New model systems for RNA interference in plants to develop eco-friendly and effective antipathogenic tools

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Abstract. The methods which are used to combat pathogens of crops, in particular, chemical treatment, pose a threat to environmental safety. RNA interference could be one of the best ways to selectively control pests, but there are not enough methods for introducing dsRNA into plants without creating GMOs. The development of new methods of dsRNA delivery requires affordable and fast gene model systems, the purpose of which is our work. We selected the genes of several carbonic anhydrases functioning in the thylakoids and mitochondria of the whole plant, the suppression of which will be seen phenotypically based on the data on mutants for these genes. Specific primers have been designed, and a PCR reaction has also been carried out to obtain the product of the corresponding mRNA, which will be delivered to the plant in the future using the VIGS system.

1. Introduction

Agriculture faces a number of challenges, such as insect, disease and weed damage, that decrease productivity and keep farmers from reaching their potential maximum harvest. In an effort to reduce pest damage, manufacturers rely heavily on synthetic chemicals. Pesticides have allowed farmers to increase production, improve product quality and increase profits. However, the large number of chemicals used every year lead to an increase in pest resistance to the drugs used, and also greatly affect the environmental situation in the world. Against the background of such a situation, alternative methods of combating pathogens are acquiring special significance. One of such methods can be the approaches based on RNA interference.

RNA interference was discovered in 1998 as an effect of suppressing the manifestation of the genotype when dsRNA was introduced into the body [1]. Later, it became known that RNA silencing is a part of a complex network of interconnected pathways for cellular defense and regulation of genes, which can make it a powerful tool for the experimental manipulation of the expression of individual genes [2]. It can be used to suppress genes for susceptibility to pathogens in agricultural crops.

In plants, virus induced gene silencing (VIGS) has shown reliable results in suppressing genes, which uses modified viruses such as the tobacco rattle virus (TRV) to deliver dsRNA into the plant cell [3]. Since the delivery of viral particles occurs using bacteria of the genus Agrobacterium, random modifications occur in the plant genome [4-5]. Because of this, accidentally genetically modified plants cannot be used in agriculture, which means that this method, despite its simplicity, is prohibited for the use in treating plants from pests or to combat abiotic stress.
The development of new methods that do not modify the genome could help the introduction of RNA interference into plant biotechnology. An example of such a system could be SIGS (spray induced gene silencing), which is a promising option for a cheap, simple and safe gene silencing in plants, but is still at the very beginning of full-fledged development [6]. The development of such methods ran into difficulties, including those of a technical nature. The genetic model systems currently used for plants have their drawbacks. One of them uses the suppression of factors of susceptibility to pathogens, such as genes CESA3, DMR1, DMR6, DND1 in Arabidopsis thaliana. Their silencing makes the plant less susceptible to the late blight disease, although to check it one should infect plants of 4-5 weeks of age and after a few more weeks observe the effect of damage. The application of this method implies the presence of separate premises for the cultivation of infected and non-infected plants, which greatly complicates the research [7]. Part of these problems lacks the silencing of the phytoendesaturase (PDS) gene, suppression of its expression reduces the synthesis of carotenoids that protect chloroplasts from burning damage by sunlight. And although it is PDS that is the main marker gene for most studies at the present time [5], silencing of this gene requires at least 4-5 weeks for the manifestation of an altered phenotype, which greatly slows down the development of new effective methods for delivering dsRNA into cells.

The aim of our work is to create a new model system for RNA interference. It will allow us to quickly, efficiently and easily test developed methods such as VIGS based on other vector systems or SIGS. Three carbonic anhydrase genes were selected for testing: β-CA5, γ-CA1, γ-CA2. All of these genes were selected based on reports of a strong slowdown in the growth of mutants for these genes compared to wild-type plants [8-9]. The gamma-carbonic anhydrase genes are planned to be silenced simultaneously, so the resulting model system will be suitable for testing the possibility of parallel suppression of two genes by the simultaneous interference induction method.

2. Materials and methods
Primer design. The selection of primers was carried out using the Primer-BLAST resource. Matrix for the design was cDNA complementary to mRNA. Below there are IDs for genes in NCBI database:

- Beta-carbonic anhydrase 5 - NM_001342222.1, NM_119514.4, NM_001036707.1;
- Gamma-carbonic anhydrase 1 - NM_101815.4, NM_001198110.1;
- Gamma-carbonic anhydrase 2 – NM_103620.4.

At the same time, since all genes except for γ-carbonic anhydrase 2 have different splice variants, a region was chosen that is identical for all mRNA variants. This site was determined using the MUSCLE program. Primer variants obtained via Primer-BLAST were checked for hairpins, self- and heterodimers using the OligoAnalyzer tool. After designing of primers, recognition sites of restriction endonucleases were added to their 5’-ends, since it is planned to insert the target product into the vector using sticky ends. The addition of sticky ends was done using SnapGene software.

Purification of total RNA was performed from the leaves of A. thaliana (ecotype Columbia-0), which were kindly provided by Zulfira Bagautdinova from the Laboratory of Computer Transriptomics and Evolutionary Bioinformatics, ICG SB RAS. The tissue was collected, weighed in 300 μg, frozen at -80 ° C. Grinding was carried out using steel balls on a TissueLyser LT homogenizer for one minute and at a frequency of 50 Hz. The material with balls was pre-cooled in liquid nitrogen, the rotor of the device was previously cooled at -80 °C. Isolation was performed with the commercial Plant Mini Kit according to the attached protocol. All manipulations were performed on ice, and after isolation, RNA was stored at -80 °C.

Checking of total RNA quality was performed on a 2100 Bioanalyzer Instrument in accordance with the described method [10] at the Genomics Center of the ICBFM SB RAS. The result was obtained in the form of a graph with the concentrations of different RNA and the RIN value, characterizing on a scale from 1 to 10 the quality of RNA preservation after isolation (table 1).
Table 1: Primers for target genes.

| Gene | Forward and reverse primers | Site for endonuclease |
|------|-----------------------------|-----------------------|
| LOX2 | GGCTGgattctACGTCATGCTGCTGATGGA | EcoRI |
|      | GTTGTctagatGGTGATAGGAAATGAGGA | XbaI |
| β-CA5 | TCAAGgattctCTCTTCAGATGCTTGTGGCC | BamHI |
|      | GTCTAagatcGCAACCACATTAGCTTCTCTG | KpnI |
| gCA1 | TGTAAgattctTTATTTTGGACGCGTTC CCC | EcoRI |
|      | TCGAATctagataCATCAGTGGCCGATGGCTTGTGG | XbaI |
| gCA2 | AAAAgattctTCCTCCATCAATACGCCCCT | EcoRI |
|      | ATCGAATctagataATGCTCAGCAAGATGGCTTCT | XbaI |
| PDS  | TTTGGgattctTTCTGCGGCGAATTGCTTATCAAAAAG | EcoRI |
|      | ATTAAGctagataAGAAACTCTAAACCCGGCCATCGTATTGAG | XbaI |
| CH42 | TCACGtctagataTGCGGTCTTCTTTTGGAAATCTCTT | XbaI |
|      | ATTAAGtctagataTGCGAATACAGAAACTTGCTTCTC | KpnI |

Reverse transcription. For reverse transcription, the M-MuLV-RH Biolabmix reverse transcription kit was used.
After that, reverse transcription was performed according to the following program:

- 37 °C - 1 hour;
- 42 °C - 30 minutes;
- 50 °C - 10 minutes;
- 75 °C - 5 minutes.

The resulting cDNA was stored at -80 °C.
Real time PCR was performed on a BioRad iCyclerIQ device with a FAM490 fluorescence sampling channel. All preparation of the mixture was performed according to the standard protocol supplied with this PCR kit («PCR 2-fold SYBR-Blue» from Biolabmix). The final concentration of magnesium ions was 3 mM, cDNA obtained after reverse transcription was diluted 10 times. The primers were diluted to a working concentration of 3 mM and added to the reaction mix, 2 μl each in a reaction with a total volume of 20 μl. For each point, two technical repeats were performed with cDNA concentration not less than 10 ng / μL and one negative control with water instead of cDNA.
The specified program included:

- 95 °C - 15 sec;
- 45 cycles:
  - 95 °C - 20 sec;
  - 63 °C - 25 sec;
  - 72 °C - 30 sec;
- 95 °C - 1 min;
- 63 °C - 10 sec;
- Melting in the range from 63 to 90 °C with the addition of 0.5 °C every 10 sec.

The resulting PCR products were stored at -20 °C
Agarose gel electrophoresis. Verification of the products obtained in PCR was performed using gel electrophoresis. Gel electrophoresis was carried out on TAE buffer in 2% agarose gel at a constant tension of 5 V/cm and for one hour. Ethidium bromide was also added to stain in 100 ml of gel to a concentration of 0.5 μg/ml.
3. Results

Selection of primers for all target genes. See Table 1 above for a list of selected primers. In addition to target carbonic anhydrases, primers were also selected for mRNA of the lipoxygenase 2 (LOX2) gene, which was also considered for the role of a target gene, but was later rejected. Besides primers were not selected for the PDS and nucleoside phosphate hydrolase (CH42) genes selected as a positive control; ready-made primers from the literature were used (Burch-Smith et al., 2006). Restriction endonuclease sites were also added to the 5'-ends. This was done in order to insert the product into a vector polylinker using restriction endonucleases and ligases in the future. In Table 1, the recognition sites of restriction endonucleases are indicated in lower case.

Purification of RNA. The RIN is 7.70, which is a good indicator of RNA quality. The product concentration is 8 ng / μL and the 28s/16s RNA ratio is 1.9

Gel electrophoresis of PCR reaction products. All products of PCR reactions were tested, as well as negative controls, which contained all components of the mixture except for the matrix for PCR. After analyzing the electropherogram (figure 1), we were able to draw several conclusions.

4. Discussion

At current stage of work, we can postulate that primers for LOX2 (2a, 4a), β-CA5 (6a) have a suitable design. The product is obtained and is clearly visible on electrophoresis. The product of primers for γ-CA1 is not always clearly visible (8a, 2b), but is still noticeable. The pair for γ-CA2 (4b) and PDS (6b) does not work, the product is not visible on electrophoresis. For the CH42 (8b) gene, the product is present and is clearly visible. After analyzing the length of the products, it became clear that the template for PCR is genomic DNA, and not cDNA obtained by reverse transcription. To check this statement, we set up a PCR reaction on total RNA isolated from the plant. And since the product is obtained (2b), we made sure that RNA needs an additional DNase treatment before RT-PCR.

It is also necessary to select another pair of primers for the γ-CA2 gene, with which the product will be produced in the reaction. It was decided not to make a vector for the PDS gene, because such a vector is present in the purchased set, and we can use it for a positive control.
5. Conclusion
In the future, it is planned to insert the genetic construct obtained after PCR into the bacterial vector pTRV2-MCS using the gateway cloning approach. Transformation of Agrobacteria with the obtained vectors with inserts and further infection of A. thaliana with Agrobacteria will be performed. At the last step of our experiment phenotypic observation and analysis of gene expression in real-time PCR will be made.

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