Extracellular ssDNA from *P. tobira* Exerts Strong Insecticidal Activity on *C. hesperidum*

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Abstract

Beyond its function as a carrier of hereditary information, recent research has uncovered novel properties for extracellular DNA, including its role in the adaptation to the environment when released from plants. The secreted DNA has been shown to exert insecticidal effect against insect pests, which plays an adaptive role in plant-insect interactions, particularly in regulating populations of economically important sap-feeding insects. The molecular mechanisms behind this insecticidal effect are underinvestigated and remain largely unknown. Therefore, there is a need for more efforts in uncovering these mechanisms to better understand the plant-pest interactions, which would provide new insights into natural pest control strategies and inspire biotechnological applications. In the current work, we show that *Pittosporum tobira* (*P. tobira*) secretes single-stranded DNA (ssDNA) that exerts an insecticidal effect on *Coccus hesperidum* (*C. hesperidum*). We collected extracellular DNA from *P. tobira* leaves and tested its potential insecticidal effect by applying it to *C. hesperidum*, which is a well-known pest that causes damage to *P. tobira*. Our results reveal that the outermost layer of leaf cuticle of *P. tobira* predominantly contains single-stranded DNA of approximately 100 bases in length, originating from both chloroplast and nuclear genomes. This DNA exhibits pronounced insecticidal activity against *C. hesperidum*, with chloroplast-derived sequences significantly enriched compared to total DNA in intact plant cells. These findings suggest that the microevolution of the *P. tobira* nucleome and plastome contributed to the formation of extracellular DNA with insecticidal properties (eci-DNA) as part of its defence strategy against insect pests. Notably, the laboratory-developed 'genetic zipper' technology, designed for sustainable pest management, possesses characteristics similar to eci-DNA found in nature, highlighting a potential natural parallel to this biotechnological approach.

Keywords: ssDNA, eci-DNA, *P. tobira*, *C. hesperidum*, pest control, 'genetic zipper' technology.

Introduction

The recent advancements in molecular biology techniques shed more light on the structure and functions of biomolecules such as DNA, RNA and proteins. DNA in particular has been shown recently to be an active tool for communication and interaction between cells and different organisms, including plants and insects. We are gradually beginning to understand the importance of DNA's role as a programmable molecule for the interactions of organisms with each other, for the

functioning of distinct ecosystems, and for maintaining the balance of the biosphere as a whole. Plants, as the main primary producers of the planet, use their extracellular DNA (ecDNA) as a versatile protective macromolecule to counteract environmental factors. Although it is not entirely clear how exactly ecDNA is formed in cells, its secretion mainly results from either cell lysis or active release. Secreted ecDNA exists in more or less fragmented forms and can be found as both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) froms. Current knowledge suggests that the formation of a nanolayer of ecDNA by plants on the surface of their leaves, stems, and roots could be a vital adaptation developed during evolution to increase the general fitness of a particular species (Oberemok et al., 2022).

Naked ecDNA, most of which is released as a result of cell death, is nearly ubiquitous in the environment. Its concentration can reach up to 2 μg/L in the soil, and up to 88 μg/L in natural aquatic environments (Tani and Nasu, 2010), where it plays multiple intracellular and extracellular roles. At the intracellular level, it has been shown to be involved in functions including, horizontal gene transfer (Vlassov et al., 2007), providing nutrients (Finkel and Kolter, 2001); or act as a buffer to recruit or titrate ions or antibiotics (Mulcahy et al., 2008). At the extracellular level ecDNA has been shown to be a component in the biofilms of several bacterial species, where it plays different roles such as regulating the attachment and dispersal of specific cell types in the biofilm (Berne et al., 2010), contributing to biofilm formation and providing it with physical strength and resistance to biological stress (Whitchurch et al., 2002, Hu et al., 2012). More recently, ecDNA has been documented to be associated with DAMP (damage-associated molecular pattern) that can cause species-specific plant damaged self-recognition (Barbero et al., 2016; Duran-Flores et al., 2018).

In 2008, our laboratory discovered the insecticidal potential of contact unmodified antisense DNA (CUAD), opening a novel dimension for DNA-based plant protection. CUAD biotechnology (briefly CUADb or ‘genetic zipper’ technology) has since emerged as an efficient and environmentally friendly platform targeting hemipterans, thrips and mites. This approach leverages pest rRNA as a molecular target, significantly enhancing both efficiency and selectivity. Ribosomal RNA constitutes approximately 80-85% of total cellular RNA, whereas mRNAs account for only 5% (Palazzo and Lee 2015). Targeting mature rRNA and/or pre-rRNA increases the signal-to-noise ratio (~10^5:1; rRNA vs. random mRNA), improving specificity while reducing the likelihood of off-target effects. CUADb-based oligonucleotide insecticides, designed with species-specific sequences, exhibit both selective efficacy and resistance avoidance by targeting conserved rRNA regions. On average, pest mortality rates range between 80–90% within 3-14 days following a single treatment with a 100 ng/μL solution, resulting in application of 1 mg of DNA per m^2 of plant leaves (Kumar et al., 2025). This research underscores the potential of CUADb as an innovative, sustainable pest control strategy and highlights its parallels with naturally occurring extracellular DNA with insecticideal properties (eci-DNA), further supporting the role of ecDNA in plant defence mechanisms.

Oligonucleotide insecticides function through a conserved DNA containment (DNAc) mechanism, which can be triggered by exogenously applied antisense DNAs in insects. The DNAc mechanism operates in two steps: (1) the arrest of target rRNA, followed by its hypercompensation via rDNA transcription and (2) target rRNA degradation. The interaction between target rRNA and an oligonucleotide insecticide, in the presence of DNA-guided rRNase, such as RNase H1, mimics a zipper-like mechanism facilitated by a DNA-RNA duplex (‘genetic zipper’ technology) (Oberemok et al., 2025). This ‘genetic zipper’ mechanism, induces metabolic shifts toward lipid-based energy synthesis, enhancing biogenesis of ribosomes and ATP production. Ultimately, widespread kinase downregulation through mTOR, which regulates ribosome biogenesis via mTORC1 causes ‘kinase disaster’ due to ATP insufficiency, while significant RNase H1 upregulation occurs during DNAc (Oberemok et al., 2025).

The primary objective of oligonucleotide insecticides in agriculture is to produce crops free of organic xenobiotics. While these insecticides may not serve as a universal solution at the moment, they could still be highly effective in controlling several dozen economically important insect pest species, representing a major advancement in sustainable plant protection. Current estimates suggest that the CUADb-based 'genetic zipper' technology has the potential to effectively manage up to 50% of the most serious insect pests on the planet using a simple and adaptable algorithm.

Recently, our group documented a naturally occurring insecticidal mechanism relying on unmodified DNA fragments (Oberemok et al., 2022), whether RNA could have a similar effect is not known yet. Notably, RNA interference has a limited success in insect pest control, largely due to the poor understanding of its role in natural pest regulation mechanisms (Cedden and Bucher, 2024). In our previous work (Oberemok et al., 2022), we investigated the effects of eci-DNA fragments from the evergreen shrub *Pittosporum tobira* on the pest *Coccus hesperidum* revealing a pronounced insecticidal effect. In natural conditions, ecDNA concentrations on *P. tobira* leaves were measured at 24.37 ± 0.97 ng per cm^2 (Oberemok et al., 2022). Unexpectedly, later we found that eci-DNA is not exclusively composed of double-stranded fragments but also contains single-stranded DNA in the outermost layer of leaf cuticle and is substantially enriched with chloroplast DNA. This study aims to address this critical knowledge gap highlighting a natural parallel to CUADb-based oligonucleotide insecticides.

Materials and Methods

Origin of P. tobira and C. hesperidum

We identified *P. tobira* Thunb. (Apiales: Pittosporaceae) with *C. hesperidum* L. larvae (Hemiptera: Coccidae) on its leaves at the Nikita Botanical Garden (Yalta, Republic of Crimea, Russia).

Collection of eci-DNA from P. tobira leaves

Eci-DNA was collected during summer from the adaxial (AD) and abaxial (AB) surfaces of around 2400 *P. tobira* leaves gently using wet cotton swabs and filtered through a 0.2-micron syringe filter (Whatman, Maidstone, UK) (Figure 1). The total 10 mL filtrate was concentrated to 400 µL under vacuum (Heidolph, Schwabach, Germany) and used for subsequent experiments.

Gel electrophoresis

To assess the quality and consistency of extracted eci-DNA, 25 µl of filtrate was loaded per well onto a 1.8 % agarose gel, stained with 1% ethidium bromide, and subjected to electrophoresis in Tris-borate-EDTA (TBE) buffer at 10 V/cm for 45 min. The primary eci-DNA fraction was approximately 100 bp in length, as determined using a 100 bp+ DNA ladder (marker) (Figure 1).

Purification of eci-DNA from agarose gel

Eci-DNA was extracted from agarose gel using the ColGen kit (Synthol, Moscow, Russia). Gel fragments containing DNA were excised using a sterile scalpel and transferred to 1.5 mL Eppendorf tubes. The gel mass was determined, and a 3:1 volume of binding buffer was added (e.g., 600 µL for 200 mg of gel). The mixture was incubated at 55 °C for 10 minutes, until complete dissolution. Purification was performed using the Isolation DNA kit (Roche Diagnostics GmbH, Mannheim, Germany) and MagNA Pure Compact Instrument (Roche Diagnostics GmbH, Mannheim, Germany). The purified DNA was concentrated to 30 µL under vacuum (Heidolph, Schwabach, Germany) (Figure 1).

Nuclease Treatment

The RNase A (Vazyme Biotech, Nanjing, China), DNase (Roche, Mannheim, Germany) and exonuclease I (ExoI) (GenTerra, Moscow, Russia) were used according to the manufacturer's instructions. For ExoI treatment, 10 µL of eci-DNA was mixed with 1 µL of ExoI and 2 µL of 1X ExoI buffer. For DNase treatemnt, 10 µL of eci-DNA was combined with 3 µL of DNase in the buffer. Both reactions were incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes. RNase A, DNase and ExoI were topically applied to *P. tobira* leaves by placing a 20 µL droplet on the adaxial side of the leaf and gently distributed by pipette tip. ExoI nuclease treatment was used to calculate percentage of single-stranded DNA in eci-DNA.

PCR analysis of P. tobira eci-DNA

P. tobira eci-DNA was analysed using real time PCR with the fluorescent dye SYBR Green I. The reaction mixture contained 3 µL of DNA, 5X qPCRmix-HS SYBR master mix (Evrogen, Moscow, Russia) and specific primers (Table 1). PCR amplification was performed on a LightCycler® 96 instrument (Roche, Basel, Switzerland) using the following thermal cycling conditions: an initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 54°C for 15 seconds, and elongation at 72°C for 10 seconds. Each reaction was performed in triplicate. Melt curve analysis was conducted to confirm amplification specificity and detect potential nonspecific products.

Table 1. Primer sequences for real time PCR using SYBR Green I system.

Gene	Primer	Primer sequence (5′-3′)	Tm	PCR product	GenBank Sequence ID
23S rRNA, plastome	Pittoplast-F Pittoplast-R	CAGGAATATTCACCTGTTG TAAGATCAGGCCGAAAG	54 °C	50 bp	MN968282.1
5.8S rRNA, nucleome	Pittonucleus-F Pittonucleus-R	ACTTGGTGTGAATTGCAG CGTTCAAAGACTCGATGG	54 °C	47 bp	LC545472.1

Application of eci-DNA as a contact insecticide in lab contidions

Eci-DNA (1.2 ng/µL in nuclease-free water) was topically applied to *C. hesperidum* larvae by placing a 1 µL droplet on the dorsal surface of each larva (average body size: 2–2.2 mm²) using a pipette. As a scrambled control ssDNA fragment, we used 56 base nucleotide long random primer, containing 14 consecutive ACTG sequences $(ACTG)_{14}$, in the same concentration. The control group received nuclease-free water. Experiments were conducted in triplicate, with around 100 larvae per treatment group, which were included in the statistical sample to assess the survival rate. Mortality was calculated as the percentage of dead larvae relative to the total number of individuals treated. Insects were counted using a Nikon SMZ745 microscope (Nikon Inst., Gyoda, Japan) and images were captured with a Toupcam-UCMOS-05100KPA camera (Asmetec, Kirchheimbolanden, Germany).

Application of oligonucleotide insecticides (Coccus-11 and Coccus(5mC)-11) in field conditions

Oligonucleotide insecticide sequences were designed based on the 28S rRNA sequence of *Coccus hesperidum* (isolate S6A395) retrieved from the GenBank database (https: //www.ncbi.nlm.nih.gov/nuccore/MT317022.1, accessed on 2 December 2025). Two short oligonucleotides Coccus-11 (5′–CCA–TCT–TTC–GG–3′) and Coccus(5mC)-11 with methylated cytosines (5′-(5mC)(5mC)A-T(5mC)T-TT(5mC)-GG-3′) were synthesized by Syntol (Moscow,

Russia) and were used for the experiments. All oligonucleotides were dissolved in nuclease-free water at a concentration of 100 ng/µL and applied to plant leaves at a rate of 5 mL per m^2. A control group treated with water was included for comparison. The application rate was 1 mg of DNA in 10 mL of solution per m^2 of foliage infested with the pest. Oligonucleotide insecticides were directly applied to first- and second-instar larvae. To ensure full coverage, the sprayer angle was adjusted so that the oligonucleotides reached the entire leaf surface harboring the pests. During the treatment, the daytime air temperature was 17 °C, humidity was 84%. Over the two-week experimental period (in November 2025), the average daytime temperature was 16.27 ± 2.11 °C, at night – 13.07 ± 2.41 °C, humidity – 76.94 ± 7.12%. Mortality was calculated by dividing the number of dead individuals by the total number of individuals per leaf and multiplying by 100 to express the result as a percentage. Insects were counted using a Nikon SMZ745 microscope and images were captured with a Toupcam-UCMOS-05100KPA camera.

Microscopy of leaf surfaces for nucleic acid detection

The studies were performed on temporary anatomical preparations of *P. tobira* (Thunb.) Aiton leaves. For staining nucleic acids, the method of R. Riegler was modified (Rigler, 1966) using acridine orange fluorochrome.

Microscopy was performed using a MIKMED-2var.26 fluorescence microscope (LOMO-Microsystems, Saint Petersburg, Russia) and objectives: PLAN F 10x/0.30 and PLAN F 20x/0.50. A block of filters "B" (BP460-490/DM500/BA520) was used, which corresponded to an excitation illumination spectrum of 460-490 nm. A 500 nm barrier filter was used; the spectrum of the recorded fluorescence was 520-700 nm. Photographic recording was performed using a digital camera "MC-6.3" (LOMO-Microsystems, Saint Petersburg, Russia), integrated with the software "ToupView" (ToupTek Photonics, Zhejiang, China).

Statistical Analyses

The non-parametric Mann–Whitney U test was used to evaluate the significance of the difference in gene expression. The non-parametric Pearson's chi-squared test (χ^2) with Yates's correction was performed to evaluate the significance of the difference in mortality between control and experimental groups; $p < 0.01$ was considered significant. All calculations were performed using Prism 9 software (GraphPad Software Inc., Boston, USA). All experiments were conducted in triplicate.

Results

Eci-DNA released from P. tobira consists of short DNA fragments

Before testing the insecticidal activity of the released DNA, we first isolated eci-DNA from the leaf surfaces of *P. tobira* and purified it. Next, we loaded it onto 1.8% agrose gel to check size distribution. Contrary to what expected, the size of eci-DNA was short and homogenous, with the majority of fragments it being under 100 bp (Figure 1). This suggests that eci-DNA is a result of an active fragmentation rather than being derived from dead and damaged cells, which would result in a DNA smear with different lengths, ranging from a few base pairs to mega base pairs.

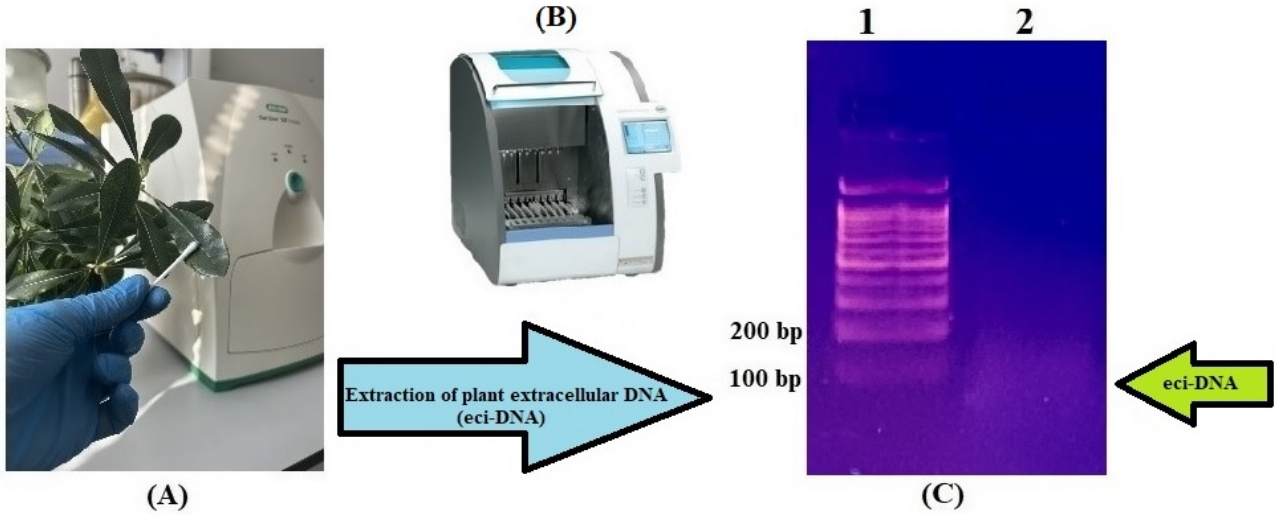

Figure 1. General scheme of collection and extraction of eci-DNA from the leaf surfaces of *P. tobira* (**A**) Collected eci-DNA from the leaf surfaces with wet cotton balls; (**B**) MagNA Pure Compact Instrument was used for the extraction of DNA; (**C**) Electropherogram (1.8 % agarose gel): 1 – DNA ladder 100 bp+; 2 – eci-DNA of *P. tobira* leaves.

Eci-DNA shows pronounced insecticidal effect after topical application

Next, to check whether this uniformally secreted DNA could exhibit an insecticidal effect as an eci-DNA, we topically applied it on *C. hesperidum* larvae (1.2 ng of eci-DNA per larva of 2–2.2 mm^2 body size). Four days post treatment, we assessed the mortality rate and found that it killed about 80% of the larvae (eci-DNA vs. control; χ^2 = 312,225; p <0.001; N = 633; df = 1), while the scrambled control containing a random single-stranded oligonucleotide $(ACTG)_{14}$ did not show any toxicity when compared with the water-treated control group, which both showed 10% mortality $(ACTG)_{14}$ vs. control; χ^2 = 0,006; p >0.05; N = 645; df = 1) (Figure 2).

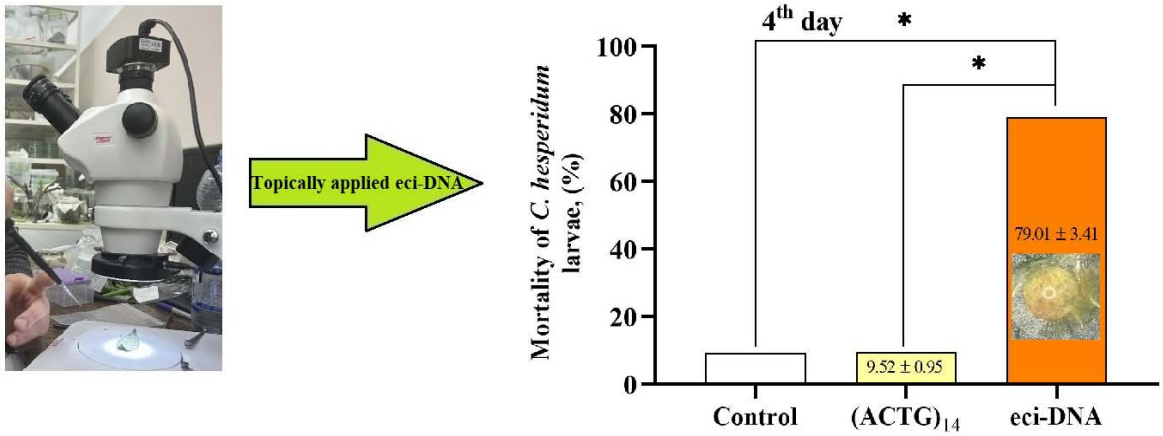

Figure 2. The isecticidal effect of the eci-DNA on *C. hesperidum* larvae. Mortality of *C. hesperidum* larvae after topical treatment with eci-DNA, $(ACTG)_{14}$ and water-treated control. The significance of difference in the eci-DNA group compared to the $(ACTG)_{14}$ and water-treated control is indicated by * at p < 0.01.

It is known that the genomic DNA of insects is predominantly methylated (Glastad et al., 2014; Li et al., 2020). Methyl groups make DNA less immunogenic to the organism itself (Li et al., 2021) and also more resistant to nucleases (Tate and Bird, 1993; Campoy et al., 1995; Diede et al., 2010). We studied the insecticidal activity of the methylated oligonucleotide insecticide Coccus(5mC)-11 and found its high insecticidal potential (76.82%) compared to unmethylated Coccus-11 (64.28%) on 14th day (Coccus(5mC-11 vs. Coccus-11; $\chi2$ = 8.549; p <0.004; N = 420; df = 1) (Figure 3). The

oligonucleotide insecticide Coccus-11 has been well characterized in our earlier studies (Oberemok et al., 2022), has a high insecticidal potential and is a reliable reference oligonucleotide insecticide. The results indicate that DNA methylation increases its insecticidal potential (or at least does not reduce it) and methylated eci-DNA is also capable of possessing insecticidal effect on pests.

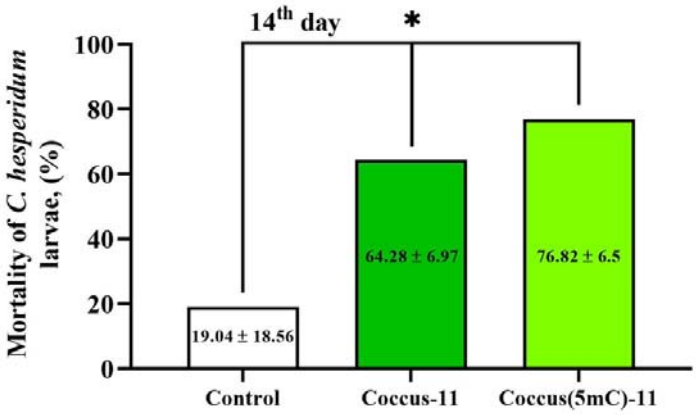

Figure 3. The isecticidal effect of the unmethylated Coccus-11 (5′–CCATCTTTCGG–3’) and methylated Coccus(5mC)-11 (5′–(5mC)-(5mC)-AT-(5mC)-TTT-(5mC)-GG–3’) on *C. hesperidum* larvae. Mortality of *C. hesperidum* larvae after topical treatment with Coccus-11, Coccus(5mC)-11, and water-treated control. The significance of difference in the Coccus-11 and Coccus(5mC)-11 groups compared to the water-treated control is indicated by * at $p < 0.01$; experiments were carried out in the field, the average daytime temperature during the experiment was 16.27 ± 2.11 °C, at night – 13.07 ± 2.41 °C, humidity – 76.94 ± 7.12%.

Eci-DNA mainly contains nuclear DNA

To understand the origin of the eci-DNA and whether it is derived from nuclear or chloroplast DNA, we amplified either 5.8S rDNA (nuclear) or 23S rDNA (chloroplast) using specific primers (Table 1). As a negative control, we measured the concentration of nuclear rDNA and chloroplast rDNA in intact *P. tobira* leaves. The quantitative PCR results showed that the concentration ratio between nuclear rDNA and chloroplast rDNA is 7.7 to 1 in the intact *P. tobira* leaves ($p < 0.01$). Surprisingly, in the eci-DNA samples, the ratio between nucleus rDNA and chloroplast rDNA was 1.6 to 1, which represents about five fold (4.8 fold; $p < 0.01$) increase in chrloroplast rDNA (Figure 4). This suggests that the eci-DNA is substantially enriched by chloroplasts DNA of the affected leaves.

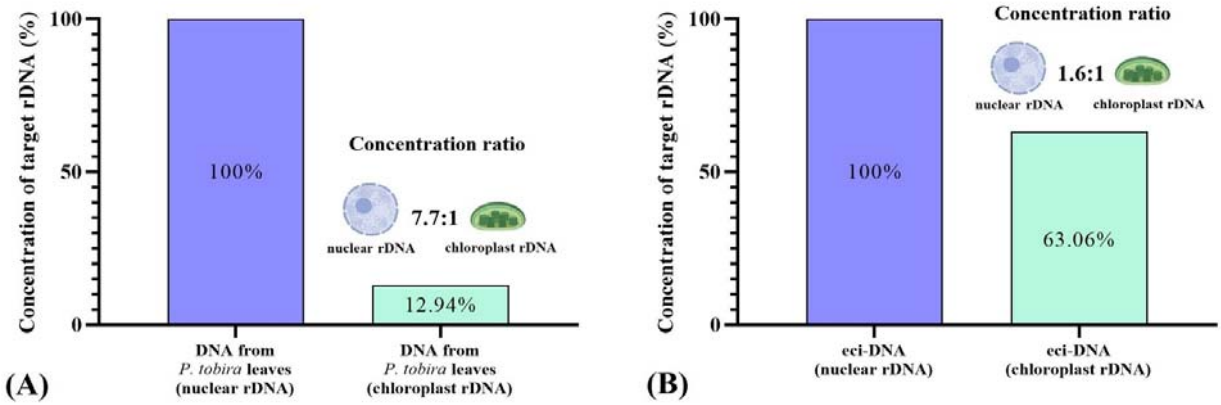

Figure 4. Relative concentration of nuclear rDNA and chloroplast rDNA in total DNA from intact *P. tobira* leaves (A) and eci-DNA (B). Nuclear rDNA was taken as 1 (100 %).

Eci-DNA is predominantly single-stranded

Next, we decided to investigate the nature of the DNA fragments in eci-DNA, whether they were predominantly ssDNA or dsDNA. To this end, we first treated DNA in intact *P. tobira* leaves with ExoI nuclease (degrades ssDNA), which specifically cleaves ssDNA, and followed with qPCR quantification. We found that 99% of the DNA was double-stranded from both nuclear and chloroplast fractions ($p < 0.01$) (Figure 5).

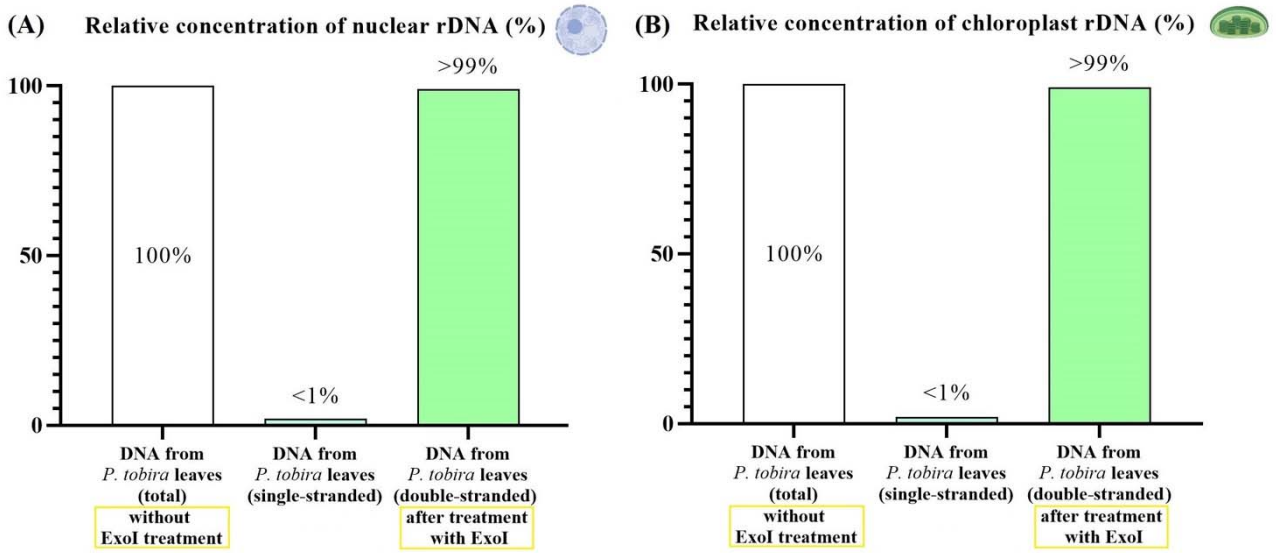

Figure 5. Relative concentration of nuclear rDNA (A) and chloroplast rDNA (B) in intact *P. tobira* leaves after using ExoI nuclease. Total DNA from intact plant leaves (non-treated with ExoI nuclease) is taken as 1 (100 %).

Surprisingly, eci-DNA treatment with ExoI nuclease completely reversed the previous profile and showed that it mainly consisted of single-stranded DNA: 24.8:1 (ssDNA:dsDNA; $p < 0.01$) for nuclear DNA and 103.1:1 (ssDNA:dsDNA; $p < 0.01$) for chloroplast DNA (Figure 6).

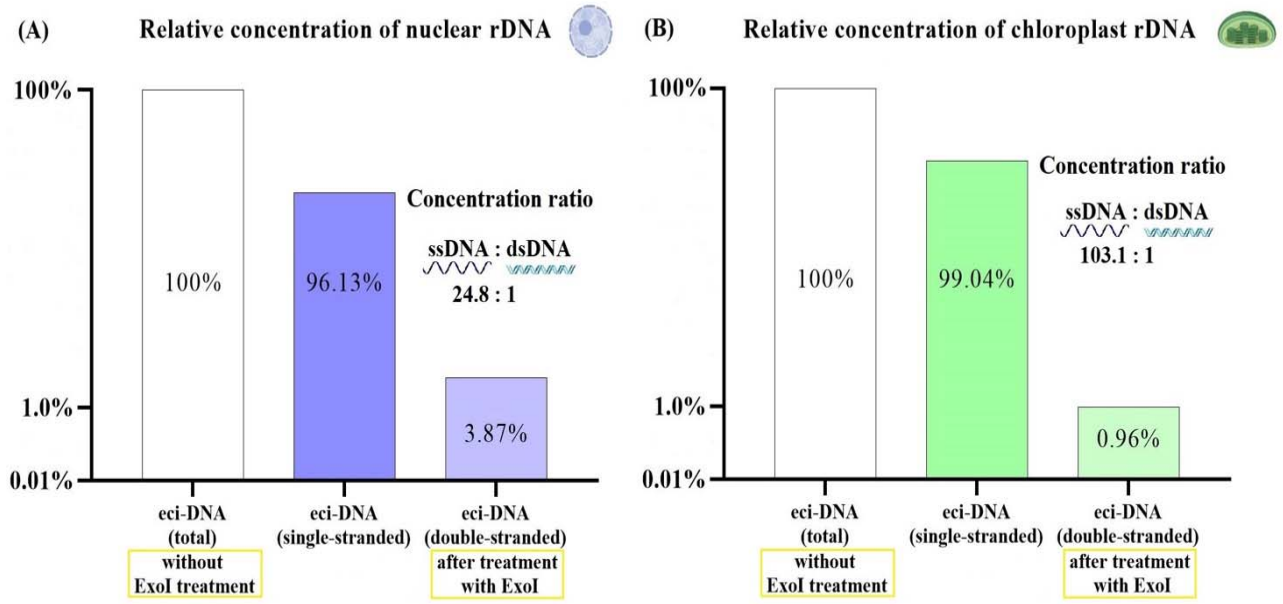

Figure 6. Relative concentration of nuclear rDNA (A) and chloroplast rDNA (B) from eci-DNA after treatment with ExoI nuclease. Eci-DNA (non-treated with ExoI nuclease) is taken as 1 (100 %).

P. tobira leaves show a positive staining for eci-DNA

To confirm the presence of eci-DNA on *P. tobira* leaves, we performed histological analyses where we stained both ssDNA and dsDNA at different parts of the leaves. The stainings show that in the control section (without staining), a number of structures of the anatomical preparation of the leaf had autofluorescence (Figure 7, A). However, after staining with fluorochrome acridine orange, we found that the cuticle on the adaxial part of the leaf fluoresced predominantly in green, indicating the presence of double-stranded nucleic acid. A thin layer of the peripheral part of the cuticle fluoresced clearly in red-orange, confirming the presence of single-stranded nucleic acid in this layer (Figure 7, B). Interestingly, the *C. hesperidum* pest predominantly settled on the abaxial part of *P. tobira* leaves, where the nucleic acid layer was less pronounced. Further studies were conducted to understand which nucleic acid (DNA or RNA) was present and fluoresced on the leaf surface in the layers fluorescing green and red-orange (Figure 7, C). For this purpose, before staining with fluorochrome, *P. tobira* leaves were pre-treated for 20 minutes with RNase A (Figure 7, D), DNase (Figure 7, E), and ExoI (Figure 7, F) nucleases and then washed out with water.

The results reveal that treatment of the adaxial side of leaves with RNase A (degrades ssRNA and dsRNA) did not induce significant differences from the control group, indicating the absence of both single-stranded and double-stranded RNA on the surface of *P. tobira* leaves. The green cuticle layer and the red-orange cuticle layer remained unchanged (Figure 7, D). On the contrary, under DNase treatment (degrades ssDNA and dsDNA), the green fluorescence of the cuticle on the adaxial side of the leaf changed to yellow, confirming that the double-stranded nucleic acid found on the leaves was dsDNA. The clearly structured red-orange layer of single-stranded nucleic acid also disappeared, indicating that it was formed by ssDNA. These effects led to the formation of a loose layer characteristic of unstructured and partially degraded ssDNA (Figure 7, E). Upon ExoI treatement (degrades ssDNA), the red-orange layer of single-stranded acid became noticeably thinner, which also confirms the presence of single-stranded DNA on the leaf surface (Figure 7, F). Also, as expected, the green layer of double-stranded nucleic acid remained unchanged. The results obtained are consistent with those obtained by PCR and confirm the presence of single-stranded DNA in the outermost layer of the cuticle of *P. tobira* leaves.

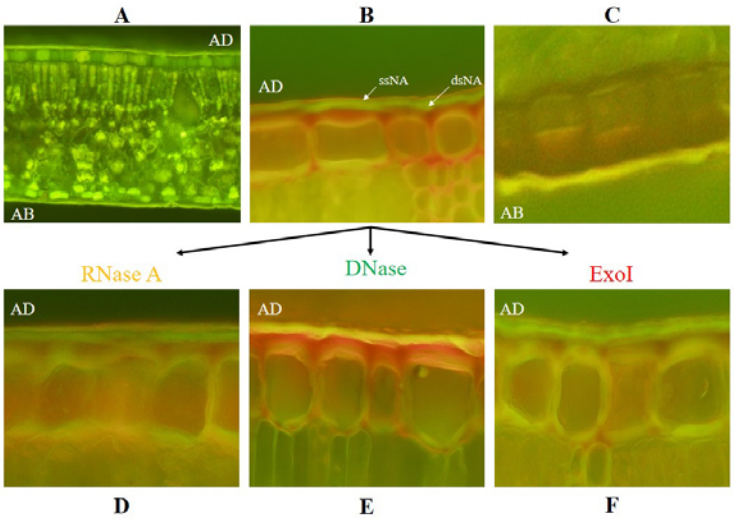

Figure 7. Anatomical preparations of *P. tobira* leaf cross-sections stained with acridine orange fluorochrome: A – temporary preparation in native state, not treated with anything; B – preparation stained with fluorochrome (adaxial surface, AD); C – preparation stained with fluorochrome (abaxial surface, AB); D – treated with RNase A before staining with fluorochrome; E – treated with DNase before staining with fluorochrome; F – treated with ExoI before staining with fluorochrome; ssNA – layer of single-stranded nucleic acid, dsNA – layer of double-stranded nucleic acid (explanation is in the text).

Discussion

The results obtained in this study suggest that *P. tobira* cells possess a mechanism for fragmenting genomic DNA into smaller fragments and converting them into single-stranded DNA (ssDNA). This phenomenon is particularly intriguing in the context of eci-DNA, which appears to modulate gene expression in *C. hesperidum* larvae through sequence complementarity to their mRNAs and rRNAs causing death of the pest. Similar to CUADb-based oligonucleotide insecticides, eci-DNA likely functions via targeted gene silencing, acting on both protein-coding and ribosomal RNA sequences. This is supported by the discovery of two 11 nucleotides (nt) sequences in the plastome of *P. tobira* that exhibit perfect complementarity to *C. hesperidum* rRNA (28S, MF594311.1; 18S, MF594274.1), suggesting a potential mechanism for DNA containment-mediated insecticidal effects (Oberemok et al., 2025). Given that the rDNA of *C. hesperidum* is only partially sequenced, additional complementary sequences may exist, further reinforcing this hypothesis. Thus, eci-DNA can also act through the targeting of pest rRNAs in a way similar to CUADb-based oligonucleotide pesticides (Kumar et al., 2025).

In our opinion, single-stranded methylated (5mC) eci-DNA can also serve as a complex of primers for chain extension on complementary mRNA and rRNA templates inside insect cells. For this reason, the most critical role will be played by the 3'-end of the DNA sequence, which should be complementary to the RNA target, based on literature data, by at least 6 nt (Ryu et al., 2000). We assume that, during DNA containment mechanism, DNA elongation (signal amplification) based on eci-DNA can be complemented in some insects by host reverse transcriptases, with characteristics similar to retroelements found in some insect genomes (Gong and Han, 2018). This suggested response of the cells can be explained by their pursuit to strengthen (amplify) the signal and get a longer DNA strand that can find more parts of the target RNA for their degradation by RNase H (obviously existing also as a defence against overreplication of retroelements). Probably, that is why we found importance of complementarity of 3′-end of olinscides for insecticidal effect in scale insects, since 3′-end nt is important for further DNA synthesis in 5′-3′ direction (Gal'chinsky et al., 2024). This hypothesis requires further investigation (Figure 8).

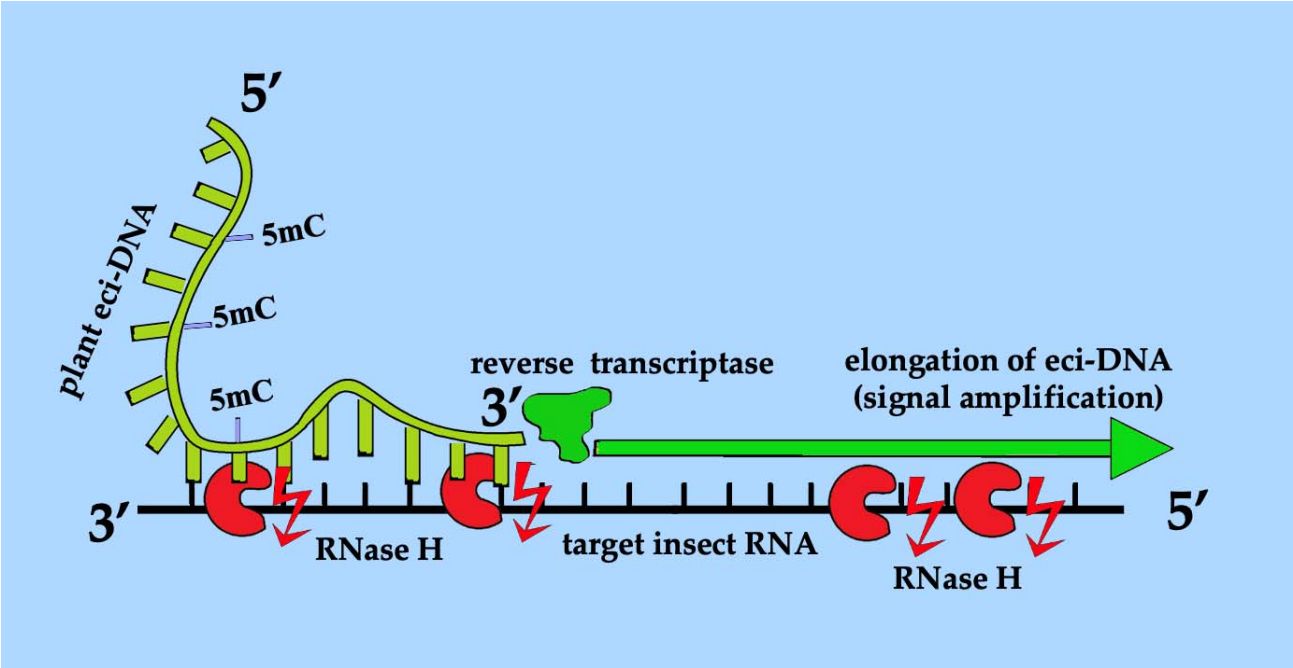

Figure 8. Hypothetical mechanism of action of eci-DNA in insect cells: reverse transcriptase elongates plant eci-DNA (signal amplification) and enhances degradation of insect RNA by RNase H.

A striking observation in our study was the shift in the ratio of nuclear to chloroplast rDNA in eci-DNA compared to intact leaf DNA. While intact *P. tobira* leaves exhibited a nuclear-to-chloroplast rDNA ratio of 7.7:1, this ratio shifted to 1.6:1 in eci-DNA, indicating a substantial enrichment of chloroplast-derived DNA. This finding suggests that *P. tobira* may selectively incorporate chloroplast DNA into eci-DNA. One plausible explanation is that chloroplasts, which are essential for photosynthesis, may become less vital in cells covered by sedentary insect pests due to the accumulation of honeydew (Douglas, 2009; Ali et al., 2024). Deprived of direct sunlight, these cells may repurpose chloroplast components to produce eci-DNA as a defensive adaptation. This would be a novel adaptation, allowing plants to utilise existing cellular components for pest defence without significant metabolic trade-offs. This hypothesis aligns with previous reports on chloroplast degradation in senescing or stressed plant cells (Ono et al., 1995), suggesting a broader role for plastid dynamics in plant-insect interactions. Additionally, our results indicate that outermost layer of ecDNA (eci-DNA) predominantly consists of ssDNA, as confirmed by nuclease treatment and PCR analysis. The conversion of plant-derived ecDNA into ssDNA may enhance its stability or facilitate its interaction with pest nucleic acids, thus increasing its effectiveness as an insecticidal agent (Kumar et al., 2020). It remains unclear whether this ssDNA formation is an active regulatory process by the plant or a consequence of enzymatic degradation by microbial nucleases. Further research is needed to determine whether different environmental or biotic factors influence this ssDNA predominance.

The ecological implications of eci-DNA extend beyond *P. tobira* and *C. hesperidum*. Given the widespread occurrence of sternorrhynchans (including scale insects, mealybugs, and psyllids) in tropical and subtropical regions, it is conceivable that eci-DNA-mediated pest control is a common strategy in evergreen plants. This study provides new insights into a naturally occurring defence mechanism that parallels synthetic CUAD biotechnology, reinforcing their potential safety and efficacy (Kumar et al., 2025). Future investigations should explore whether eci-DNA production varies among plant species, whether different pests elicit distinct DNA fragment compositions, and whether environmental conditions influence eci-DNA efficacy. Overall, our findings suggest that eci-DNA represents a previously unrecognized form of plant defence, where selective DNA fragmentation and chloroplast DNA enrichment play a role in mitigating herbivory by sedentary insect pests. Understanding the molecular underpinnings of this process could pave the way for novel bioinsecticidal applications, leveraging natural plant defence mechanisms for sustainable pest management similar to ‘genetic zipper’ technology.

It is important to note that this study examined the outermost layer of ecDNA (eci-DNA) of the plant itself on the leaf cuticle. We found that plant eci-DNA is single-stranded and enriched in chloroplast DNA compared to genomic DNA in intact *P. tobira* cells. However, we understand that microbial and insect DNA may also contribute to the formation of eci-DNA. The form in which their DNA may present in eci DNA is still unknown and will be the subject of further research.

Conclusion

This study demonstrates that the outermost layer of ecDNA (eci-DNA) of *P. tobira* leaves is predominantly single-stranded and exhibits a significant insecticidal effect on the pest *C. hesperidum*. Under natural conditions, plant DNA is unwound, partially degraded, and forms a previously unrecognised nanolayer of ssDNA on leaf. This adaptation may serve as a natural defence mechanism against sap-feeding insects. Notably, chloroplast DNA disproportionately enriched in eci-DNA compared to total cellular DNA, suggesting an active release mechanism for chloroplast-derived sequence in eci-DNA formation. The insecticidal activity of natural DNA appears to function through a DNA containment mechanism, wherein eci-DNA interferes with the expression of plant mRNAs and non-coding RNAs (including rRNAs) that share full or partial sequence complementarity. Intriguingly, the ‘genetic zipper’ technology, developed in the

laboratory for pest control, has a strikingly similar analogue in nature, underscoring the evolutionary convergence of DNA-based defence strategies. The findings of this study support that CUAD biotechnology is a nature-based pest control solution, reinforcing its safety and potential for agricultural applications. CUADb-based oligonucleotide pesticides hold promise as standalone treatments or as components of integrated bioformulations for the selective management of a wide range of insect pests, ushering in a new era of DNA-programmable plant protection.

Conflict of Interest

The authors declare no conflict of interest.

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