A Bireporter Vector System for Assaying Translational Activity of Regulatory Elements

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A bireporter vector system for assaying translational activity of regulatory elements

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Abstract

Background: Molecular biology has always shown some similarities with computer science. So, considering transient expression, one can see an analogy with a DDoS attack on a computing system. Like the DDoS attack, transient expression can carry a payload. In particular, analysis of the structure of cell mechanisms and signal amplification in the study of very subtle mechanisms of regulation.

Results: A new vector system for transient expression in plants is described; this system is intended for quantitative analysis of the contribution of regulatory elements to transcription and translation efficiencies. The proposed vector comprises two expression cassettes carrying reporter genes (of the *Clostridium thermocellum* thermostable lichenase and *E. coli* β -glucuronidase) under the control of different promoters. Herewith we also propose a new method for quantification of the effect of tested regulatory elements on expression, which relies on assessment of the enzyme activities of reporter proteins taking into account the transcription of their genes.

Conclusions: In our view, this approach makes it possible to precisely determine the amounts of reporter proteins and their transcripts at all stages of expression. The efficiency of the proposed system has been validated by the analysis of the roles of known translation enhancers at the stages of transcription and translation.

Keywords: high throughput screening; vector; lichenase; β -glucuronidase; regulatory elements; transient expression

Introduction

The recent advance in the technologies for obtaining omic data has allowed for accumulation of tremendous array of information. Correspondingly, molecular biologists frequently need experimental verification of the biomolecular data. A wide range of reporter systems working in most different organisms has been designed for this purpose. For plants, the most relevant method is transient expression–agroinfiltration; it consists in the transfer of a large number of copies of vectors via the infiltration of the agrobacteria (*Agrobacterium tumefaciens*) carrying these vectors to the mesophyll of a model plant. The apparent advantages of this approach are rapidness, simplicity, and availability. The vector systems optimized for the transient expression in plants commonly have the following specific features: the absence of a selective marker, the presence of silencing suppressor genes, and the fact that the reporter genes code for rapidly maturing proteins. This tool makes it possible to

get precise data on the expression of target polypeptides in plants. Nonetheless, transient expression is the approach that depends on manifold parameters. A considerable set of factors, such as plant age and the conditions of plant growth, can add noise to the corresponding data [1].

In order to level the dependence of expression level on the uncontrolled conditions, we propose using the bireporter vector pLAUMe, carrying the genes of two reporter proteins, namely, *Clostridium thermocellum* thermostable lichenase (LicBM3) [2] and *E. coli* β -glucuronidase (GUS) [3, 4], which have shown a good performance as reporter proteins with different sensitivities being simple in use. This approach allows for a concurrent assessment of the expression levels of both the reporter gene under the control of a tested element and the gene being a kind of internal control. Thus, the presence of internal control module allows for assessment of the expression level of a gene from the target cassette despite the changes in external conditions. In addition, this makes it possible to avoid co-transformation, which considerably simplifies the experiment. The main advantages of the proposed vector system include simplification of the analysis of regulatory elements (no need in co-transformation) and a direct quantification of reporter proteins.

In this work, we have designed and tested a vector system for assessing the efficiency of translation enhancers. Known and well-characterized enhancers were used for the analysis. In addition, we propose a new method for quantification of the effect of individual regulatory elements on expression based on assessment of enzyme activities of reporter proteins. We have demonstrated that it is feasible to measure not only a relative amount, but also an absolute amount of reporter proteins and, as a consequence, to quantify the contribution of each enhancer to transcription and translation. The general plot of the experiment is shown on the Figure 1.

Materials and methods

Plant growing

Nicotiana benthamiana plants were used in the work; the plants were grown on mineral wool using Knop's solution according to the earlier described protocol [5]: photoperiod, 14 h of light/10 h of darkness and illumination, 130–150 $\mu\text{Em}^{-2}\text{s}^{-1}$. The plants aged 6 weeks were used for agroinfiltration.

Bacterial strains

The *A. tumefaciens* strain GV3101 transformed with individual constructs was grown for 48 h in the LB medium supplemented with 50 mg/L rifampin, 50 mg/L carbenicillin, and 100 mM of acetosyringone. In order to obtain a standardized lichenase preparation, *E. coli* strain XL1 Blue was transformed with the vector pQE30-LicBM3 (earlier developed by the team of the authors [6]).

Construction of vectors

Standard molecular cloning procedures and PCR protocols were used. Restriction endonucleases, T4 DNA ligase, *Taq* and *Pfu* DNA polymerases, and phosphatases were used according to the manufacturers' protocols (Promega, United States; Fermentas, Lithuania). The basic vector, named pLAUMe, was constructed in several steps. Initially, the *SacI*/*SmaI* fragment carrying the reporter gene of thermostable

lichenase, *licBM3*, was cloned from the vector pQE-LicBM3 [6] to the vector pPGG 1A [7] hydrolyzed with *SacI* and *SmaI* to get an intermediate vector, pPGG-L. The *SpeI/XhoI* fragment of pPGG-L, carrying the reporter gene *licBM3* under an enhanced CaMV 35S RNA promoter and terminator, was cloned into the vector pVIG-T [7] hydrolyzed with *SacI* and *SmaI* to form the intermediate vector pGLR. At the next stage, the pACT-*uidA*-Tnos cassette, comprising *A. thaliana* actin promoter, *E. coli* β -glucuronidase gene (*uidA*), and the *A. tumefaciens* termination sequence of the nopaline synthase gene, was synthesized (see Table S1 for the used primers). The pACT-*uidA*-Tnos cassette was cloned into the vector pGLR preliminary hydrolyzed with *XhoI* to get the vector pLAUMe. The last vector was further used to construct the vectors pLAUMe-SynM, pLAUMe-GGR, pLAUMe-AT30, and pLAUMe-AT65 by cloning the regulatory sequences between the CaMV 35S RNA promoter and *licBM3* reporter gene sequences using SLiC method [8]. A correct fusion of the genes with the corresponding regulatory sequences in the plant expression vectors pLAUMe-SynM, pLAUMe-GGR, pLAUMe-AT30, and pLAUMe-AT65 was confirmed by sequencing (Evrogen, Russia). See Supplementary Materials (Table S1) for detailed information.

Agroinfiltration

Agroinfiltration followed the earlier described protocol [9]: *A. tumefaciens* cells of an overnight culture were centrifuged and suspended in the infiltration buffer (10 mM MES pH 5.5, 10 mM MgSO₄, and 100 mM acetosyringone). For a typical assay, the leaves of greenhouse-grown *N. benthamiana* plants were infiltrated with the *Agrobacterium* mixture (50 mL/leaf) using a syringe without a needle. After the infiltration, the plants were further grown under greenhouse conditions. All experiments were performed in four to six replicates.

Analyzing reporter gene transcription

The transcription of reporter genes was assessed using quantitative PCR in one experiment in five biological replicates and three technical replicates.

Total RNA from the majority of samples was extracted using TRIzol reagent (Evrogen, Russia) according to the manufacturer's protocol. Prior to cDNA synthesis, RNA was treated with RNase-free DNase I (Thermo Scientific, United States) according to the manufacturer's protocol to ensure no DNA contamination; then, the first-strand cDNA synthesis was carried out with approximately 2 μ g RNA using a Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, United States) and oligo-dT primers according to the manufacturer's protocol. The primers were designed using PrimerBLAST (Table 1) with melting temperatures of 60°C and amplicon lengths of 159 and 143 bp, respectively.

RT-qPCR was conducted in an Applied Biosystems QuantStudio 5 (Thermo Scientific, United States) using qPCRMix-HS SYBR (Evrogen, Russia). The reactions were performed in a total volume of 20 μ l of the reaction mixture containing 1 μ l of the template, 5 μ l of 5 $\times$ SYBR mix, 1 μ l of each specific primer to a final concentration of 200 nM under the following conditions: initial denaturation at 95°C for 180 s followed by two-step thermal cycling profile of denaturation at 95°C for 15 s, and 40 cycles of combined primer annealing/extension at 60°C for 30 s. No-template controls were included for each primer pair and each PCR reaction was

completed in triplicate. To verify the specificity of the amplicon for each primer pair, a melting curve analysis was performed ranging from 60 to 95°C with the temperature increasing steps of 1.6°C /s at the end of each PCR run.

Constructing standard curves for copy number determination and absolute quantification

The pLAUMe (described above) vector carrying the *uidA* and *licBM3* genes was used as a standard. The standard sample was tenfold diluted to cover the concentration range of 0.2 to 200 ng/15 µL. The absolute quantitative assay was performed using the Design Analysis Software v. 2.5.1 and Standard Curve v. 1.5.1 (Thermo Scientific, United States).

Preparing lichenase standard

An overnight *E. coli* strain XL1-Blue (Stratagene, United States) culture carrying the earlier produced vector pQE-LicBM3 [6] was diluted (1 : 50) with LB medium (Amresco, United States) and grown at 37°C to an OD₆₀₀ of 0.5. Then, the gene expression was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) to grow the culture at 28°C for 48 h. The cells were separated from the medium by centrifugation for 15 min at 3160×g, washed twice with 50 mM Tris-HCl buffer pH 8.0, and suspended in the buffer containing 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.1% Triton X-100, 5 mM DTT, 0.01% SDS, and 10 mM NaCl. The cells were incubated at 65°C for 30 min and clarified by centrifugation for 30 min at 16 000×g and 4°C. The supernatant was purified on a HisTrap HP column according to the manufacturer's protocol (GE Healthcare, 17-5247-01). The eluted proteins were dialyzed against 5 mM Tris-HCl pH 8.0 at 4°C and the purified thermostable lichenase protein was diluted in the buffer containing 20 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 1 mM DTT, 200 µg/mL BSA, 50% glycerol, and 100 mM KCl to a final concentration of 1 µg/µL to use in the further experiments. The protein amount in preparations was determined using bicinchoninic acid (Sigma, United States) [10]. The proteins were separated by 12% SDS-PAGE according to Laemmli [11]. The molecular weight of proteins was determined using a Thermo Scientific PageRuler Unstained Protein Ladder (Thermo Fisher Scientific, Inc., United States). See Supplementary Materials (Figure S1) for electrophoretic pattern.

Preparing plant protein lysates

The leaves sampled from *N. benthamiana* plants on days 4–7 after agroinfiltration were pulverized in liquid nitrogen to a fine powder. Each powdered sample was suspended in three volumes of the 1× PBS containing 0.5% Triton X-100 and incubated for 15 min at 4°C and for 15 min at 50°C. Cell debris was removed by centrifuging twice for 5 min at 16 000×g. The concentration of the samples was adjusted with 1× reaction buffer. Translational activities of the reporter genes were measured in two independent experiments (eight to ten biological replicates each).

Quantification of β-glucuronidase

β-Glucuronidase was quantified in plant extracts according to Jefferson et al. [3]. The amount of β-glucuronidase in preparations was determined using the calibration

plot and expressed in nanomoles (4-MU) per unit volume per minute. To assume the amount of GUS protein in the plant samples we used curve constructed by standart β -glucuronidase diluted with factor 0.5.

Quantification of LicBM3 lichenase

For this purpose, lichenan at a concentration of 125 $\mu\text{g/mL}$ (if not stated otherwise) and Congo red solution at a final concentration of 0.005% were used. The fluorescence was assessed in a Synergy H1 (BioTek, United States) multimode microplate reader using 96-well microtiter plates [12].

Statistical data processing and analysis

For this purpose, we wrote a special Python [13] script using several libraries, namely, pandas [14] for table data; NumPy [15] for data arrays; SciPy [16] for statistical data processing; math for mathematical functions; and seaborn and matplotlib [17,18] for data visualization. Data processing comprises the following stages: (i) Normalization of the samples according to dilution; (ii) Normalization of the samples according to volume; (iii) Construction of calibration curves; derivation of regression equation; (iv) Computation of the equation of dependence (inverse to regression equation); (v) Computation of absolute amounts of reporter proteins in unit volume; (vi) Derivation of linear regression equation for the dependence of amount of one reporter protein on the amount of another one for each tested enhancer; (vii) Regression analysis of quantitative PCR data; and (viii) Representation of the results of analysis as tables and plots. Construction of calibration curves for determining the amount of reporter proteins is described in the corresponding section of Materials and methods. The data were processed using linear regression. The equations for straight lines and calibration curves were optimized by least square technique with the help of Levenberg–Marquardt algorithm (using the Python SciPy library).

Results

Model of vector system

Two reporter systems were united in the designed vector, namely, the *uidA* gene under the control of arabidopsis actin promoter, which acts as the internal control module, and the *licBM3* gene under the control of enhanced CaMV 35S RNA promoter, the test module for assessing the contribution of the studied regulatory elements (Figure 2). In addition, the vector carries the cassette with the tombusvirus p19 silencing suppressor under the control of arabidopsis translationally controlled tumor protein promoter [19]. Being united in one vector molecule, these structural modules make it possible to avoid the co-transformation with individual vectors. The castor bean catalase intron and potato ST-LS1 intron were integrated into the *uidA* and *licBM3* genes, respectively, to prevent unauthorized expression in the intermediate prokaryotic systems. See Supplementary materials (Figure S2) for the detailed map of the vector. The vector thus constructed was tested in *N. benthamiana* (the corresponding data are shown below with the results of testing of the remaining vectors). To verify the designed test system, we decided to use the already described and characterized enhancers (listed in table). Note that

for better coverage, we selected four short plant enhancers contrasting in their expression levels (deletion variants AT30, At65, AT100, and AT208: AT5G46430, AT1G07260, AT1G67090, AT1G58420 of *A. thaliana*, respectively [19]), one long plant enhancer (GGR, geranyl-geranyl reductase enhancer) [20], and two synthetic enhancers (SynJ and SynM (MsynJ and SynM) [21]. The enhancers were integrated using CPEC method [22], which allowed for a seamless integration of target fragments alone. Thus, the following expression vectors were obtained using the pLAUMe vector as the major component: pLAUMe-AT30, pLAUMe-AT65, pLAUMe-AT100, pLAUMe-AT208, pLAUMe-SynJ, pLAUMe-SynM, and pLAUMe-GGR, respectively. In these vectors, the sequence of a tested enhancer is located upstream of the *licBM3* reporter gene. Each of the constructed vectors was used to transform *A. tumefaciens* strain GV3101, which were further used to agroinfiltrate *N. benthamiana* plants.

A comparative analysis of the functional activity of translation enhancers

Total mRNA and total soluble protein were isolated in parallel from the agroinfiltrated leaf fragments to assess the expression of the reporter gene under the control of a tested regulatory element at the stages of transcription and translation. The transcription of reporter genes was assessed by qPCR in one experiment. The translational activities of reporter genes were analyzed according to the enzyme activities of reporter proteins. Using the above-described (Materials and methods) pipeline for data processing, we determined the regression slopes to construct the rating of the tested enhancers (Table 2). The column Normalized lists the ratios of reporter proteins (*LicBM3/GUS*) normalized by the ratio $RNA_{enhancer}/RNA_{pGLR}$, where $RNA_{enhancer}$ is the ratio of *licBM3/uidA* mRNAs for a selected enhancer and RNA_{pGLR} is the control variant without any translation enhancer, pGLR. Analysis of the transcriptional (RT-qPCR; Figure 3.1) and translational (enzyme activities of reporter proteins; Figure 3.2) activities of the tested enhancers showed a linear dependence between the transcription and translation levels of the reporter genes. Table 2 lists the comparative data and statistical tests.

The numerical data listed in table and visualized in plots suggest the following inference: the distribution of measurements corresponds to the proposed model described below (see Discussion). The absolute contribution of a tested enhancer to the translation level (Q_{abs}) is determined by the ratios of coefficients A (slope angle of regression line) from the equation $f(x) = Ax$: $Q_{abs} = A_{protein}/A_{RNA}$; $f(x)$, a dependent variable, is the level of lichenase under the control of a tested element (protein or mRNA) and x , an independent variable, is the level of β -glucuronidase. The relative contribution requires normalization by the selected internal control, i.e., the enhancer playing the role of standard, but does not require the precise amounts of enzymes in samples to be calculated.

Discussion

Model of the contribution of regulatory elements

First and foremost, it is necessary to consider the main question potentially arising when reading this paper: What is the purpose of the internal control? Since the vector contains two genes of reporter proteins at once, the ratio of the copies

(*licBM3/uidA*) in T-DNA will always be 1 : 1. In the expression under controlled conditions (since the reporter genes are under the control of constitutive promoters), the ratio of expression levels (*licBM3/uidA*) at the stage of transcription expressed as the mRNA copy number is also constant and reflects a certain linear dependence (determined by the strength of promoters). It is reasonable to assume that, other conditions being equal, the same ratio will be observable for the target proteins (LicBM3/GUS). However, if one of the reporters is placed under the control of a regulatory element that influences translation process, this ratio (LicBM3/GUS) will change in the direction of the effect of this regulatory element. With a set of assumptions, this dependence may be considered linear. Then the ratio of the slopes of lines, which reflect the ratios of mRNAs of reporters and their protein products, will show the contributions of a given regulatory element to the control of translation. We propose to assess the contribution of particular translation enhancers (both known and potential) using the following scheme:

(i) Analysis of the transcription of reporter genes with qPCR for determination of the absolute copy number of the corresponding mRNAs; (ii) Analysis of the enzyme activities of reporter proteins using the calibration curves constructed according to purified standardized enzymes for determination of the absolute copy numbers of the corresponding enzymes; and (iii) Statistical analysis of the data and comparison of the slopes of the lines reflecting the ratios of mRNAs (*licBM3/uidA*) and proteins (LicBM3/GUS).

Thus, the system of internal control physically linked to the main reporter gene and a tested regulatory element provides the possibility to more precisely measure the expression level of the reporter under the control of this element as well as to considerably accelerate and simplify the very process of testing thanks to avoiding the stage of co-transformation. As for the currently existing vectors with more than one reporter, bicistronic systems have been widely used recently. These systems have been applied to localize pathogens [23–25]; study enzyme activities [26, 27], heat shock proteins [28], and transcriptional and posttranscriptional modifications [29, 30]; and assess the activity of bacterial promoters [31] and the cell cycle [32]. Of special interest is the role of bicistronic systems in IRES analysis [33].

However, a bicistronic system is not completely adequate for analyzing the cis-regulatory elements.

It is reasonable to turn back to the stage of qPCR analysis of transcription; this stage is necessary since several studies [34, 35] demonstrate that some functional elements of promoter can be located downstream of the transcription start site; correspondingly, these elements reside in the 5'UTR. The opposite situation is equally true: if a 5'UTR in the distal region contains the motifs similar to functional elements of promoter, such 5'UTRs can be involved in the regulation not only of translation, but also of transcription. Thus, we believe that a more precise characterization of a tested translation enhancer requires analysis of its role at the stage of transcription as well.

Statistical data model

Before describing the very results of experiment, we would like to consider a potential model of the data obtainable when implementing the described scheme. It

is important to answer the question on how our data will look like in an ideal experiment. We assume that the measured expression level at the stages of both transcription and translation reflect the linear dependence of LicBM3 on GUS (naturally, with a certain noise level).

In this work, we propose to perform a relative analysis of the role of translation enhancers by arbitrarily selecting one of them as a control. It is necessary to take into account that the 5'UTRs frequently contain proximal promoter regulatory elements (boxes) [34,35], thereby also influencing the process of transcription. Correspondingly, we propose to assess the changes in the ratio of the products of the reporter genes at the stages of both transcription and translation to more accurately determine the contribution of translation enhancers to the expression level. We assume that the enzyme activity (U_e) is associated with the number of protein molecules (N_e) as $N_e = k_e \times U_e$ (k_e , coefficient); thus, the ratio of the normalized amounts of reporters in absolute units (moles or the number of molecules in a unit volume or a unit weight of a sample) is expressed as

$$Q_{Lic/Gus} = \frac{N_{Lic}}{N_{Gus}} = \frac{k_{Lic}U_{Lic}}{k_{Gus}U_{Gus}} \quad (1)$$

Then, assuming that $K = \frac{k_{Lic}}{k_{Gus}}$ and normalizing by the contribution of enhancer at the stage of translation ($R_{abs} = \frac{RNA_{Lic}}{RNA_{Gus}}$) we get

$$Q_{abs} = K \frac{U_{Lic}}{U_{Gus}} \frac{1}{R_{abs}} \quad (2)$$

Thus, a mere comparison of the normalized ratios of reporter enzyme activities allows us to make the conclusions on a relative contribution of enhancers at the stage of translation.

It is reasonable to separately discuss the possibilities of an absolute estimation of the contribution of a tested regulatory element. In our view, the processes of transient expression are rather variable; correspondingly, it is not realistic to assess the changes in the amount of protein expressed under the control of a certain enhancer in terms of weight units, i.e., relative estimates are sufficient. Nonetheless, an absolute estimate is not too complex in terms of methodology provided that the k_{Lic} and k_{Gus} coefficients are determined from Eq. (1).

Advantages and disadvantages of the proposed vector system

The proposed vector system for assessment of the role of translation enhancers have some shortcomings. As is mentioned above, the use of the vector with physically linked genes coding for reporter proteins provides the possibility to obtain the absolute contributions of a studied cis-enhancer to expression at the stages of transcription and translation. Nonetheless, this works only for the samples harvested at the same time moment. Since reporter proteins, as we believe, can have different lifetimes and their activity ratios can change with time, we have not studied this issue in sufficient detail. However, we assume that construction of the time series for

the ratios of reporter protein activities/amounts can make it feasible to determine the absolute contribution of an enhancer to the translation efficiency. Depending on the variant (absolute or relative), the proposed approach makes it possible to obtain the data on the contribution of individual enhancers to the regulation of translation. It is important to understand that the absolute contributions to expression levels for each reporter at each stage should be measured in absolute units—moles or number of copies (molecules).

Appendix

Text for this section. . .

Acknowledgements

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Abbreviations

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Availability of data and materials

All datasets generated for this study are included in the paper/supplementary information.

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

All authors agree with submission of this version.

Authors' contributions

Contributions The work was designed and planned by AAT, IVGP, and AVS. The experiments were conducted and data were acquired by AAT, AVS, IGS, and OSP. Data were interpreted by ONM, AVS, and IVGP. The paper was written by AAT, OAG, and AVS and reviewed by IVGP. All authors read and approved the final manuscript.

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Figures

Figure 1 Scheme of the experiment

Figure 2 Scheme of the pLAUMe vector. p19 – Silencing supressor from tombusviruses. TCTP – arabidopsis translationally controlled tumor protein promoter. en35SCaMV –enchanced 35S CaMV promoter. LicB – lichenase gene. pAct – arabidopsis actin promoter. uidA – gene of beta-glucuronidase.

Figure 3 Trancriptional and translational stages of the expression ef the tested enhancers

Figure 4 Map of the pLAUMe vector. OCS – octopin synthase terminator. p19 – Silencing supressor from tombusviruses. TCTP – arabidopsis translationally controlled tumor protein promoter. en35SCaMV –enchanced 35S CaMV promoter. LicB – lichenase gene. pAct – arabidopsis actin promoter. uidA – gene of β -glucuronidase. T-Nos – nopaline synthase terminator. CBCI – castor bean catalase intron. Data are based on [12]

Figure 5 Standart curve for lichenase by enzyme

Figure 6 Standart curve for β -glucuronidase by 4-Methylumbelliferyl- β -D-glucuronide

Figure 7 Standart curve for β -glucuronidase by enzyme

Tables

Additional Files

Additional file 1 — Sample additional file title
Additional file descriptions text (including details of how to view the file, if it is in a non-standard format or the file extension). This might refer to a multi-page table or a figure.

Additional file 2 — Sample additional file title
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Table 1 Primers for qPCR

Gene	Sequence (5'-3')
<i>uidA</i>	F: CGGCAATAACATACGGCGTG; R: ATACCGAAAGGTTGGGCAGG
<i>licBM3</i>	F: GGACCTTCGGACAACAATCCA; R: TCCTGGGAAGCATCGAATCC

Table 2 Ranked expression quotion *licBM3/uidA* as a normalized slope of regression line

Enhanser	Slope	qPCR	Normalized on tran- scription impact	Normalized on the impact of <i>uidA</i> 5UTR
AT30	1.3479	923.9530	0.0015	0.1711
AT208	9.8941	1241.3535	0.0080	0.9347
pGLR	4.1858	490.8870	0.0085	1.0000
AT65	7.4723	521.4279	0.0143	1.6806
SynJ	6.9351	413.8579	0.0168	1.9652
SynM	6.3721	314.1038	0.0203	2.3791
AT100	20.1628	907.7488	0.0222	2.6049
GGR	48.6790	1095.2883	0.0444	5.2122

Table 3 Regression parameters

Enhanser	slope	intercept	rvalue	pvalue	stderr
AT30	1.3479	248.5330	0.1161	0.7340	3.8454
AT208	9.8941	189.6554	0.5142	0.3754	9.5270
pGLR	4.1858	-45.3358	0.7204	0.0438	1.6453
AT65	7.4723	13.6183	0.9472	0.0000	0.8945
SynJ	6.9351	-11.1162	0.6310	0.0504	3.0149
SynM	6.3721	51.4474	0.8279	0.0016	1.4387
AT100	20.1628	180.8762	0.6508	0.0416	8.3168
GGR	48.6790	-40.7414	0.9729	0.0000	4.3721

Figure 1

Figure 3

beta-glucuronidase

Supplementary Files

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