental stage, and stress conditions influence the specific cleavage of tRNA that generates these fragments. (Zahra et al. 2021). These tRFs can be produced in both DICER-dependent and -independent manner. RNase P eliminates the leader sequence and RNase Z cleaves the trailer sequence precisely at the discriminator base of the precursor-tRNA leading to the production of leader-tRF and tRF-1/tsRNA, respectively (Kim et al. 2020). Processing of certain types of tRNA halves requires both Dicer and angiogenin (Liu et al. 2018). The regulatory role of tRFs includes 1) regulation of mRNA stability as well as their potential to induce mRNA cleavage (Yu et al. 2021), 2) translation inhibition (Kumar et al.

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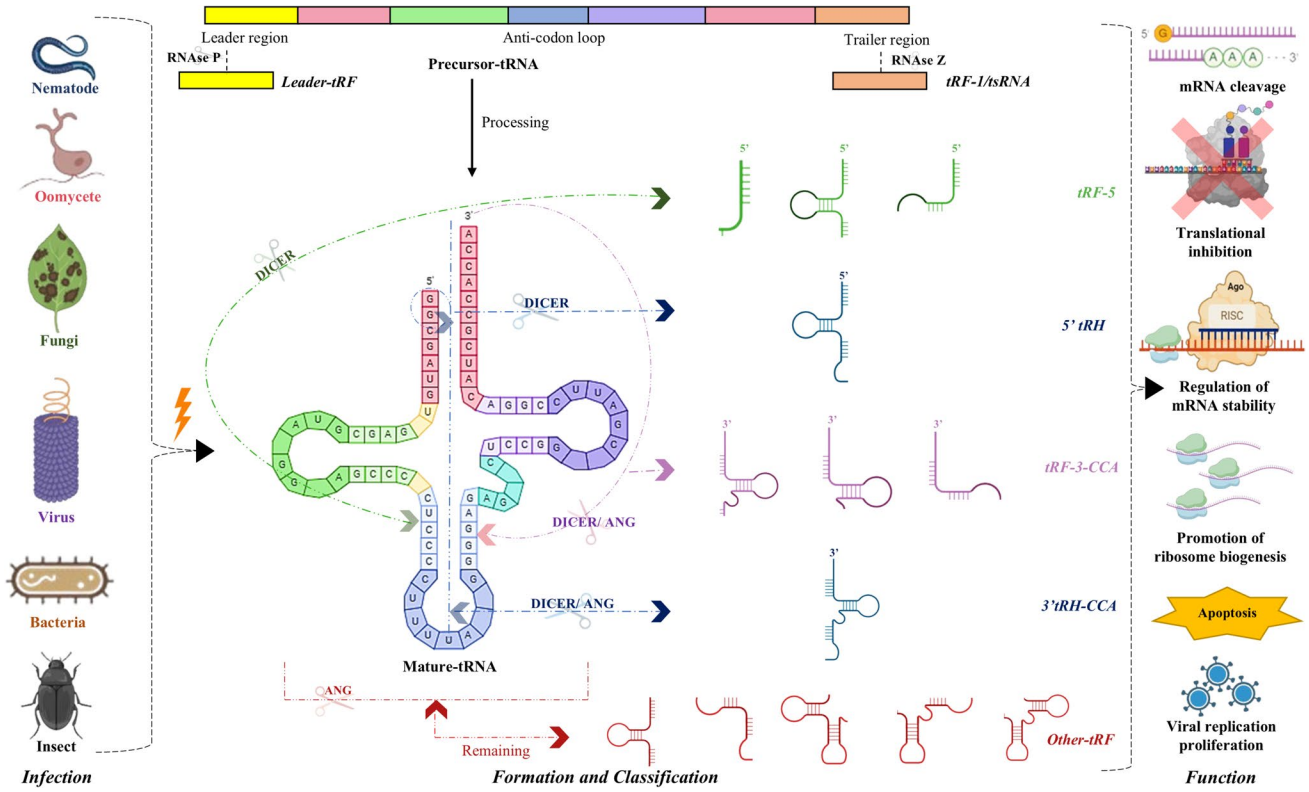

Fig. 1 Classification and biogenesis of tRNA-derived fragments (tRFs). These fragments are generated through specific cleavage mechanisms involving enzymes such as DICER and angiogenin (ANG). tRFs are produced under various biotic conditions, including those caused by nematodes, fungi, viruses, bacteria, and insects, which influence tRNA cleavage and tRF production. The resulting tRFs play diverse biological roles, such as association with RISC complexes, regulation of translation, and apoptosis

2016), 3) regulation of ribosome production (H. K. Kim et al. 2017), 4) control on apoptosis of cells depending on the diseases and target mRNA (Cui et al. 2022; Gong et al. 2023), and 5) facilitates amplification of viral propagation (Fu et al. 2015).

Exploration of tRFs in plants has been growing rapidly due to the exposure of plants to various biotic and abiotic stresses, which significantly impact their development (C. Wang et al. 2023). To manage these challenging conditions, plants have developed complex response mechanisms that involve multiple gene regulatory processes, including transcriptional (Chang et al. 2020) and post-transcriptional regulation (Jones-Rhoades et al. 2006). It is shown that tRFs in plants operate similarly to those in animals which could be due to their similarity in biogenesis, suggesting an evolutionary connection (Cao et al. 2022; Lalande et al. 2020). The studies on tRFs related to biotic stresses are relatively less developed compared to research on abiotic stresses (C. Wang et al. 2023). Nonetheless, the biological functionality of tRFs produced by biotic stress is better understood than that of abiotically induced tRFs (C. Wang et al. 2023). Their functional roles were thoroughly investigated in both the leaves and roots of black pepper affected by *Phytophthora capsici* (Asha & Soniya 2016). In *A. thaliana*, *Botrytis cinerea* infection was seen to be significantly downregulating the 5' tRNA-derived small RNAs (Gu et al. 2022) whereas in *S. lycopersicum*, differentially expressed tRFs were characterized in plants subjected to *Tomato Mosaic Virus* (*ToMV*) (Zahra et al. 2021). Identification and characterization of tRFs and their specific possible targets in plants, particularly under biotic stress conditions, alongside an in-depth understanding of tRF-guided stress regulatory networks, can greatly contribute to enduring agricultural production. In this study, we identified a diverse range of tRFs across three angiosperms viz. *A. thaliana*, *O. sativa*, and *S. lycopersicum* using publicly available small RNA-seq (sRNA-seq) datasets of plants under various biotic stresses. Differential expression (DE) analysis revealed stress-specific tRFs, and common tRNA sources, followed by their mRNA targets, complemented by functional enrichment and pathway analysis. Analysis of RNA-Seq datasets further highlighted the DE patterns of mRNA targets, and interactions between tRFs and TEs, offering insights into their roles in stress adaptation. These findings were integrated into PbtRFdb, a comprehensive database with advanced search features, visualization tools, and dynamic graphs for

exploring tRFs under biotic stress conditions. An overview of each step followed during the study is shown in Fig. 2.

Methodology

Data retrieval and processing

A total of 112, 188, and 44 sRNA-seq samples of *A. thaliana*, *O. sativa*, and *S. lycopersicum*, sequenced under various biotic stress conditions, were downloaded from the NCBI SRA Toolkit (v3.0.2) (S. Sherry et al., 2023). Genome files for the respective plants viz. *A. thaliana* (TAIR10.1), *O. sativa japonica* (Build 4.0; organellar: IRGSP-1.0), and *S. lycopersicum* (nuclear: SL4.0; organellar: SL3.0), were obtained from EnsemblPlants (https://plants.ensembl.org/index.html) (Yates et al. 2022). To facilitate efficient secondary analysis, the headers for the corresponding plant genome files were substituted with "chr" followed by the chromosome numbers (Zahra et al. 2021). The raw FASTQ reads were processed by Trim Galore v0.6.6 (https://github.com/FelixKrueger/TrimGalore) to eliminate the low-quality bases and adapter contaminations (J. Wang et al. 2022). The FASTQ data quality comparison was then performed with FastQC v.0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

tRF identification and filtration

For each sRNA-Seq sample, the tncRNA toolkit (https://github.com/skbinfo/tncRNA-Toolkit.git) was employed to identify the tRFs and their associated tRNAs (Zahra et al. 2023). The genomes were indexed using the integrated Bowtie v.1.3.0 tool in this toolkit, and this indexed genome was utilized to further locate the tRFs in the sequencing samples. The tRFs identified from each sample of each plant were concatenated, resulting in three merged files, one for each species. The tRFs with individual expression values of ≥ 10 per million reads were shortlisted (Alves et al. 2017). Unique sequences were subsequently identified, with the criterion that tRFs with identical sequences but differing modification sites were treated as distinct tRFs. The tRFs were named according to their respective species i.e. *A. thaliana* tRFs were designated as tRFs; *O. sativa* tRFs were labeled as OStRFs; and *S. lycopersicum* tRFs were referred to as SLtRFs (Table S1).

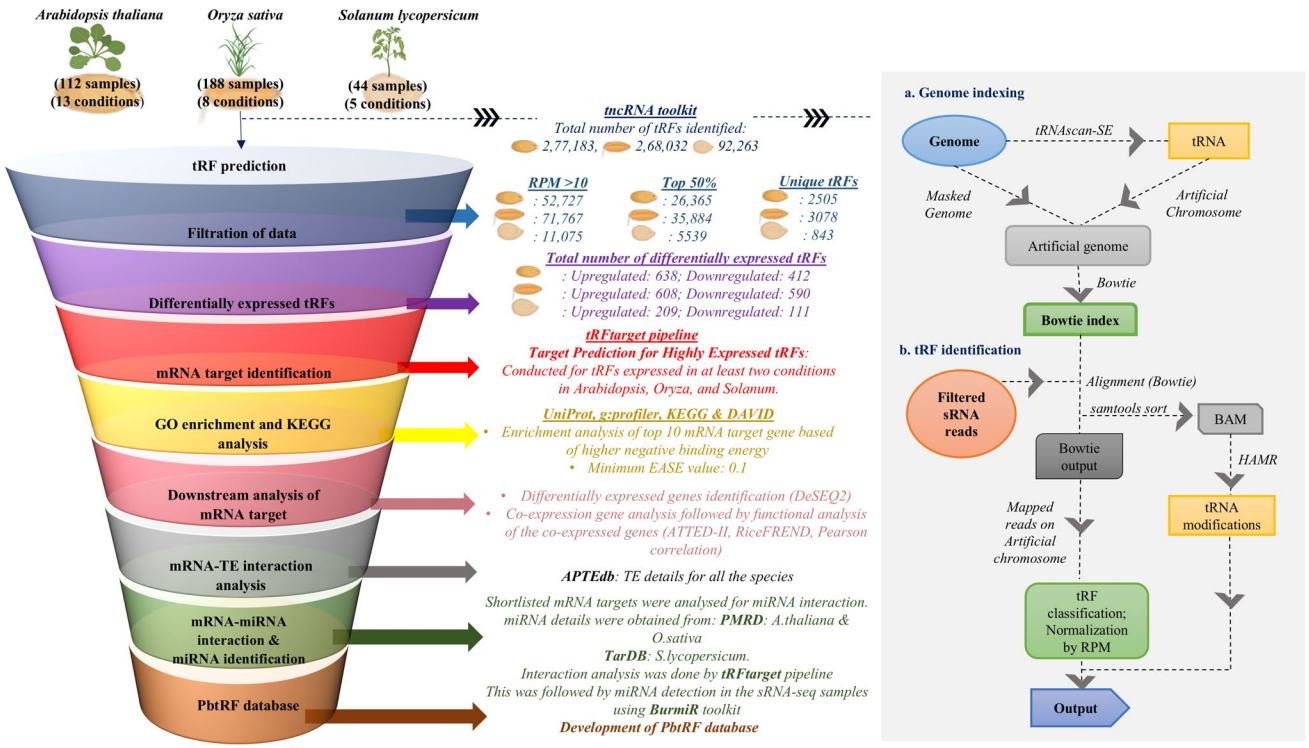

Fig. 2 Workflow for the Identification and Analysis of tRNA-derived Fragments (tRFs). The workflow outlines a systematic pipeline for identifying and analyzing tRFs under biotic conditions. Initial datasets consist of 112 samples across 13 biotic conditions for *Arabidopsis thaliana*, 188 samples across 8 biotic conditions for *Oryza sativa*, and 44 samples across 5 biotic conditions for *Solanum lycopersicum*

Differential expression of tRFs

A matrix was constructed in R, comprising tRFs and their corresponding read counts across the various samples (Table S1). This matrix was utilized as "countData" for DE analysis, with sample names and their descriptions designated as "colData", as detailed in Table S2. For the identification of differentially expressed tRFs under various stress conditions, the DESeq2 R package was used (Love et al. 2014) with the R script mentioned in the Supplementary file 2. Only samples with corresponding treated and control pairs for each condition were considered. The threshold to identify the DE genes was an absolute fold change (|log2FC|) ≥ 1 or ≤ 1, along with an adjusted *P*-value < 0.05 (Kim et al. 2024a, b) by using the Benjamini–Hochberg correction. The bar plots and heatmaps were created using the "ggplot2" package in R.

Target prediction and functional enrichment analysis

The highly, differentially expressed tRFs that were present in at least two infections were considered as queries for target prediction against the cDNA sequence of the considered plants. The tRFtarget-pipeline v0.3.0 was used to detect the respective transcripts for the tRFs at default parameters (https://github.com/ZWang-Lab/tRFtarget-pipeline.git) (N. Li et al. 2021). Moreover, it also facilitates the computational calculation of the binding energy between the tRFs and their target transcript (Tan et al. 2024). Based on minimum free energy (mfe) for each tRF, the top 10 targets were subjected to GO and pathway enrichment followed by further characterization. Functional enrichment of the target genes was done on g:profiler (https://biit.cs.ut.ee/gprofiler/gost) (Raudvere et al. 2019); Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Dennis et al. 2003) (Saif et al. 2022); UniProt (https://www.uniprot.org/uniprotkb/) (Bateman et al. 2021), and KEGG (https://www.genome.jp/kegg/) (Kanehisa 2000). The significance threshold while using g: profiler was set to Benjamini–Hochberg FDR with a user threshold of 0.05 (Garay-Baquero et al. 2020). The DAVID analysis parameters were configured with an EASE score threshold of 0.1, the inclusion of FDR for result display, and a requirement for at least two hub genes in the analysis (Mukherjee & Sudandiradoss 2021). We employed a combination of databases for functional analysis since many tomato genes have not yet been annotated in g: profiler and DAVID. To address this, individual genes were further analyzed using UniProt which also helped in minimizing false positives. Only the most highly enriched terms across the databases were selected for downstream analysis (Saif et al. 2022).

RNA-Seq analysis and co-expression profiling of differentially expressed genes

The reference genome was indexed using HISAT2 (D. Kim et al. 2019), which was also utilized to align the filtered reads to the indexed genome. The aligned reads were assembled into potential transcripts using StringTie (Pertea et al. 2015), and the transcript abundance was quantified as FPKM values [total exon fragments/mapped reads (millions) x exon length (kB)]. The number of mapped reads and transcript length in the samples were normalized to accurately reflect each transcript's expression level and a gene count matrix consisting of raw read counts was constructed (Kim et al. 2024a, b). To evaluate whether the target genes were upregulated or downregulated during the infection, differential expression analysis was performed using DESeq2, employing the same parameters previously used for the DE tRF study. The volcano plot was generated using the SRplot web server (https://www.bioinformatics.com.cn/srplot) (Tang et al. 2023). The target genes that were downregulated in the samples were shortlisted and further underwent co-expression analysis. The Venn diagrams were generated using the jvenn web server (https://jvenn.toulouse.inrae.fr/app/index.html) (Bardou et al. 2014). The Pearson pairwise correlation analysis was conducted on the normalized data obtained from the differential expression study using the "corrplot" and "hclust" R packages. The threshold for the Pearson correlation coefficient was set at ≥ 0.9 between genes with a significance *P*-value of 0.05 to ensure that only highly co-expressed genes are considered (Pirona et al. 2023). The functional correlation of the co-expressed genes in *A. thaliana* and *O.sativa* was investigated using ATTED-II (https://atted.jp/) (Obayashi et al. 2022) and RiceFREND (https://ricefrend.dna.affrc.go.jp/) (Sato et al. 2013), respectively. Genes that were positively co-expressed with the target gene and also showed downregulation under the same infection condition in *S. lycopersicum* were selected for GO enrichment using UniProt. Cytoscape software version 3.10.2 was used to visualize and construct the various biological networks required for the study (Shannon et al. 2003).

Identification of potential transposable element targets

Details of Transposable Elements for the respective organism were obtained from the APTEdb website (http://apte.cp.utfpr.edu.br/) (Pedro et al. 2021). Only known and characterized TEs were retained for further analysis. To determine the DE status of the genes, their coordinates were cross-referenced with the DE data. The interaction of the tRFs with the TE was visualized using tRFtarget-pipeline v0.3.0. To categorize the classes of TEs involved in interactions

with tRFs, a chord diagram was created using the "circlize" package in R. (H. Wang et al. 2019).

Detection and verification of miRNAs for the target mRNA

The top 10 targets were shortlisted based on 'mfe' of each tRF and were also examined for potential miRNA interactions. The miRNA information for *A. thaliana* and *O. sativa* was sourced from the PMRD database (https://bioinformatics.cau.edu.cn/PMRD/) (Z. Zhang et al. 2010) while data for *S. lycopersicum* was obtained from the TarDB database (http://www.biosequencing.cn/TarDB/) (Liu et al. 2021a, b) respectively. After conducting the mRNA-miRNA interaction study using the tRFtarget pipeline v0.3.0, the interactions between tRFs and miRNAs with their respective target genes were compared based on binding energy and the location of binding on the mRNA. Subsequently, the presence of the miRNA in the sample was detected using the BrumiR toolkit (https://github.com/camoragaq/BrumiR) (Moraga et al. 2022).

Database development

The data analysis was compiled into a database called "PbtRFdb". Web development for this database was carried out using the XAMPP package (v.8.0.30) (https://www.apachefriends.org/), which facilitates the integration of Apache, MySQL, and PHP in a local server environment. The front-end web interface was designed using HTML, CSS, and JavaScript. MySQL handled data management and processing, providing a robust platform for storing and efficiently retrieving data. The PHP scripts interacted with MySQL to deliver query results to the user.

Results

Comprehensive profiling and differential expression of tRFs under biotic stress conditions across three plants

Using the tncRNA-toolkit, a total of 277,183 tRFs were identified in *A. thaliana*, 268,032 in *O. sativa*, and 92,263 in *S. lycopersicum*. After an initial filtration based on individual expression level (Fig. S1), and retaining the top 50% of tRFs, a final unique set of 2,505 tRFs for *A. thaliana*, 3,078 for *O. sativa*, and 843 for *S. lycopersicum* was obtained. Based on individual expression levels, other-tRFs categories were more abundant (Table S1). The number of tRFs remaining after each filtration step is shown in Fig. 2. We analyzed the expression of these tRFs under various biotic stress conditions in plants, revealing distinct variations in both expression patterns and the types of tRFs across different groups of biotic stress infections (3), and in response to each specific infection (Fig. S2). A significant number of tRFs, across various types, were predominantly upregulated in all the plants (Fig. S3). The classes tRF-5, other-tRFs and 5' tRH showed significant differential expression. Alongside the stress response, we also observed tissue-specific variations in tRFs expression. Notably, tRF-5 was highly upregulated and downregulated in leaf tissue at different developmental stages under various infections (Fig. S4). The majority of the tRF-5 fragments were found in the developmental stages ranging from mature leaf (21–30 days old) to young leaf (15 days). Additionally, other tRFs were found to be DE across major tissue types under prominent biotic stress conditions, including various developmental stages of leaves and in 15-day-old roots of *O. sativa*.

GluTTC exhibits maximum tRNA cleavage in response to biotic stress

All the DE tRFs were analyzed for the tRNA origin information. It has been found that the GluTTC generated the highest number of tRFs across various biotic stress conditions in all studied plants (Fig. S5). Different isoacceptors produced various types of tRFs and showed differences between plants (Zahra et al. 2021). Specifically, the isoacceptors of tRNA-Glu i.e., TTC and CTC, contributed to the majority of DE tRF production across all plants, with GluTTC producing the highest amount of tRF-5 in *A. thaliana*, *O. sativa,* and *S. lycopersicum*, respectively (Fig. S6). Other tRFs were also observed to be produced from Glu-TTC in both *A. thaliana* and *S. lycopersicum*. Various anticodons contributed to the production of tRF-5 across all plants. For example, in *A. thaliana*, tRF-5 was also generated by GlyTCC, AspGTC, GlyGCC, and MetCAT, among others. To assess the impact of tRFs generated from GluTTC, their mRNA targets were identified and shortlisted based on binding energy. The identified targets were then subjected to GO enrichment analysis, revealing a strong enrichment for the terms "response to heat" and "cellular component organization" in *A. thaliana* (Fig. S7). These terms fall under the broader categories of "response to stimulus" and "cellular process". The targets of tRFs generated by GluTTC in *O. sativa* were found to play a role in "galactose metabolism", suggesting that these tRFs may regulate genes involved in this metabolic pathway. None of the genes showed enrichment in *S. lycopersicum.*

Enrichment of mRNA targets in distinct stress response mechanisms

tRFs may function as post-transcriptional regulators by binding complementarily to their mRNA targets, leading to the degradation of these mRNAs (Alves et al. 2017; Kuscu

et al. 2018). To explore the functional roles of tRFs, we conducted target predictions (Fig. 3, Table S3) and pathway enrichment analyses, for tRFs expressed in at least two infectious conditions in plants. Our analysis revealed that the majority of target genes were associated with responses to various stress types, including cold stress (abiotic) and bacterial stress (biotic) (Fig. S8). The cellular mechanisms activated to cope with stressors, such as "signal transduction", "transport", and various "metabolic processes", were found to be enriched across all plant species analyzed**.** Within the transport processes, "microtubule-based movement" was particularly prominent. Among biological processes, the term "biosynthetic process" was observed to be the most highly enriched across the plants. Under molecular functions, "catalytic activity" was extensively abundant. Consistent with the findings of Zahra et al. 2021, our tRF targets were also prominently associated with "transferase" and "kinase" activities. Additionally, "binding" was highly enriched in *A. thaliana* and *O. sativa*. "ATP binding" was significantly enriched and the most prevalent term, while "microtubule binding" exhibited the highest level of significance. In *A. thaliana* and *O. sativa*, the term "membrane" was highly and significantly enriched, whereas in *S. lycopersicum*, the term "nucleus" was extensively enriched. These findings suggest distinct functional adaptations among plant species, reflecting their specific stress response mechanisms and highlighting the role of tRFs in regulating various biological processes (Fig. S9).

Identification of differentially expressed mRNA targets

RNA-seq analysis followed by DE gene identification was conducted to examine the expression of target genes in the samples under biotic stress conditions. This approach also aimed to assess the potential influence of tRFs on regulating these genes within the samples. In *O. sativa* and *S. lycopersicum*, most of the genes were observed to be downregulated, whereas, in *A. thaliana*, the majority of the genes were predominantly upregulated (Fig. 4). No common genes were identified across all conditions in all plant species; however, overlapping genes in at least two conditions are evident in Fig. S10. Most of the 76 genes in *A. thaliana* were found to overlap between those upregulated during *Tobacco Mosaic Virus* (TMV) infection and those downregulated in response to *Turnip Crinkle Virus* (TCV). A similar trend was observed in *O. sativa* and *S. lycopersicum*, where gene overlaps were detected in at least two infection conditions. In *O. sativa*,

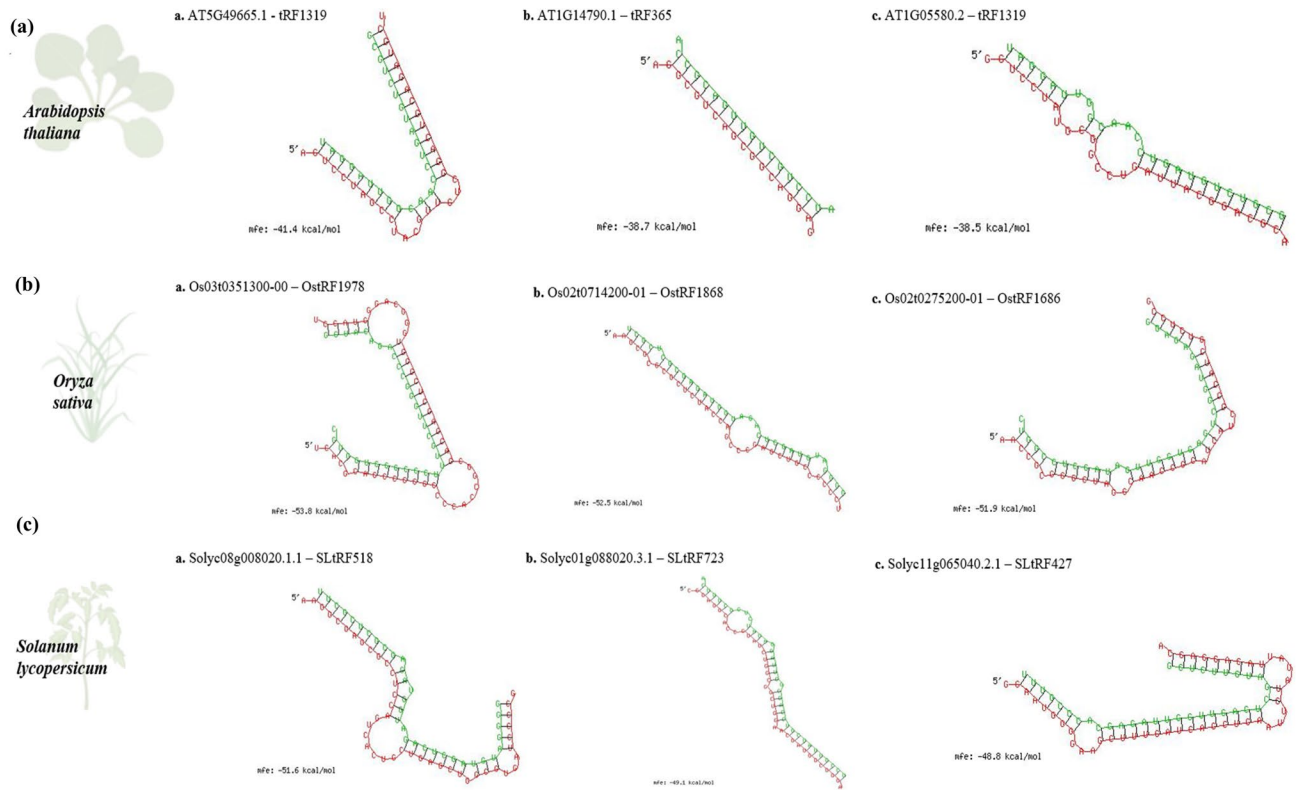

Fig. 3 mRNA targets of the most recurrent tRFs identified through tRFtarget-pipeline v0.3.0 (https://github.com/ZWang-Lab/tRFtarget-pipeline.git). The figure highlights the top three mRNA targets for each species with their minimum free energy (mfe): **a** *Arabidopsis thaliana*, **b** *Oryza sativa*, and **c** *Solanum lycopersicum*

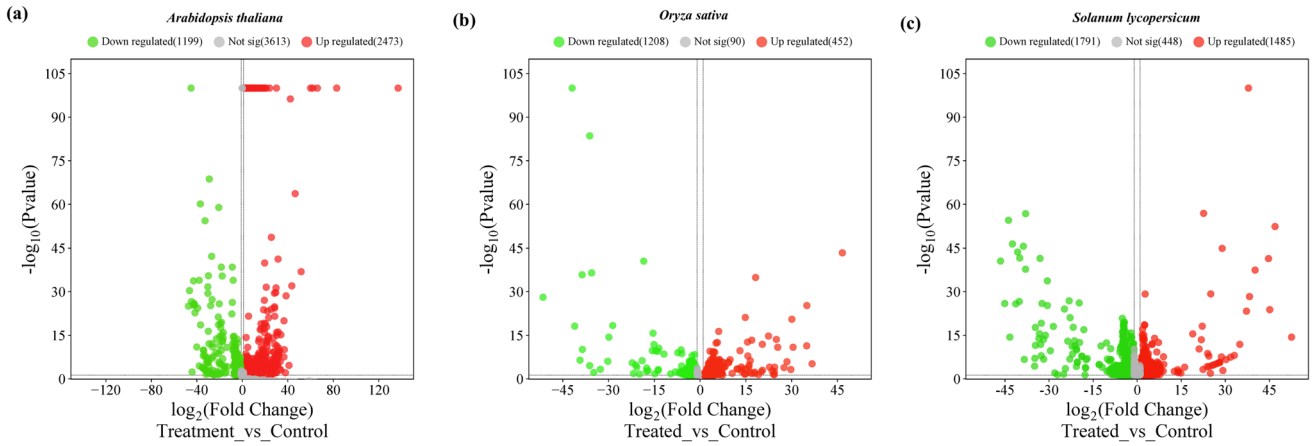

Fig. 4 Volcano plots showing the gene expression in response to biotic stress conditions. The plots illustrate the upregulation and downregulation of genes for each species: **a** *Arabidopsis thaliana*, **b** *Oryza sativa*, and **c** *Solanum lycopersicum*

11 genes predominantly overlapped between those downregulated by *Brown Planthopper* (BP) infection and *Xanthomonas oryzae* (XO) infection. Meanwhile, in *S. lycopersicum*, a substantial overlap of 150 genes was noted between those downregulated during *Meloidogyne incognita* (MI) infection and those upregulated in response to *ToMV*. In *A. thaliana*, the overlapping genes were significantly enriched in pathways related to "intracellular protein transport," "ARF protein signal transduction," and "post-transcriptional gene silencing" (Fig. S11). These terms generally fall under the category of stress response. In *O. sativa*, "monoterpenoid biosynthesis" was prominently featured, while in *S. lycopersicum*, the "ubiquitin conjugation pathway" was notably highlighted which also indicated the stress-related process.

Upregulated tRFs may contribute to the downregulation of target mRNAs and co-expressed genes, thereby influencing shared biological pathways

We observed that the majority of shortlisted mRNA targets were downregulated during DE analysis. With the hypothesis that the upregulated tRF could be a contributing factor to the downregulation of its target mRNA (Zong et al. 2021), we selected the downregulated target mRNAs from the list of DE genes in each plant species. The co-expression analysis revealed that genes co-expressed with the target gene were also downregulated in response to the infection. AT2G39950, a flocculation protein and a potential target mRNA for tRF1492 in *A. thaliana*, was significantly downregulated during infection with *Alternaria brassicicola* (AB), showing a log2FC value of −1.66. Additionally, its co-expressed gene AT5G60170 was also found to be downregulated during the same infection, showing a log2FC value of −1.05 (Table S4). The functional analysis revealed that genes AT5G60170 and AT2G28540 both belong to the RNA-binding protein family. At the same time, AT3G22270 is associated with topoisomerase II which may facilitate mRNA decay and enhance post-transcriptional reprogramming https://www.arabidopsis.org/locus?name=AT3G22270 (Fig. 5a). In *O. sativa*, the gene OsCesA5 (Os03g0837100), which encodes a cellulose synthase A5 protein, was significantly downregulated during the BP infection with a notable negative log2FC value of −3.075. This gene is targeted by OstRF1920 and OstRF1928, which were significantly upregulated during the same infection. All the co-expressed genes, including CESA6 (Os07g0252400), OsGT47A (Os01g0926600), and OsFLA3 (Os08g0321000), were significantly downregulated during BP infection; however, the Os07g0633600 gene did not show any DE in the samples. The CESA6 and OsGT47A are involved in “plant-type cell wall biogenesis” and “polysaccharide biosynthetic process” (Fig. 5b). These genes along with the target mRNA, OsCesA5, play a role in the biosynthesis and modification of the plant cell wall. The other co-expressed gene sets for the mRNA targets OsWD40-125 (Os06g0143900) and Os09g0283600 did not show DE under any of the infectious conditions. In *S. lycopersicum*, the majority of significant co-expression was observed for the target gene *Solyc02g080340.3* with SLtRF725 and SLtRF26 under *ToMV* infection, specifically in processes related to “fatty acid metabolism and lipid degradation” and “defense response to other organisms”. The target gene was downregulated with a log2FC value of −0.96, while the tRFs were upregulated with log2FC values of 28.054 and 36.855, respectively, during the infection. All the co-expressed genes were downregulated under *ToMV* stress. Among them, the *Solyc01g104210.2* gene is also involved in “fatty acid metabolism (elongation)” and the “sphingolipid biosynthetic process” (Fig. 5c). Two of the co-expressed

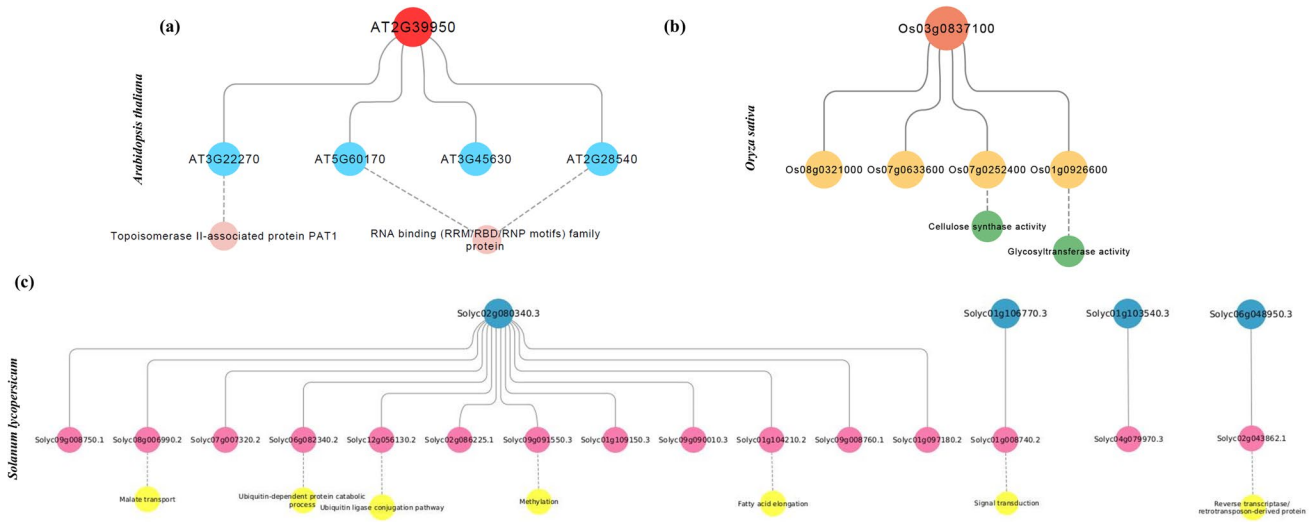

Fig. 5 Flowchart illustrating the co-expression of mRNA targets with other genes and their functional annotation for each species: **a** *Arabidopsis thaliana*, **b** *Oryza sativa*, and **c** *Solanum lycopersicum*

genes, *Solyc06g082340.2* and *Solyc12g056130.2,* are involved in ubiquitin-dependent pathways, specifically the ubiquitin-dependent protein catabolic process and the ubiquitin ligase conjugation pathway, respectively. In contrast, most of the remaining genes were not found to be significantly enriched. Moreover, the gene *Solyc06g048950.3*, which encodes a non-specific serine/threonine protein kinase (STPK), was significantly downregulated during infection by the *Alternaria* fungus (AF). This gene showed considerable co-expression with a retrotransposon-derived protein that was also downregulated under the same infection. Additionally, the target gene *Solyc01g106770.3*, another STPK-TOR, which is targeted by the upregulated SLtRF723, was downregulated under ToMV infection, along with its co-expressed gene *Solyc01g008740.2*, a calcium-dependent kinase involved in intracellular signal transduction.

miRNAs did not influence target mRNA expression

Fig. S12a illustrates that several mRNA transcripts in *A. thaliana* have the potential to be regulated by specific miRNAs capable of binding to them. In contrast, in *O. sativa*, two genes were identified to bind to miRNAs, as depicted in Fig. S12b potentially. No miRNAs were found to potentially bind to any predicted target genes in *S. lycopersicum*. A comparison of the binding energies and binding coordinates between miRNA and tRF reveals that, although both exhibited highly negative binding energies, the binding coordinates on the target gene differed (Table 1). Additionally, these mRNA targets did not show DE under any infection conditions in either *A. thaliana* or *O. sativa*. Furthermore, none of these miRNAs were detected in the samples considered in this study. Moreover, this supports the previous hypothesis that the downregulated potential target mRNA and its corresponding co-expressed downregulated mRNA may be DE primarily due to their interaction with the tRFs, which are upregulated during various biotic stresses.

tRFs regulate the activity of the transposable elements

The chord diagram in Fig. 6 collectively demonstrates that most tRFs across all plant species exhibited a strong affinity for binding to LTR/gypsy TE, supporting the findings of(Zahra et al. 2021). In *A. thaliana*, tRF-5 was predominantly associated with LTR/gypsy elements, while tRF-3-CCA primarily interacted with SINE/Unknown elements (Table S5). It was observed that TE transcripts were predominantly downregulated during TCV infection, with tRF-5 and 5'tRH showing strong interactions with TE transcripts in *O. sativa*. Notably, tRF-5 mainly interacted with Helitron/Helitron elements, while 5'tRH was primarily associated with TIR/Dada elements. Significant downregulation of TE transcripts was observed during BP infection, with some downregulation also occurring in XO and *Meloidogyne graminicola* (MG) infections. In *S. lycopersicum*, most tRF-5 s and other-tRFs showed a preference for interacting with LTR/gypsy elements, with some interaction observed with LTR/Copia and LTR/Unknown elements. The strongest downregulation of TE transcripts was observed during AF infection, with notable downregulation also seen in MI and ToMV infections. This supports the conclusion highlighted in the review article of (Y. Zhang et al. 2024), that tsRNA can regulate cellular responses by affecting the activity of retrotransposons. It was observed that tRNAMetCAT generates

Table 1 Comparative analysis of miRNA and tRF targets in *Arabidopsis thaliana* and *Oryza sativa*

Species	Target	miRNA	SubseqDP	BE miRNA	Start target	End target	tRF ID	SubseqDP	BE tRF	Start target	End target
Arabidopsis thaliana	AT3G10290.1	ath-miRf10578-akr	AUAUAAGCG AAGAGUGCA GU&ACUGCA ACUGCUUGUAU	−23.2	1413	1432	tRF1319	UCCUUUAAUCAA GCCGUUGGU GCUACGACG C&GCGUCUGUA GUCCAACGG UUAGGA	−36.8	451	481
	AT5G58190.2	ath-miRf10373-akr	AGCUCUUCU UUGGGAGGU UA&UGACCU GCCAAAGGA GAGUU	−34.3	1474	1493	tRF365	UGGCGUCCAACA GCAGG&CCU GCUGUUGAC GCCA	−33.3	81	97
	AT1G05580.1	ath-miR414	GCGGUGAUGGUC UGAA&UUCAUC AUCAUCGU	−20.6	1154	1169	tRF1319	GUCCUAUGCGGC CUGAUUACG GACGC&GCG UCUGUAGUC CAACGGUUA GGAU	−38.5	874	899
	AT1G05580.2	ath-miR414	GCGGUGAUGGUC UGAA&UUCAUC AUCAUCGU	−20.6	1077	1092	tRF1319	GUCCUAUGCGGC CUGAUUACG GACGC&GCG UCUGUAGUC CAACGGUUA GGAU	−38.5	874	899
	AT1G72120.1	ath-miRf11033-akr	GAUGUUGAA GAUGCCACA GC&GCUGUG GCAUCAUCA AGAUU	−32.8	1212	1231	tRF1492	UUGAGCUAGAGG CAAAUACAU CCCC&GGGGAU GUAGCUCAA	−28.5	821	845
	AT3G04200.1	ath-miR163	AUCGAAGGGUUC GAUCCAAAA ACCGCGUCU GCAA&UUGAAG AGGACUUGG AACUUCGAU	−21.5	217	250	tRF1319	AUCCUGGUACCA UUAGGAUUG CAGACGC&GCG UCUGUAGUC CAACGGUUA GGAU	−38.5	545	572
	AT1G56080.1	ath-miRf10720-akr	UCCGCGGUAUAC CGCGGG&CCC GCGGUAUAC CACGGG	−37.7	111	128	tRF1492	GAGCUGCAUCUC C&GGGGAUGUA GCUC	−29.4	614	626

Table 1 (continued)

Species	Target	miRNA	SubseqDP	BE miRNA	Start target	End target	tRF ID	SubseqDP	BE tRF	Start target	End target
Oryza sativa	Os05g0590000	osa-miR2103	UGGGUGCGCACG GCCUUGGAG AAGGCGGCG GGGAGAG&UUU CCCUCUCCG UGCGCGCUC	−36.8	1526	1547	OstRF1274	GCGACGUUCUAC UACGUGCAC CUCCUGGGC UUCAGCGUG GGC&GCCCAC UUAGCUCAG AGGUUAGAG CAUCGC	−45.4	961	1002
	Os05g0590000	osa-miR2103	UGGGUGCGCACG GCCUUGGAG AAGGCGGCG GGGAGAG&UUU CCCUCUCCG UGCGCGCUC	−36.8	1526	1547	OstRF1530	CCUACGCCGCGG UGGACUGCG UCGCGC&GCG UUUGUAGUC CAACGGUUAGG	−33.2	530	556
	Os06g0611000	osa-miRf11622-akr	GGCUGCUCCGGU GAA&UGCCAU CACCGGUAG CAGCACUU	−30.2	4090	4112	OstRF2652	GACCAGCUA GCUGCAGAG CAGGA&UCC GUUGUAGUC UAGCUGGUU	−34.1	1060	1082

The table displays target gene identifiers, miRNA and tRF ID, and the subsequence of duplex pairing (SubseqDP), their binding energy (BE), and start and end target positions within the gene

Fig. 6 Chord diagram shows that the majority of tRFs across all plant species display a strong binding affinity to transposable elements, LTR/Gypsy TEs

tRF-s which specifically targets the LTR retrotransposon, Athila6A (Martinez et al. 2017). Since both tRFs and TEs are known to be expressed and accumulate under stress, it would seem that the regulation of tRF production is connected to TE activity.

PbtRF database

A total of 637,476 entries are stored in PbtRFdb, comprising 277,181 entries for *A. thaliana*, 268,032 for *O. sativa*, and 92,263 for *S. lycopersicum*. For differentially expressed

tRFs, *A. thaliana* has 2,260 entries, *O. sativa* has 6,663, and *S. lycopersicum* has 901 entries. Detailed information on tRFs can be retrieved through two main modules: the "Search" and "Browse" modules. The Search module allows users to extract detailed information based on sequences, tRF types, amino acids, anticodons, modifications, and differentially expressed tRFs. The "DE tRFs" section enables users to query biotic stress-specific studies across the three species, with filters for various stress conditions and expression levels. The Browse module allows users to extract information across the three species via four categories: tissue type, amino acid, anticodon, and stress. Each query provides users with specific details such as SRA accession, organism, tissue, developmental stage, tRF type, locus, position, length, stress, sequence, and modification. The query also provides log2fold change data and expression information for differential expression, including upregulation and downregulation. Each SRA accession is hyperlinked to the NCBI SRA page, offering additional information. Users can visualize tRFs using the "JBrowse" module for the species of interest. The module allows users to select tracks, including the reference sequence, general feature file track (GFF), and tRF track. Users can zoom in on specific locations to view tRFs and gene features. The tRF track includes sequence information, tRF type, tRNA derivatives, and the length of the tRF. PbtRFdb also includes a "Statistics" module, which allows users to visualize tRFs across various stress conditions. The most abundant classes of tRFs and parental tRNAs under different conditions are displayed using graphs. DE tRFs are presented with user-friendly visualizations such as line graphs, bar plots, and pie charts. Additional features include a "Download" module, allowing users to download results for each plant species separately. A "Help" module is also provided to guide users through navigating the database.

Discussion

tRNA-derived fragments are emerging as essential regulators of plant defense responses and stress-related adaptations. However, their functional roles in biotic stress responses remain underexplored. In this study, we address this gap by using computational methods to comprehensively profile tRFs across the plant species—*A. thaliana*, *O. sativa*, and *S. lycopersicum* under biotic stress conditions. Furthermore, we studied the regulatory networks of the majority of recurring tRFs, by examining how they interact with mRNA targets, miRNA, and TEs to gain a better understanding of their functional importance in plant stress response.

A significant number of other-tRFs amongst the tRF subclasses may indicate that less-defined tRFs are important mediators of plant responses to different biotic stresses. The findings of Thompson et al. 2008, showcased that oxidative stress causes the endonucleolytic cleavage of tRNAs in plants, was consistent with the significant differential expressions shown in the tRF-5, other-tRFs, and 5'tRH classes. Likewise, Alves et al. 2017 emphasized how tRF-5 is linked to certain plant AGOs, promoting its synthesis under abiotic stress. Thus, it is reasonable to assume that during biotic stress conditions, tRF-5 actively participates in post-transcriptional gene regulation. Plant immunity is based on the different pathways that it may activate during infection.

In our study, for differential expressions we considered biotrophic pathogens (*Xanthomonas oryzae, Heterodera schachtii, African Xanthomonas, Meloidogyne graminicola, Meloidogyne incognita*) (Galeng-Lawilao et al. 2018; Haidar et al. 2016; Panthapulakkal Narayanan et al. 2020), necrotrophic pathogens (*Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Alternaria brassicicola*, *Fusarium oxysporum*) (Cho 2015; Pandey et al. 2016; Panthapulakkal Narayanan et al. 2020; Petrasch et al. 2019) and hemibiotrophs (*Phytophthora capsici, Magnaporthe oryzae*) (Barraza et al. 2022; Panthapulakkal Narayanan et al. 2020). Biotrophic pathogens activate salicylic acid (SA)-dependent immunity (Yang et al. 2015) and necrotrophic pathogens activate jasmonic acid (JA)- and ethylene (ET)-dependent immunity (Macioszek et al. 2023; Rahman et al. 2012; Sanchez et al. 2012). NRP1 is a gene that regulates salicylic acid-mediated immunity in plants (Mishra et al. 2024). In one of the studies, cleavage mediated by the 5′Ala^{CGC} tRF was experimentally mapped at the tRF binding sites on the mRNA targets of non-expresser of pathogenesis-related protein (NPR1), which showed a down-regulation during Phytophthora capsica infection (Asha & Soniya 2016). Another study demonstrates that 5′ tRNA-Ala-derived tRFs regulate the defense against fungal responses, possibly due to the upregulation of jasmonic acid-mediated immunity (Gu et al. 2022). These pathways can function both synergistically and antagonistically, adding complexity to plant immune responses (Ghozlan et al. 2020; N. Li et al. 2019; Mur et al. 2006). Our results highlighted that tRF-5, tRF-3-CCA, and other-tRFs were major subclasses that were differentially expressed in all the infections suggesting that they are involved in the specific adjustments of plant immunity (Figure S1). As viruses are obligate biotrophs, their relationship with SA-dependent defense may influence tRF biogenesis. The increased levels of specific tRFs in *A. thaliana* as a response to bacterial, fungal, and viral infections could indicate a potential activation of SA-mediated immunity. In *O. sativa*, the decrease of specific tRFs in fungi and virus infections might suggest a suppression or diminished activation of JA/ET-mediated immunity. In *S. lycopersicum*, the increase of specific tRFs during fungal and viral infections could indicate a response similar to that seen in *A. thaliana*. Gaining insight into the responses of tRF subclasses to biotrophic and necrotrophic pathogens within these different immune networks may help

us understand their possible regulatory functions in plant defense.

The regulatory role of tRF-5 is evident, as a large amount of tRF-5 is cleaved from tRNA-GluTTC across all species (Figure S6). tRNA-GluTTC translates to the glutamate (Glu) amino acid, which is a key metabolite in stress-related pathways such as signaling and metabolic regulation. Therefore, tRNA-GluTTC is highly abundant during abiotic stress across species (Qiu et al. 2020; Zahra et al. 2021). tRNA-GluTTC serves as a stress-responsive biomarker in human gastric and thyroid cancers (Mao et al. 2024; Shan et al. 2021; Zhu et al. 2019a, b). Using GO enrichment analysis, we derived some functional relevance of tRFs cleaved from tRNA-GluTTC. In *A. thaliana*, the tRFs were linked with UDP glucose 4-epimerase activity (essential for cell wall biosynthesis), ATP-dependent chromatin remodeling activity (related to plant development and stress response), and cytoplasmic stress granule (early stress response), all of which contribute to stress adaptation (Guo & He 2024; Maruri-López et al. 2021; Reuper et al. 2021; Rösti et al. 2007) (Figure S7a). In *O. sativa,* tRFs cleaved from tRNA-GluTTC were associated with galactose metabolism (Figure S7b), a process that plays a role in cellular homeostasis during stress conditions (Wang et al. 2024; Höftberger et al. 2022; Liu et al. 2021a, b). However, no significant enrichment patterns were observed for *S. lycopersicum*, possibly due to the limited functional annotation data. This indicates that tRFs derived from tRNA-GluTTC may play an active role in responses to biotic stress by regulating metabolic pathways through gene expression or interactions with proteins that respond to stress, as part of a larger plant defense system (Yagyu & Yoshimoto 2024).

Apart from their function in the metabolic process, tRFs also affect gene expression via interacting with splicing factors and mRNA regulation (George et al. 2022; Y. Li et al. 2023). Differential gene expression patterns were observed across species in response to biotic stress. During *Alternaria brassicicola* infection, the target mRNA and co-expressed genes were downregulated in *A. thaliana*, whereas during *Brown planthopper* infestation, the target mRNA co-expressed genes showed a comparable downregulation in *O. sativa*. The target mRNA and co-expressed genes in *S. lycopersicum* were upregulated after *ToMV* infection and downregulated in *Meloidogyne incognita*. The upregulation of the target genes under specific conditions may enhance stress responses, by increasing the metabolism activity and increasing the production of stress-related proteins (Hillwig et al. 2008). Conversely, the downregulation of target genes may suppress certain stress responses therefore increasing the susceptibility to pathogens (Krishna et al. 2021; Park & Kim 2018). The regulatory patterns observed indicate that tRFs might play a role in stress adaptation by modulating mRNA expression and the co-expressed gene network, possibly via RNA interference or splicing modulation. Further experimental validation is, however, required to understand gene regulation mediated by tRFs and its conservation among species.

Considering the established function of small RNAs in gene regulation, it is crucial to observe how tRFs compare or interact with other small RNA pathways, like miRNA-mediated regulation. Mechanisms like translation repression of mRNAs and their degradation have defined the role of miRNAs in regulating mRNA stability (Dalmay 2013). In *A. thaliana,* studies reveal miRNAs play a role in post-transcriptional regulation by targeting the mRNAs of transcription factors (Bartel 2004). The co-expression of the target genes and miRNA has been linked to various metabolic processes and signaling systems. For instance, ath-miR414 participates in the phosphatidylinositol signaling system (Xie et al. 2015), ath-miR163 is potentially linked to adaptations in nutrient-deficient environments (Fasani et al. 2021), and osa-miR2103 was found to play a critical role in rice immunity against *Magnaporthe oryzae* (Fan et al. 2020). The co-expression of tRFs and miRNAs hypothesizes that tRFs can function in miRNA canonical pathways by competing with the AGO association acting as global regulators of RNA-mediated silencing (Alves et al. 2017; Alves & Nogueira 2021). However, despite the well-documented role of miRNAs in gene regulation, our study did not detect miRNAs in the analyzed samples. The absence of detectable miRNAs in our datasets may be due to a reduced count of highly abundant miRNAs. We can hypothesize the possibility that tRFs might serve as alternative regulators of mRNA stability and gene expression. Since tRFs are known to interact with AGO proteins and affect translation in a manner comparable to miRNAs, they could play a role in mRNA silencing via different or independent mechanisms. It is necessary to conduct further research to establish whether tRFs and miRNAs work together in post-transcriptional regulation or if tRFs function as independent regulators under certain stress conditions.

Given the ability of tRFs to regulate gene expression through the interactions with mRNAs, and miRNAs, it is important to extend its regulatory to non-coding elements such as TEs. The interaction between tRFs and TEs has been explored in only a few studies across both plants and mammals, yet their functional relationship remains unclear (Martinez 2018; Slotkin et al. 2009; Zahra et al. 2021). TEs engage with transcripts, modifying expression levels through their role as new promoters or by affecting regulatory pathways (Hirsch & Springer 2017). Our results showed various TE families among species, with some directly associated with stress responses and adaptations to challenging environments (Bolger et al. 2014; Pietzenuk et al. 2016). The findings suggest that tRFs could play a role in the regulation

of TEs, potentially by influencing their expression or affecting their function as regulatory elements.

We also developed a database to catalog and analyze tRF profiles across *A. thaliana, O. sativa*, and *S. lycopersicum,* which allows for detailed investigations of tRFs under different biotic stresses. This resource offers a foundation for future investigations into tRF-mediated regulatory mechanisms during biotic stress conditions.

The insights gained from our *in-silico* analysis provided an emphasis on the regulatory roles of tRNA-derived fragments in plants under biotic stress. The identification of distinct tRF profiles and their potential interactions with mRNA targets, miRNAs, and transposable elements highlights the complexity and specificity of stress response pathways across various plant species. The results emphasize the possible function of tRFs in cellular adaption under biotic stress. To verify these interactions and clarify the molecular pathways by which tRFs aid in cellular adaptation to biotic stress conditions, additional experimental validation and thorough mechanistic investigations are necessary.

Conclusion

tRFs are small non-coding RNAs that can act as novel regulators, impacting transcriptional and post-transcriptional processes (Martinez et al. 2017). Several studies have characterized tRFs in viruses, fungi, and plants (Asha & Soniya 2016; Gu et al. 2022; Gupta et al. 2018; Zahra et al. 2021, 2022), making it a critical regulators of stress mechanisms in plants. Our study emphasizes the function of tRFs in biotic stress across three species- *A. thaliana, O. sativa, and S. lycopersicum* as well as their regulatory network with mRNA, microRNAs, and transposable elements.

Notably, our findings reveal that tRNA-GluTTC is extensively cleaved in response to biotic stress and plays a unique role in stress-response pathways. Most recurrent tRFs show interaction with mRNA targets essential for the plant adaptation to stress, indicating that differential tRF expression may influence mRNA regulation. Additionally, post-transcriptional gene silencing may be facilitated by tRF-mediated stress regulation mechanisms that function independently of miRNA pathways. A complex regulatory network affecting stress adaption may result from the interaction of tRFs and TEs. This study's database is accessible at http://www.nipgr.ac.in/PbtRFdb and serves as a valuable resource for future studies on tRFs in plant biotic stress response and provides a background for further experimental studies for their regulatory role.

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Declarations

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