Silencing of *Nicotiana benthamiana* phytoendesaturase using dsRNA synthesized in vivo

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Abstract. RNA interference (RNAi) using exogenous double-stranded RNA (dsRNA) has been used to silence the model gene of *Nicotiana benthamiana* phytoendesaturase. Here we report on an efficient technique for dsRNA synthesis using *E. coli* HT115 strain. This strain is deficient in RNase III, an enzyme that normally destroys most dsRNA in a bacterial cell and has been engineered to produce big quantities of dsRNA. We also used root treatment for dsRNA delivery to *N. benthamiana* plants. We found this method to be one of the most efficient ways to deliver dsRNA for plants.

1. Introduction

Modern approaches of plant protection from pests and pathogenic microorganisms mainly involve the use of chemicals, which often have insufficient selectivity and can harm human health and the environment. With the growing global demand for sustainable food production, one of the biggest challenges for agriculture is associated with crop losses due to parasitic nematodes, fungi and other pathogens [1]. In addition, there is a constant threat of pest resistance to the used control methods (mainly chemicals), so it is necessary to search for alternative strategies of struggle [2-3]. The development of new selective non-toxic agents is required for the transition to a highly productive and environmentally friendly agricultural sector.

Classical approaches to regulating gene activity using RNA interference are accompanied by the creation of genetic constructs that are introduced into the genome of a host organism, followed by the expression of small interfering RNAs. However, currently the use of genetically modified organisms (GMOs) is significantly limited by legislation, in particular, their use is prohibited in agriculture. Since RNAi is a very powerful tool for silencing genes, including those responsible for the colonization of plants by various pathogens, there is a need for alternative approaches that allow avoiding the creation of GMOs, for example, by delivering of exogenous dsRNA in various ways. At present, the consequences of manipulation with the genome are still not well understood, which raises public concern about the safety of GMOs [4] and therefore there are legal restrictions on the cultivation of transgenic plants in particular [5]. One of the alternatives may be the use of technologies based on RNAi, for the induction of disease resistance in agricultural plants.

The use of RNAi methods in plants is a new approach for increasing the resistance of important crops to various diseases or pests by suppressing the gene at the translation stage using small dsRNAs. At present, this is one of the most promising approaches for enhancing crop resistance and combating plant pathogens. RNAi is induced either through the creation of transgenic plants, or through the introduction of exogenous dsRNA, which results in suppression of target genes and, as a result,
enhances immunity. Exogenous dsRNAs used for plant protection have recently been widely investigated as a potential alternative to chemical insecticides and pesticides. In addition to eliminating the need to create GMOs, this approach also avoid many problems associated with the use of various chemicals.

From a practical point of view, one of the main obstacles to using dsRNA to suppress genes through RNAi is the development of a cost-effective system for the production of big quantities of dsRNA. Thus, the development of effective methods for the production of exogenous dsRNA is a very urgent problem [6]. Also, improved delivery systems could increase the use of dsRNA as a bioinsecticide in fields. In our work, we created a genetic construct for the production of dsRNA in \textit{E. coli} cells in order to turn off the phytoendesaturase gene in \textit{N. benthamiana} plants. Decreasing in the phytoendesaturase gene expression has a distinct phenotypic manifestation in the form of whitening of young leaves. Root treatment of plants with a rude lysate of a bacterial suspension was used as a delivery system for dsRNA [11]. This approach is approved in model experiments on the regulation of gene expression in plants using exogenous dsRNA [4; 7-8].

2. Materials and methods

Characteristics of bacterial strain. We used the \textit{E. coli} HT115 strain (DE3) [F-, mcrA, mcrB, IN(rrnD-rrnE)1, rnc14::Tn10(DE3 lysogen: lacUV5 promoter-T7 polymerase) (IPTG-inducible T7 polymerase) (RNase III minus)], kindly provided by Benoit Alluni (University Paris-Sud, Orsay, France). This strain is deficient in RNase III, which makes it possible to use it for the production of dsRNA. Cultivation of the strain was carried out on standard liquid LB medium or LB agar with tetracycline.

Plasmid characteristics. We used the L4440 plasmid (Plasmid #1654, Addgene, USA). A feature of this plasmid is the presence of two strong T7 promoters in opposite directions, due to which it is used for the production of dsRNA fragments. The plasmid contains the ampicillin resistance gene.

\textit{N. benthamiana} was used as a model plant for experiments on phytoendesaturase silencing by exogenous dsRNA produced in \textit{E. coli} HT115 (DE3) cells. The plants were taken from the plant collection of the genetic engineering laboratory and kindly prepared for processing by S. R. Popova. A week before the experiment, laboratory plants were planted in peat pots to adapt to room air. The tobacco was kept under 24-hour lighting and temperature of 17 °C.

Primers designing was carried out using the resource Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) to \textit{N. benthamiana} mRNA template (409 bp). The selected primer variants were checked for the presence of hairpins (self- and heterodimers using the OligoAnalyzer tool):

- Forward primer 5’-GGCACTCAACTTATAAACC-3’
- Reverse primer 5’-CTTCAGTTTTCTGTCAAACCATATATGGAC-3’

Purification of total RNA was performed using the RNasy Plant kit (QIAGEN, USA) containing phenol and guanidine isothiocyanate (according to the manufacturer's protocol); per 100 mg of \textit{N. benthamiana} leaf tissue. For a more accurate determination of the concentration of the obtained mRNA, control of the purity of isolation, and control of the integrity of RNA, were carried out on a BioAnalyzer (Agilent) device. Samples with RIN almost 8 were used.

Genomic DNA digestion from total RNA preparations and reverse transcription. Genomic DNA impurities were removed from total RNA samples, for which DNase I without RNases (Thermo Fisher Scientific, Finland) was used according to the manufacturer's protocol. The synthesis of cDNA was carried out using a reverse transcription reaction using a kit with genetically modified M-MuLV – RH reverse transcriptase (BiolabMix, Russia) according to the manufacturer's protocol.

qRT-PCR was performed using HS-qPCR SYBR Blue technology (Biolabmix, Russia) in a Bio-Rad IQ thermal cycler (BioRad,USA). The master mix was prepared using SYBR Blue quantitative PCR mix as the main PCR reagent. 1.0 pg of cDNA and 0.1 ng of each of the primer pair were added in a final volume of 20 μl. The amplification reactions were carried out at 95 ° C for 5 min, 40 cycles
at 95 °C for 30 sec, and then at 60 °C for 1 min. The specificity of the amplification was assessed by dissociation or melting curve analysis at 61–95 °C after 40 cycles. A temperature gradient of 61–66 °C was set. A negative control containing water instead of cDNA was set for every 2 samples. After completion of qRT-PCR, data was analyzed using Bio-Rad IQ software.

Isolation of plasmid L4440 was performed using the BiolabMix kit. To isolate plasmid DNA, 3 ml of bacterial cell suspension of *E. coli* DH5α strain was used. All procedures were carried out at 22°C.

The integrity of the isolated plasmid DNA can be checked by gel electrophoresis in 1% agronomical gel. The amount of DNA isolated can be estimated using UV spectrometry. Typical absorption maximum for DNA at λ = 260 us.

Cloning of phytoendesaturase gene fragments into plasmid L4440. For cloning, a region between T7 promoters, 2000 bp and 2186 bp, was selected. PstI (2067 bp) and NcoI (2098 bp) restrictases for cloning were chosen. The phytoendesaturase gene fragments obtained using qPCR-RT were cloned into a T-vector, which allows insertion of the PCR product into this vector without pretreatment with restriction enzymes. Successful cloning is achieved by selection of blue-white clones due to the presence of a gene in the T vector of the lac Z gene. The resulting fragment can then be easily recloned into the final vector, since control of the complete restriction of the gene by the enzymes selected for the final cloning is provided. The results of the successful creation of the genetic construct were confirmed by sequencing. The plasmids were transformed into *E. coli* HT115 (DE3) using the standard CaCl₂ transformation protocol [9]. *E. coli* HT115 (DE3) strain is an RNA-deficient strain that has been modified to express T7 RNA polymerase from the inducible IPTG promoter [10].

Rude bacterial lysate for phytoendesaturase gene silencing. To obtain samples, *E. coli* HT115 cells were induced with IPTG, granulated by centrifugation and resuspended in lysate buffer (50 mM Tris HCl, 10 mM EDTA, pH 7.5) [9]. The cell suspension was sonicated (20 kHz, 15 min) and then centrifuged at 9000 rpm for 20 min. Supernatant was used as root treatment. Rude lysates of bacterial suspensions after induction of dsRNA synthesis can be used as a source of exogenous dsRNA [11]. Root treatments are also used to deliver target molecules for PTGS in plants [4, 7, 8].

In this study, *N. benthamiana* was used as a model plant for the experiment.

For each type of root treatment (water, Tris HCl / EDTA buffer, *E. coli* HT115 without plasmid, *E. coli* HT115 with plasmid L4440, *E. coli* HT115 with plasmid L4440 with inserted phytoendesaturase gene fragment) 3 plants are used. Plants are treated 3 times a week (Monday, Wednesday, Friday), the duration of the experiment is 4 weeks.

3. Results

Analysis of the isolated plasmid L4440. Isolation of plasmid DNA was performed using the BiolabMix kit. To isolate plasmid DNA, 3 ml of bacterial cell suspension of *E. coli* DH5α strain was used. The amount of isolated DNA was assessed using a spectrophotometer at λ = 260 nm. The concentration was 20 ng (A₂₆₀ / A₂₈₀ ratio = 1.9).

The integrity of the isolated plasmid DNA was verified by 1% agarose gel electrophoresis (figure 1).

The gel electrophoresis data indicate the successful purification of plasmid DNA isolated from *E. coli* DH5α strain. This strain is suitable for the production of big quantities of the required plasmid DNA with subsequent isolation for cloning.

Root treatment of *N. benthamiana* with a rude lysate containing dsRNA. After 4 weeks of treatment, leaves of *N. benthamiana* treated with bacterial lysate with insertion of a phytoendesaturase gene fragment exhibited photobleaching phenotypes of young leaves characteristics of phytoendesaturase gene silencing (figure 2). The phenotype recovered after photobleaching for 28 days after treatment.

4. Discussion

In the present study, we have demonstrated that dsRNA can be produced in vivo in *E. coli*. It has been shown that phytoendesaturase dsRNA products synthesized in *E. coli* HT115 are effective in silencing the selected gene when applied exogenously to *N. benthamiana* plants. These results demonstrate that
dsRNA root treatment is a simple and practical method for dsRNA delivery. This approach can also be used to deliver dsRNA molecules as a fungicide and insecticide.

**Figure 1.** Gel electrophoresis of plasmid DNA. 1-3: plasmid DNA L4440 of *E. coli* strain DH5α; 4: DNA ladder 1000bp.

**Figure 2.** Whitening of *N. benthamiana* leaves after induced phytoenesaturase silencing by exogenous dsRNA. A) water; B) Tris-EDTA buffer; C) rude *E. coli* HT115 lysate; D) *E. coli* HT115 with plasmid L4440; E) positive control for RNAi using VIGS construction against phytoenesaturase gene; F) *E. coli* HT115 with plasmid L4440 with inserted phytoenesaturase gene fragment; G) experimental plant (F) against negative control (D).
5. Conclusion
Our experiment showed a simple, fast, safe and inexpensive way to synthesize big amounts of dsRNA against phytoenodesaturase mRNA, in order to provide a practical approach to silencing our selected targets with a homologous sequence. RNAi technology can be widely applied and available to protect plant crops from various pathogens. This strategy also provides a reliable tool for studying the mechanisms of post-transcriptional suppression of genes in fungal plant infections.

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