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IDENTIFICATION OF AN EXOIII DOMAIN OF A PUTATIVE PLANT ERI-1 HOMOLOGUE

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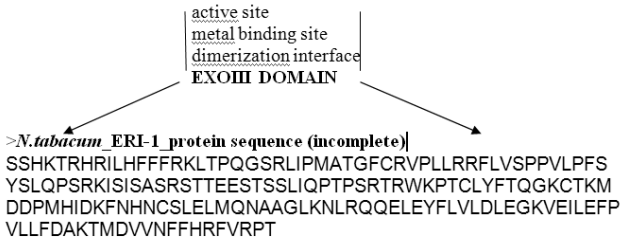
One of the most eminent endogenous suppressors of RNA silencing is the exonuclease protein ERI-1. The ERI-1 family has been implicated in a variety of short RNA pathways in different model organisms, with negative regulation of RNA silencing. So far no plant homologue of ERI-1 has been characterized. In the present study we report identification and characterisation of a plant ERI-1 homologue in tobacco, as the closest plant homologue of *Caenorhabditis elegans* ERI-1.



INTRODUCTION

The importance of RNA has been boosting significantly by the finding that double-stranded RNA is a trigger of gene silencing, providing a negative feedback mechanism independent of protein synthesis; a process termed RNA interference or RNAi.[1] During RNAi, foreign dsRNAs are processed into small RNAs of approximately 21 nucleotides, termed small interfering RNAs (siRNAs), which guide the destruction of complementary target mRNAs.[2]

In addition to the numerous viral suppressors of silencing, intensive research has been undertaken to identify endogenous proteins that may be regarded as suppressors of the RNA silencing system. The first report of a putative *bona fide* endogenous suppressor of silencing came from work in *Caenorhabditis elegans*, where the 3'-5' exonuclease ERI-1 (Enhanced RNAi-1) had been found to negatively affect RNAi through a siRNA-degrading functionality.[3] This function was established by the observations that RNAi is significantly enhanced in *eri-1* mutants and that recombinant ERI-1 protein is able to bind and degrade siRNAs *in vitro*.[3]

RNA silencing enhancement as a result of loss of function of the respective proteins can be attributed to suppressed default mRNA turnover and quality control mechanisms, thereby allowing 'aberrant' RNAs to accumulate and be more readily available for RDR-mediated dsRNA production. *C. elegans* ERI-1 has been shown to interact physically with DCR-1, but interestingly this is only the case for the longer of two *C. elegans* ERI-1 isoforms (ERI-1b).[4] Unfortunately, differential functional analyses of ERI-1a and ERI-1b have not been reported to date, but both ERI-1

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isoforms are predicted to fulfill different, yet related, tasks in *C. elegans* RNAi regulation.[4] In agreement with these results, *eri-1* mutants are hypersensitive towards viral infection.[5,6] Numerous studies followed that consolidated ERI-1's role as a factor with a primary role in negative RNA silencing regulation, not only in *C. elegans*, but also across kingdoms.

ERI-1 is phylogenetically conserved, and homologues were identified in many clades of the animal kingdom, including *C. elegans*, *Saccharomyces pombe*, *Drosophila*, mouse and human, as well as in fungi.[3,7-10] Mouse Eri1 has been reported to be induced by high doses of transfected siRNAs and thus being responsible for the so-called 'rebound effect'.[11] It was observed that initial RNAi-mediated suppression of target genes subsequent to transfection of respective siRNAs, reverts after several days, and this reversal of RNAi-targeted mRNA levels is dependent on Eri1.[11] In *S. pombe* Eri1 plays a well-defined role in repressing heterochromatin-derived siRNAs.[7,12] In *S. pombe eri1* mutants, heterochromatic siRNAs are able to function in *trans* to induce transcriptional gene silencing (TGS) of secondary homologous loci that are not the origin of the respective heterochromatin siRNAs. Such a spread of heterochromatin formation is suppressed by Eri1 in the wild type, and thus *S. pombe* Eri1 acts as a suppressor of RNA silencing by restricting heterochromatin-derived siRNAs to act in *cis*.[7,12] Also the human ERI-1 homologue Thex1/3'hExo is implicated in RNA silencing regulation, exemplified by its ability to degrade siRNAs *in vitro*.[3] *In vivo* evidence for its involvement in RNA silencing comes from work in HeLa cells, where overexpression of Thex1/3'hExo results in the efficient suppression of nonsense-mediated transcriptional gene silencing; a unique TGS pathway in which heterochromatinisation of immunoglobulin minigenes is triggered by premature termination codons.[13] While the *Drosophila* ERI-1 homologue Snipper showed high efficiency in the degradation of dsRNA and dsDNA with 3'-protruding ends *in vitro*, a function for Snipper in RNA silencing regulation could not be identified *in vivo*.[8] It was proposed that Snipper belongs to a group of ERI-1 homologues not implicated in RNA silencing regulation based on its phylogenetic grouping with ERI-1 homologues characterized by the lack of a SAP domain[14] which was shown to be essential for RNA binding site.[7,9,15]

Apart from the ever-growing data on RNA silencing regulation across kingdoms, additional conserved functions have been appointed to ERI-1 homologues, revealing an interesting versatility of this 3'-5' exonuclease protein. Here we present data on the identification and functional characterization of the *Nicotiana tabacum* 3'-5' exoribonuclease protein ERI-1, the closest plant homologue of *C. elegans* ERI-1.

RESULTS

1. Identification of a plant ERI-1 homologue

Identification of an ERI-1 protein in *N. tabacum* (accession number: BP529372) has been determined by PCR assays using a specific set of primers designed to conserve type-specific sequences. The sequences of *C. elegans* ERI-1 gene and *Tobacco* genomic databases were accessed via National Centre for Biotechnology Information-NCBI gene database (http://www.ncbi.nlm.nih.gov/dbEST/) and JCVI-J. Craig Venter Institute *N. tabacum* ESTs (http://www.jcvi.org/cms/research/platforms/sequencing). Using the DNAMAN program the nucleotide sequences of *C. elegans* ERI-1 gene were blast searched with *N. tabacum* ESTs database for identification homologous sequences. The gene specific primers were designed for an EXOIII conserved domain existing in the *C. elegans* and *N. tabacum* genomic counting, using the software of OLIGO program. Based on this software program the following primers were assigned:

(F: 5'-CTTCAGGAAAACTCATACCT-3' /
R: 5'-TGCAATTCTTCATAAGAAC-3').

The primers designed yield a PCR product of about 600 bp, then the recombinant pGEM plasmid was digested with *Eco*RI to verify the presence of the cloned *ERI-1*fragment and further sequenced to confirm the presence of EXOIII domain in tobacco (*NtERI-1* gene) (**Fig. 1**). Highly conserved ERI-1 homologues (>80 % similarity on the protein level) were readily identified in all presently sequenced plant genomes. Despite their strong similarities, ERI-1 homologues also exhibit striking differences. *Sorghum bicolor* ERI-1 contains a large deletion of approximately 35 amino acids within its EXOIII domain. Similarities are most pronounced in the conserved exonuclease domain, while the amino- and carboxy- terminal regions exhibit stronger variability between species (**Supplementary Fig. 1.a**). *Populus trichocarpa* and *S. bicolor* ERI-1 homologues exhibit large amino-terminal deletions within their EXOIII domains (**Supplementary Fig. 1.b**). The functional significance of this deletion could not be interpreted yet, suggesting a functional diversity of ERI-1 proteins in different plant lineages. This work was done together with Schumacher H.T. as part of his PhD thesis.[16]

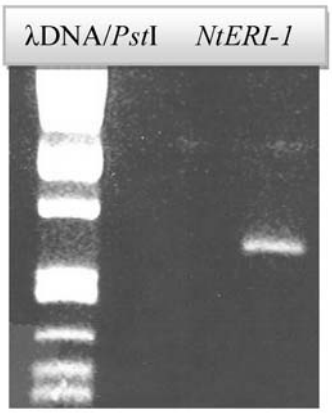


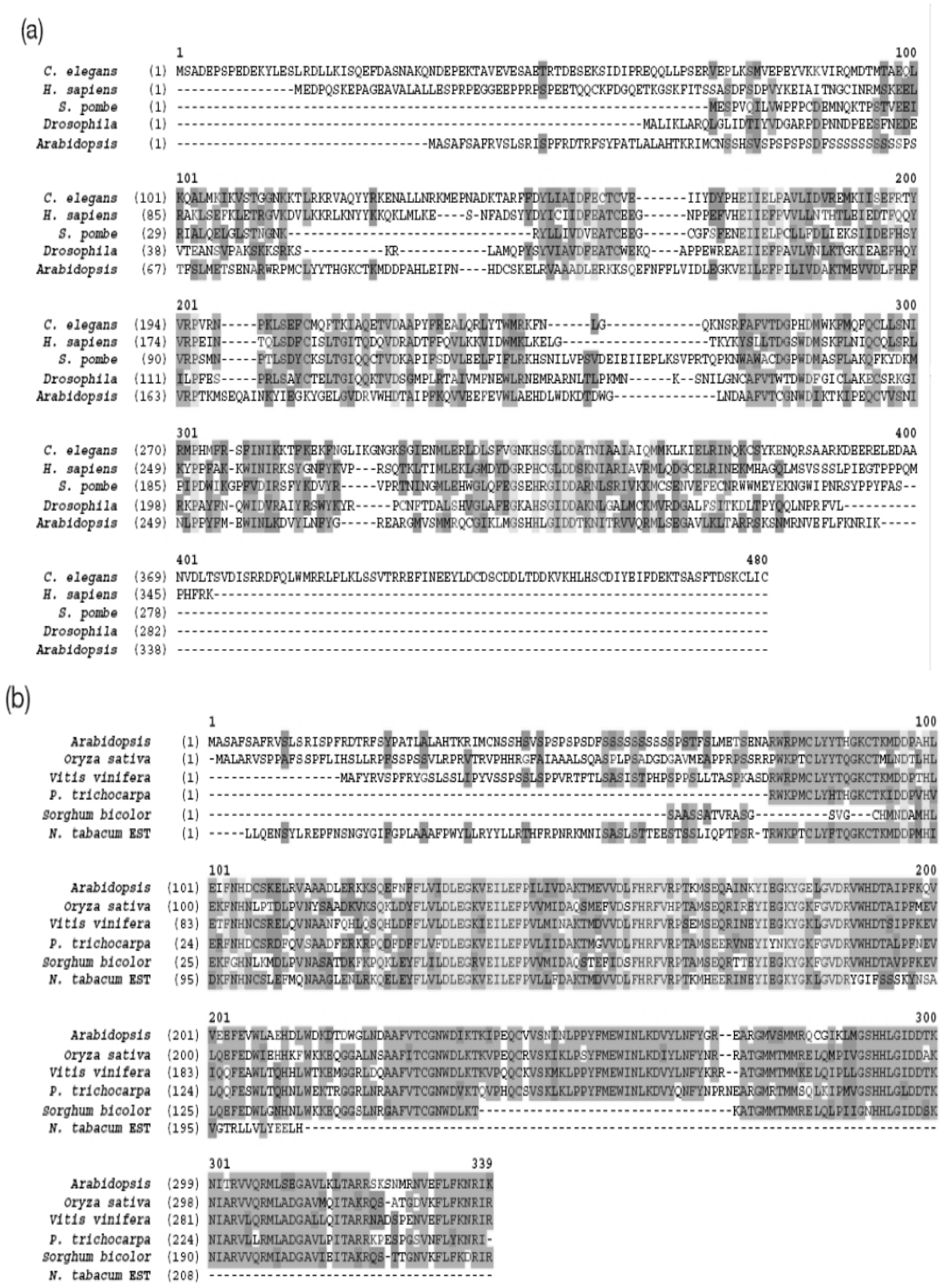
Fig. 1 – Detection of the ERI-1 gene homologue in N. tabacum. Restriction endonuclease digestion of pGEM-T Easy/*NtERI-1* recombinant plasmid with *Eco*RI enzyme, showing an expected fragment of 600bp. Lane 1: λDNA/*Pst*I ladder, lane 2: restriction digestion fragments of recombinant pGEM/*NtERI-1* with *EcoRI.*

2. Molecular analysis of ERI-1 in plant tissue

In this experiment a tobacco *ERI-1* cDNA-derived probe was used for the detection of ERI-1 gene in plant samples by Southern hybridization analysis. Agarose gel electrophoresis and Southern blot analysis were carried out using methods described by Maniatis.[17] Plant tissue from each sample of *Arabidopsis thaliana*, *Nicotiana benthamiana*, *N. tabacum* and *Lycopersicon esculentum* were subjected to genomic DNA extraction.[18] Genomic DNA was cleaved with *EcoR I* and *BamH I* enzymes for comparison and in both cases molecular analysis implies that *ERI-1* is a single copy gene in *Arabidopsis*, *N. tabacum*, and tomato (*L. esculentum*), while *N. benthamiana* may contain a duplication of the *ERI-1* gene (**Fig. 2**). Due to its low expression, ERI-1 mRNA can be detected *via* RT-PCR, but not in northern hybridization blots. Plant tissue from *N. tabacum* and *N. benthamiana'* flowers and leaves were subjected to RNA extraction. The highest ERI-1 expression can be detected in flowers, whereas expression levels drop in fully differentiated tissues. As a control an Actin gene, was amplified in the same time (**Fig. 3**).

DISCUSSION

As yet the roles of ERI-1 homologues in small RNA pathways have been investigated in a number of model species including *C. elegans*, *S. pombe*, *Drosophila*, mouse and human.[3,7-10] So far no plant homologue of ERI-1 has been characterized. In the present study we reports that the cloned *N. tabacum ERI-1* fragment encoded an EXOIII domain and has maximum homology to *C. elegans* gene. Also, molecular analysis implies that *ERI-1* is a single copy gene in *Arabidopsis*, *N. tabacum* and tomato (*L. esculentum*), while *N. benthamiana* may contain a duplication of the *ERI-1* gene while a tobacco *ERI-1* cDNA-derived probe was used for the detection of *ERI-1* gene in plant samples by Southern hybridization (**Fig. 2**). One kind of view is that a duplication of ERI-1 gene in *N. benthamiana*, allows the new copy of the gene to mutate without damaging consequence to the plant. This freedom from consequences allows for the mutation of novel genes that could potentially increase the fitness of the plant or code for a new function. The ERI-1 family members share a common 3'-5' exonuclease domain (EXOIII: SMART accession number SM00479)[19] containing a highly conserved DEDD motif.[20] DEDD domain proteins include the bacterial oligoribonuclease and RNase T that degrade small RNA oligonucleotides and are involved in maturation and 3' end processing of small stable RNAs, respectively.[20] In the NCBI Conserved Domain Database[21] ERI-1-type EXOIII domains have recently been annotated as a distinct subfamily termed ERI-1_3'hExo_like (CDD domain cd06133). A DNA/RNA-binding SAP domain[22,23] is present at the amino-terminus of several but not all of the reported ERI-1 homologues. If present, the SAP domain conveys binding specificity to short double-stranded RNA substrates. It was proposed that the *Drosophila* ERI-1 homologue (Snipper) belongs to a group of ERI-1 homologues not implicated in RNA silencing regulation based on its phylogenetic grouping with ERI-1 homologues characterized by the lack of a SAP domain[14] which was shown to be essential for RNA binding site.[7,9,15]



Supplementary Fig. 1a/b – Phylogenetic alignments of ERI-1 homologues. **(a)** Full protein alignment of ERI-1 homologues from animals, fungi, and plants revealing the conserved EXOIII domain. Amino and carboxy-terminal parts in comparison exhibit a higher degree of variability between species. **(b)** The protein sequences of plant ERI-1 homologues are highly conserved. Important differences are found in the amino-terminal regions, where the *P. trichocarpa* and *S. bicolor* ERI-1 homologues harbor large deletions. These areas correspond to chloroplast localisation signals. An approximately 35 amino-acids (aa) deletion inside the EXOIII domain of *S. bicolor* ERI-1 could not be functionally interpreted yet (modified after Zuo and Deutscher, 2001).

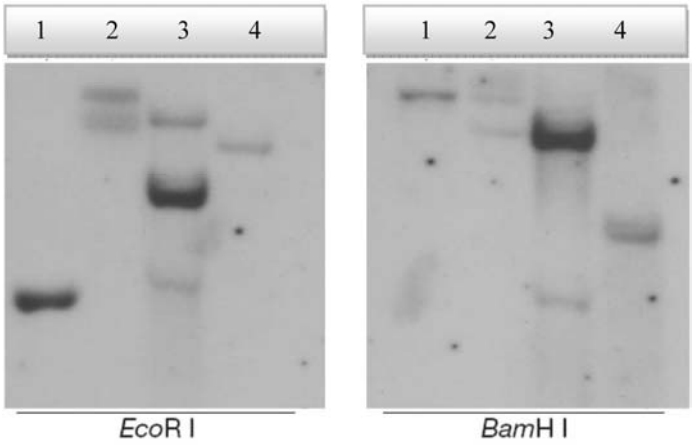


Fig. 2 – Southern analysis of ERI-1 in different Solanaceae species. A tobacco *ERI-1* cDNA-derived probe was used for the detection of *ERI-1* genes by Southern hybridization. Genomic DNA was cleaved with *EcoR I* and *BamH I* for comparison. In both cases single major bands were detected in *Arabidopsis*, tobacco, and tomato, while 2 distinct bands were detected in both *N. benthamiana* samples. Where, lane 1: *Arabidopsis*, lane 2: *N. benthamiana*, lane 3: *N. tabacum* and lane 4: *L. esculentum*.

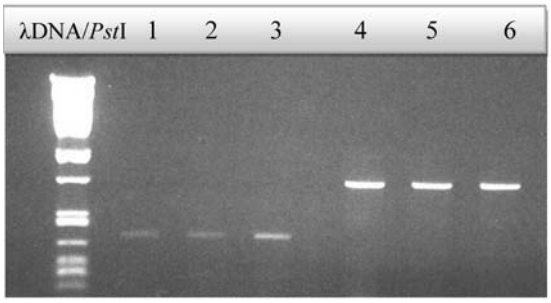


Fig. 3 – Detection of ERI-1 mRNA by RT-PCR analysis. Plant tissue from *N. tabacum* flowers/leaves and *N. benthamiana* leaves were subjected to RNA extraction followed by RT-PCR. The amplicons were analyzed by agarose gel electrophoresis. The highest ERI-1 expression can be seen in flowers (lane 3: *N. tabacum* flowers), whereas expression levels drop in fully differentiated tissues (lane 1: *N. benthamiana* leaves/lane 2: *N. tabacum* leaves). As a control an Actin gene was amplified in parallel (see lanes 4, 5 and 6); λDNA/*Pst*I is the loading ladder.

Phylogenetic analysis of multiple characterized and non-characterized ERI-1 homologues from diverse eukaryotic lineages revealed two phylogenetically distinct ERI-1 subclasses.[14] The chief differences between Group I and Group II ERI-1 homologues lie in the domain compositions of the respective proteins. Group I ERI-1 homologues contain two functional domains: a conserved ERI-1_3'hExo_like EXOIII domain responsible for 3' to 5' exonucleolytic cleavage and an amino-terminal SAP domain conferring nucleic acid binding capability in a sequence-independent manner. These SAP/EXOIII proteins include the well characterised *C. elegans* ERI-1, human Thex1/3'hExo, mouse and *S. pombe* Eri1, as well as *N. crassa* QIP. They stand in contrast to Group II ERI-1 homologues that are exemplified by *Drosophila* Snipper, *Tribolium castaneum* Tnc-Snp, and *Dictyostelium discoideum* eriA that all lack the aforementioned SAP domain and solely consist of an ERI-1_3'hExo_like EXOIII domain. Tnc-Snp and eriA have not been functionally characterised yet, but based on results from *Drosophila* Snipper [8] they are expected to be involved in pathways distinct from RNA silencing regulation.[14] Moreover, due to its low expression, tobacco *ERI-1* mRNA can be detected only *via* RT-PCR; since *ERI-1* mRNA is transcribed below detection limit in standard northern hybridization blots. The highest ERI-1 expression can be detected in flowers whereas expression levels drop in fully differentiated tissues (**Fig. 3**). ERI-1 is transcribed at exceedingly low levels, which by itself suggests a comparatively minor regulatory impact. It could hence be expected that partial loss of ERI-1, even upon constitutive RNAi-mediated suppression, would not cause discernable alterations in RNA silencing phenotypes. On the protein level tobacco ERI-1 shares significant homology with animal and fungal ERI-1 homologues (**Supplementary Fig.1.a/b**), which lead to the working hypothesis that ERI-1 may be a

functional ERI-1 orthologue similarly involved in the negative modulation of plant RNA silencing pathways.

MATERIALS AND METHODS

1. Identification of a plant ERI-1 homologue

1.1. PCR analysis

Total RNA was isolated from *N. tabacum* flowers, using Trizol reagent according to manufacturer's directions (Invitrogen, USA), followed by DN*ase*I treatment. THERMOSCRIPT™ RT-PCR System (Invitrogen) was used to generate cDNA synthesis following the manufacturer's instructions. About 1μg of total RNA and 1mM/μL reverse primer were denaturing in a final volume of 10 μL at 65°C for 5 min. A master mix reaction was prepared with 1X cDNA synthesis buffer, 10mM/μL DTT, 2u/μL RNaseOUT, 1mM/μL dNTPs Mix and 0.75u/μL THERMOSCRIPT RT. The samples were transferred to a PTC-200 Peltier Thermal Cycle (MJ Research) and incubated for 60 min at 50°C followed by incubating at 85°C for 5 min. As control, reactions were run in parallel that excluded reverse transcriptase. cDNA synthesis reaction was used for PCR immediately as follows: 3μL cDNA was mixed with 47 μL master mix containing 1X PCR buffer with 1.25mM/μL MnCl 2 (Minotech), 0.2 mM/μL dNTPs mix, 0.2 mM/μL of each forward and reverse primers, 2u/μL *Taq* polymerase (Minotech), and completed with double distilled water for each separate sample. Amplification reaction conditions were: one cycle at 94°C /5min; 30 cycles at 94°C / 1 min; 52°C /30 sec.;72°C /30 sec. and 72°C /10 min. PCR products were separated on 1.2 % agarose gel.

1.2. DNA sequencing

The PCR amplified product was purified through GEANCLEAN® III Kit (Q-Biogene) and cloned with pGEM-T Easy vector system kit (Promega). Sequencing was performed by the Institute for Molecular Biology and Biotechnology (IMBB-FORTH, Greece). The protein query sequence submitted to search against the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST) using the BLASTP program (P value = 0.001) was scanned for conserved domain signatures and results showed that an EXOIII domain was present in the translated protein sequence.

2. Analysis of ERI-1 copies in plant genomic DNA

2.1. Southern analysis

Agarose gel electrophoresis and Southern blot analysis were carried out using methods described by Maniatis.[17] 1g of plant tissue from each sample of *A. thaliana*, *N. benthamiana*, *N. tabacum* and *L. esculentum* were subjected to genomic DNA extraction,[18] suspended in 15 *mL* DNA extraction buffer (100mM Tris-HCl, pH 8.0, 500mM NaCl, 50mM EDTA and 10mM β-mercaptoethanol), 2% CTAB and 600 μL of a 15 % sarkosyl solution. An extra 1X SDS was added and incubated in water bath at 65ºC for 10 min, followed by 20 min on ice with 1M potassium acetate. The purification and precipitation of DNA were carried out using methods described by Maniatis et. al (1989). 15-20 µg of genomic DNA were digested with 50-80u of *EcoRI* and *BamHI* (Minotech), restriction enzymes in 150 μL reaction buffer at 1x concentration and 100 µM spermidine. The reactions were incubated at 37°C for 5 hours. Additional 50-80u of restriction enzymes were added after 2.5 h to compensate for loss of active enzyme during the incubation time.

2.2. Capillary blotting procedure

After incubation the samples were isopropanol-precipitated and resuspended in 40 μL of DEPC-treated water. The samples were loaded on 1% agarose gel and run at 22 V over night. The gel was subsequently depurinated in 0.2 N HCl for 10 minutes denatured in 1.5 M NaCl/0.5 N NaOH for 45 minutes and neutralized in 1 M Tris (pH 7.5)/1.5 M NaCl for 30+15 minutes (10 minute washes with water between all steps). After equilibrating the gel in 10x SSC, the DNA was transferred onto a Nytran N nylon membrane (Schleicher and Schuell) as described by Sambrook and Russel (2001). After transfer, membrane was briefly rinsed in 2x SSC and subsequently UV crosslinked at 120 mJ/cm² using a Stratalinker® (Stratagene, La Jolla, USA) device.

2.3. Random-prime labelling of DNA probe

A tobacco *ERI-1* cDNA-derived probe was used for Southern hybridizations using Invitrogen's RadPrime DNA Labelling system according to the manufacturer's recommendations (Invitrogen, USA). 50 ng of template DNA were denatured at 95°C for 2 minutes and quick-chilled on ice,

followed by the addition of 20 μL 2.5x RadPrime Buffer, 1 μL of 0.5 M dTTP and 0.5 M dGTP each, 1 μL random primers (3 μg/μL), 2 μL [α-32P]ATP and [α-32P]CTP each (3000 Ci/mmol), 40u Klenow Fragment, and water to a final volume of 50 μL. The reaction mixture was incubated in a water bath at 37°C for 1 hour and then purified using Amersham's MicroSpin™ S-200 spin columns according to the manufacturer's protocol. Purified random-primed DNA probes were denatured in a water bath at 95°C for 5 minutes and quick-chilled on ice before adding them to the hybridization buffer.

2.4. Hybridisation, washing, and exposure of Southern membrane

Membrane was pre-hybridised in 10-15 *mL* pre-warmed hybridisation buffer (5X SSC, 1% SDS, 250$\mu g/mL$ tRNA, 1X Denhard's solution) for 2 hours at 65°C. The probe was denatured and added to the hybridisation buffer over-night at 65°C. The following day, membrane was washed with 10-15 *mL* of warmed washing solutions in the following order: 1. rinse with 2x SSC/0.1 % SDS; 2. wash 2x 15 minutes with 2x SSC/0.1 % SDS; 3. wash 2x 10 minutes with 1x SSC/0.1 % SDS; 4. wash 2x 5 minutes with 0.5 x SSC/0.1 % SDS. All washing steps were performed at hybridization temperature. Washed membrane was rinsed in 2x SSC and subsequently sealed in plastic bag while still wet. Membrane was exposed to X-Ray films in appropriate exposure cassette. Exposed X-Ray film was developed automatically using a Curix 60 developer (Agfa).

3. Detection of ERI-1 mRNA by RT-PCR

3.1. RT-PCR analysis

Total RNA from *N. tabacum* leaves and flowers and *N. benthamiana* leaves, was isolated using TRIZOL® Reagent (Invitrogen, USA) following the kit instructions. 1μg *N. tabacum* and *N. benthamiana* RNAs leaf/ flower tissue was subjected to an RT reaction following the kit instructions: THERMOSCRIPT™ RT-PCR Sistem (Invitrogen) and immediately a PCR reaction [(PTC-200 Peltier Thermal Cycle (MJ Research)] was set up as follow: 94ºC, 5min one cycle; 94ºC, 1 min; 53ºC (for Actin) and 57ºC (for ERI), 30 sec; 72ºC, 30 sec., 30 cycles; 72ºC, 10 min.; 4ºC forever. 1μL of cDNA was added to the PCR mix: 1X PCR buffer (Minotech), 2.5mM/μL MnCl 2, 0.5 mM/μL of dNTPs mix, 0.25 mM/μL of each forward and reverse primers, 0.2u/μL *Taq* DNA polymerase (Minotech), and water for a final volume of 20μL. For loading control an Actin gene (*N. tabacum*) was amplified in parallel.

CONCLUSIONS

The extensive utilization of repressive small RNA pathways in many cellular functions has raised questions regarding the endogenous regulation of these mechanisms. The *C. elegans* 3'-5' exonuclease ERI-1 was the first endogenous factor to be described as a *bona fide* suppressor of silencing, based on its ability to partially degrade *si*RNA*s*.[3] Related functions have subsequently been appointed to ERI-1 homologues in *S. pombe*, *N. crassa*, and mammals.[7,11-13] Recent studies, however, revealed conserved roles of ERI-1 homologues in posttranscriptional processing of 5.8S *r*RNA.[10,24] Phylogenetic analysis of multiple characterized and non-characterized ERI-1 homologues from diverse eukaryotic lineages revealed two phylogenetically distinct ERI-1 subclasses.[14] The chief differences between Group I and Group II ERI-1 homologues lie in the domain compositions of the respective proteins (as previously discussed).

Phylogenetic analysis places *N. tabacum* ERI-1 in the same group with ERI-1 homologues from *Tribolium*, *Drosophila* and *Dictyostelium*. A unifying characteristic of these Group II ERI-1 homologues is the lack of a canonical SAP domain, which was shown to be important for small RNA binding in the *S. pombe* and human. Group II ERI-1 homologues are hence predicted to play roles distinct from RNA silencing regulation.[14] This notion is supported by the fact that no RNA silencing-related function could be appointed to the *Drosophila* ERI-1 homologue SNIPPER.[8]

To see if ERI-1 exhibits the same specificity for siRNA degradation and negative RNA silencing regulation as its homologues, further investigation is intended as suppressing or overexpressing ERI-1 and assessing the respective effects on RNA silencing and small RNA steady-state levels. Hence, despite functional differences in different model organisms ERI-1 homologues may share a common affinity for short stem structures that could account for the observed diverse functionalities in the respective cellular contexts.

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