biology and life sciences forum

MDPI

Proceeding Paper

Nicotiana benthamiana γ-Thionin Synthesis Is Induced in Response to Foreign Nucleus-Targeted Proteins †

Ekaterina Sheshukova [1], Natalia Ershova [1], Fedor Lipskerov [1] and Tatiana Komarova [1,2,*]

[1] Vavilov Institute of General Genetics Russian Academy of Sciences, 117971 Moscow, Russia; sheshukova@vigg.ru (E.S.); ershova@vigg.ru (N.E.); fedor@lipskerov.ru (F.L.)
[2] Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119991 Moscow, Russia
* Correspondence: t.komarova@belozersky.msu.ru
† Presented at the 2nd International Electronic Conference on Plant Sciences—10th Anniversary of Journal Plants, 1–15 December 2021; Available online: https://iecps2021.sciforum.net/.

Abstract: Pathogenic and symbiotic bacteria secret protein factors—nucleomodulins—to affect the host cell nucleus. During evolution, plants acquired a great variety of defense mechanisms including the synthesis of such antimicrobial peptides (AMPs) as defensins. We have demonstrated that transient production of a foreign protein containing nuclear localization signal (NLS) in *Nicotiana benthamiana* leaves leads to the increased expression of the *γ-thionin* (NbγThio) that belongs to the defensin group of AMPs. We hypothesized that NbγThio is induced by nucleomodulins of pathogenic bacteria and in particular in response to their NLSs. We used artificial nuclear proteins based on green fluorescent protein (GFP) fused with the human prothymosin *α* NLS or VirE3 NLS from *Agrobacterium tumefaciens* as mimetics of bacterial effectors. We demonstrated that superproduction of these NLS-containing reporters in the transient expression system in *N. benthamiana* leaves resulted in the increase of NbγThio mRNA level. We isolated the NbγThio gene promoter ($Pr^{\gamma Thio}$) and created an expression vector ($Pr^{\gamma Thio}$-GUS) directing GUS synthesis in agroinfiltrated leaves. Co-expression of $Pr^{\gamma Thio}$-GUS with 35S-GFP:NLS variants led to the significant stimulation of GUS synthesis. We concluded that *NbγThio* gene expression is activated in response to bacterial nucleus-targeted proteins in the cell via induction of $Pr^{\gamma Thio}$.

Keywords: defensin; γ-thionin; nuclear localization signal; nucleomodulins; *Agrobacterium tumefaciens*; transient expression

Citation: Sheshukova, E.; Ershova, N.; Lipskerov, F.; Komarova, T. *Nicotiana benthamiana* γ-Thionin Synthesis Is Induced in Response to Foreign Nucleus-Targeted Proteins. *Biol. Life Sci. Forum* **2021**, *1*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Iker Aranjuelo

Published: 1 December 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Many of the plant bacterial pathogens could affect the plant cell nucleus by means of the nucleomodulins—the effectors that reprogram the nucleus of the host cell [1]. Most of the nucleomodilins enter the host cell nucleus via the mechanism based on nuclear-localization signal (NLS) recognition [2]. Plants have numerous lines of defense among which are different antimicrobial peptides including defensins. Plant γ-thionins, or defensins, are relatively small proteins possessing antifungal or antibacterial activity [3]. These proteins are allocated to a separate group of *cis*-thionins according to their primary and secondary structure and amino acid composition: they usually have a signal peptide, contain several cysteine residues forming disulfide bonds stabilizing αβ (CSαβ) motif [4,5]. The mechanism of γ-thionins antibacterial effect is still not completely clear but their amphipathic helix and disulfide bonds are believed to play and important role [4]. Here, we propose a model system in which leaf mesophyll cells perceive the artificial NLS-containing protein as a bacterial effector and hence as a signal of bacterial invasion. In response to the bacterial pathogen attack, the plant cell switches on defense mechanisms, including

the induction of *γ-thionin* synthesis. We suggested that induction of γ-thionin is performed via activation of its transcription in response to NLS-containing foreign proteins.

2. Methods

2.1. Agroinfiltration

Genetic material was delivered into cells of the fully expanded *N. benthamiana* leaves using the agroinfiltration approach. *Agrobacterium tumefaciens* strain GV3101 was transformed with individual binary vectors and grown at 28 °C in LB medium supplemented with 50 mg/L rifampicin, 25 mg/L gentamycin, and 50 mg/L carbenicillin/kanamycin. *Agrobacterium* overnight culture was diluted in 10 mM MES (pH 5.5) buffer supplemented with 10 mM $MgSO_4$ and adjusted to a final OD_{600} of 0.1. Agroinfiltration was performed using a 2 mL syringe, after which the plants were grown under standard greenhouse conditions at 24 °C with a 16 h/8 h light/dark photoperiod.

2.2. GFP and mRFP Imaging and Fluorescence Measurement

GFP and mRFP fluorescence was detected using an AxioVert 200M light fluorescence microscope (Carl Zeiss) equipped with a Plan-Neofluar 100× NA 1.3 objective (Carl Zeiss) and an ORCA-II ERG2 digital camera (Hamamatsu Photonics). Excitation and emission wavelength for GFP or mRFP were 488 nm and 584 nm, respectively, and detection window was 493–607 nm.

Plant protein extracts were prepared as follows: leaf sections from infiltrated areas were ground in GFP extraction buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0) and clarified by centrifugation at 16,000× *g* for 10 min. GFP fluorescence was measured using a Turner Quantech fluorimeter with the following set of filters: NB390 (narrowband) excitation filter and an NB520 emission filter.

2.3. GUS Activity Measurement

Plant material from infiltrated areas was harvested and ground in GUS-extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% Triton X-100). GUS enzymatic activity in plant extracts was estimated with the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG). The fluorescence of the MUG cleavage product was analyzed with a Turner Quantech fluorimeter using an NB455 excitation filter and an NB520 emission filter. All measurements were performed according to the previously described standard protocol [6].

2.4. Quantitative Real-Time PCR (qRT-PCR) Analysis of Transcript Concentrations

Total RNA was extracted from plant tissues using TriReagent (MRC, USA) according to the manufacturer's instructions. The synthesis of the first strand, followed by real-time qPCR, was performed as described in [7]. Real-time quantitative PCR was carried out using the iCycler iQ real-time PCR detection system (Bio-Rad, USA). Target genes were detected using sequence-specific primers for 18S rRNA (for normalization) and NbγThio and Eva Green master mix (Syntol, Russia) according to the manufacturer's instructions. The results of RT-qPCR were evaluated using the Pfaffl algorithm [8].

3. Results

3.1. A Massive Synthesis of mRFP Fused with a Nuclear Localization Signal Stimulates γ-Thionin mRNA Accumulation

We selected a nuclear localization signal from human prothymosin α (pTα) ($NLS^{pT\alpha}$) to obtain an artificial model of NLS-containing reporter proteins based on mRFP or GFP sequence. We created genetic constructs encoding mRFP:$NLS^{pT\alpha}$ and GFP:$NLS^{pT\alpha}$ fusion proteins (Figure 1A). Using agrobacterium-mediated delivery of the genetic material for

the transient expression we have shown that NLS^{pTα} effectively targets mRFP and GFP to the nucleus (Figure 1B).

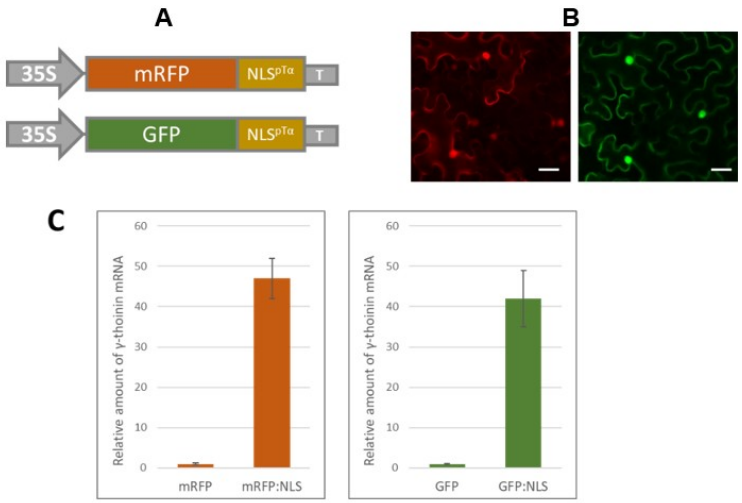

Figure 1. Foreign NLS-containing protein stimulates *γ-thionin* mRNA accumulation in *N. benthamiana* leaves: (**A**) schematic representation of genetic constructs encoding mRFP:NLS^{pTα} and GFP:NLS^{pTα}; (**B**) fluorescent images of epidermal cells of *N. benthamiana* leaves agroinfiltrated with 35S-mRFP:NLS^{pTα} (left) or 35S-GFP:NLS^{pTα} (right), scale bar = 20 μm; (**C**) relative amount of *γ-thionin* mRNA in leaves 3 days after agroinfiltration with 35S-mRFP or 35S-mRFP:NLS^{pTα} (left) and 35S-GFP or 35S-GFP:NLS^{pTα} (right) and quantified by qRT-PCR. $p < 0.001$ (Students *t*-test) for statistical significance of the difference compared to the control (mRFP or GFP).

We hypothesize that superproduction of a foreign nuclear protein can be recognized by the cell as a signal for the induction of pathogenesis-related (PR) genes expression, especially those that are activated in response to bacterial infection because NLS-containing proteins could be perceived by the plant cell as bacterial nucleomodulins, protein factors that are delivered to the nucleus to interfere with its functioning [9,10]. We demonstrated that expression of mRFP:NLS^{pTα}-encoding construct in the leaves drastically stimulated the accumulation of *N. benthamiana γ-thionin* mRNA (Acc. FR686584.1) (Figure 1C, left). To exclude the effect of the reporter protein we also checked *γ-thionin* response to GFP:NLS^{pTα} and assessed the level of *γ-thionin* mRNA in leaves 3 days after agroinfiltration with 35S-GFP:NLS^{pTα}. We observed a comparable increase in *γ-thionin* expression in response both to mRFP:NLS^{pTα} and GFP:NLS^{pTα} (Figure 1C).

3.2. Isolation of the γ-Thionin Promoter (Pr^{γThio}) and Assessment of Its Sensitivity to the Intensive Accumulation of the Model Nuclear Protein

To identify γ-thionin promoter (Pr^{γThio}) we used the "chromosome walking" approach and isolated the 1142-nucleotide sequence upstream of the *γ-thionin* gene, which we regarded as Pr^{γThio} (EMBL Acc. ERA1901858). In the next step, we created plant expression vectors containing a reporter gene encoding *Escherichia coli* β-glucuronidase (GUS) under the control of Pr^{γThio} (Figure 2). Here we used GFP fused with NLS^{pTα} or *A. tumefaciens* virulence protein E3 [11] NLS (NLS^{VirE3}) to model the entrance of bacterial NLS-containing nuclomodulins. In accordance with our hypothesis on the stimulatory role of the foreign NLS-containing protein for γ-thionin the co-expression of Pr^{γThio}-GUS with 35S-GFP:NLS^{pTα} or 35S-GFP:NLS^{VirE3} should result in increased GUS accumulation. We extracted GUS from agroinfiltrated leaves at 3 dpi and assessed its enzymatic activity that reflects Pr^{γThio}-GUS expression. Both GFP-NLS^{pTα} and GFP:NLS^{VirE3} synthesis activated Pr^{γThio} and stimulated GUS accumulation by about 20% (Figure 2) indicating that Pr^{γThio} is sensitive to the foreign nuclear proteins.

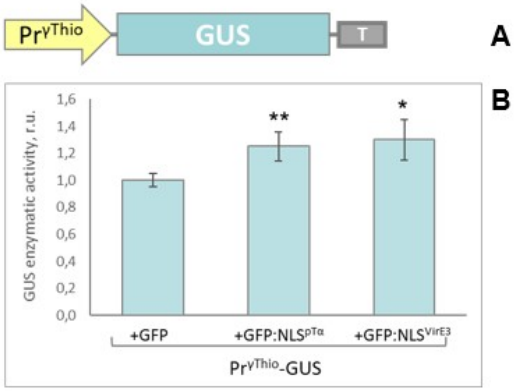

Figure 2. An artificial nuclear protein based on a GFP:NLS^{pTα} or *A. tumefaciens* VirE3 (GFP:NLS^{VirE3}) stimulates *γ-thionin* promoter (Pr$^{\gamma Thio}$)-directed GUS synthesis (Pr$^{\gamma Thio}$-GUS): (**A**) schematic representation of the 1142-nt Pr$^{\gamma Thio}$-based vector encoding GUS; (**B**) comparative analysis of GUS activity in leaves 3 days after co-agroinfiltration with Pr$^{\gamma Thio}$-GUS and 35S-based vectors encoding GFP, GFP:NLS^{pTα} or GFP:NLS^{VirE3}. The fluorescence detected for the combination of Pr$^{\gamma Thio}$-GUS and 35S-GFP is taken as 1. Standard error bars are indicated. *, $p < 0.05$, **, $p < 0.01$ (Student's *t*-test).

4. Conclusions

We concluded that *N. benthamiana γ-thionin* expression is stimulated in response to the production of the foreign NLS-containing protein in the cell at the transcription level via Pr$^{\gamma Thio}$ stimulation.

Author Contributions: E.S. and T.K. designed research; E.S., T.K., N.E. and F.L. performed research; E.S. and T.K. analyzed data and wrote the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Russian Science Foundation (project No. 19-74-20031).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Hanford, H.E.; Von Dwingelo, J.; Abu Kwaik, Y. Bacterial Nucleomodulins: A Coevolutionary Adaptation to the Eukaryotic Command Center. *PLoS Pathog.* **2021**, *17*, e1009184. https://doi.org/10.1371/journal.ppat.1009184.
2. Le, L.H.M.; Ying, L.; Ferrero, R.L. Nuclear Trafficking of Bacterial Effector Proteins. *Cell. Microbiol.* **2021**, *23*, e13320. https://doi.org/10.1111/cmi.13320.
3. Pelegrini, P.B.; Franco, O.L. Plant γ-Thionins: Novel Insights on the Mechanism of Action of a Multi-Functional Class of Defense Proteins. *Int. J. Biochem. Cell Biol.* **2005**, *37*, 2239–2253. https://doi.org/10.1016/j.biocel.2005.06.011.
4. Sathoff, A.E.; Samac, D.A. Antibacterial Activity of Plant Defensins. *Mol. Plant-Microbe Interact.* **2019**, *32*, 507–514. https://doi.org/10.1094/MPMI-08-18-0229-CR.
5. Höng, K.; Austerlitz, T.; Bohlmann, T.; Bohlmann, H. The Thionin Family of Antimicrobial Peptides. *PLoS ONE* **2021**, *16*, e0254549. https://doi.org/10.1371/journal.pone.0254549.
6. Jefferson, R.A.; Kavanagh, T.A.; Bevan, M.W. GUS Fusions: Beta-Glucuronidase as a Sensitive and Versatile Gene Fusion Marker in Higher Plants. *EMBO J.* **1987**, *6*, 3901–3907.
7. Dorokhov, Y.L.; Komarova, T.V.; Petrunia, I.V.; Frolova, O.Y.; Pozdyshev, D.V.; Gleba, Y.Y. Airborne Signals from a Wounded Leaf Facilitate Viral Spreading and Induce Antibacterial Resistance in Neighboring Plants. *PLoS Pathog.* **2012**, *8*, e1002640. https://doi.org/10.1371/journal.ppat.1002640.
8. Pfaffl, M.W. A New Mathematical Model for Relative Quantification in Real-Time RT–PCR. *Nucleic Acids Res.* **2001**, *29*, e45.
9. Bierne, H.; Cossart, P. When Bacteria Target the Nucleus: The Emerging Family of Nucleomodulins. *Cell. Microbiol.* **2012**, *14*, 622–633. https://doi.org/10.1111/j.1462-5822.2012.01758.x.
10. Bierne, H.; Pourpre, R. Bacterial Factors Targeting the Nucleus: The Growing Family of Nucleomodulins. *Toxins* **2020**, *12*. https://doi.org/10.3390/toxins12040220.
11. Lacroix, B.; Vaidya, M.; Tzfira, T.; Citovsky, V. The VirE3 Protein of Agrobacterium Mimics a Host Cell Function Required for Plant Genetic Transformation. *EMBO J.* **2005**, *24*, 428–437. https://doi.org/10.1038/sj.emboj.7600524.