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A NOVEL SELECTION SYSTEM FOR CHARACTERIZATION AND IDENTIFCATION OF *ARABIDOPSIS* MUTANTS AFFECTED IN STRESS RESPONSES

Cristina POPESCU[1], László SZABADOS[2]

[1]DEPARTMENT OF GENETICS, BANAT UNIVERSITY OF AGRICULTURAL SCIENCE, TIMISOARA, ROMANIA
[2]INSTITUTE OF PLANT BIOLOGY, BIOLOGICAL RESEARCH CENTER, LABORATORY OF ARABIDOPSIS MOLECULAR GENETICS, SZEGED, HUNGARY

ABSTRACT

In order to identify and characterize regulatory genes, able to control the expression of drought and salt responsive genes in Arabidopsis, a novel selection system has been designed.

Several ten thousand transgenic Arabidopsis plants were created by efficient vacuum infiltration method that carries both the pRD29A-Luc or pADH1-Luc constructs and the pTAc17 tagging vector. T1 seedlings were screened for luciferase activity in the absence of stress.

Our results suggests that the luciferase reporter system can be exploited for the identification and tagging of genes, the expression of which is positively or negatively regulated by ABA, salt or osmotic stress

KEYWORDS:

Activation tagging, luciferase reporter system, ABA, osmotic stress

1. INTRODUCTION

In *Arabidopsis* many mutants have been characterized in the past few years and most of them are the result of recessive or loss-of-function mutations. However, a limitation of loss-of-function screens is that they rarely identify genes that act redundantly.

After the *Arabidopsis* genomic sequence was determined it has been revealed the existence of many duplicated genes that are very similar both in their coding regions and in the non-coding regulatory regions [2]. This genetic redundancy sometimes prevents elucidation of gene function by loss-of-function approaches [11]

A number of approaches have emerged to help circumvent these potential problems. The enhanced expression of genes providing gain-of-function phenotypes has proved a productive strategy to identify gene function [10]. The first direct method for undertaking gain-of-function

mutations in plants exploited the enhancer element from cauliflower mosaic virus (CaMV) 35S gene [7]. A T-DNA vector was constructed containing four copies of this element.

These enhancers can cause transcriptional activation of nearby genes, and, because activated genes will be associated with T-DNA insertion, these approach has become known as activation tagging [11].

2. MATERIALS AND METHODS

Vector construction and plant transformation

The transgenic Arabidopsis line expressing the *RD29A::Luc* construct was obtained from Prof. ***Zhu*** (Arizona State University, USA). The *ADH::Luc* reporter gene was previously constructed by ***L. Szabados,*** by fusing the stress and ABA-regulated ADH1 promoter to the *FFluc* reporter gene in the *pBin-luc+* vector. The resulting plasmid was introduced into Arabidopsis (Col-0) by *Agrobacterium*-mediated transformation. Activity of the *ADH1::Luc* reporter gene was tested by bioluminescence imaging. Luciferase activity in several transgenic lines was highly responsive to salt, sugar, or ABA.

The ***pTAc 17*** activation vector (obtained from *N-H Chua*, Rockefeller University, USA, modified by *L. Szabados*) has the estradiol responsive XVE promoter placed at the right border. The XVE activator termed like this for LexA-VP16-ER, is well characterized and highly regulated by estradiol in transgenic plants with undetectable transactivating activity in the absence of the inducer [13]

The ***pER16*** vector [12] designed for 17^{β} - estradiol inducible expression of adjacent genes possesses a G10-90 promoter fused to XVE transcription factor gene and ribulose diphosphate carboxylase ER poly A addition sequence (TE9).The G10-90 promoter is tetramer of the G-box motif. This synthetic promoter drives constitutive non-tissue specific expression in both dicots and monocots [5]. The chimeric XVE protein consists in DNA-binding domain of the bacterial repressor LexA (X), the acidic transactivation domain of VP16 (V) and the regulatory domain of the human estrogen receptor [14] A second transcriptional unit is comprised of the NOS promoter (PNOS), a kanamycin resistance gene (NPTII) and NOS terminator. Eight copies of LexA operator sequence (O^{LexA}), a binding site for the XVE transcription factors are fused to minimal 46bp CaMV35S promoter sequence which is adiacent to LB. Addition of 17^{β} - estradiol induces the XVE binding to the O^{LexA} sites, which promotes the expression of the gene(s) adiacent to the left border.

Plant Transformation and Screening for Activation of Luciferase Reporter Gene

Transgenic *Arabidopsis* plants which carry promoters of drought-and ABA responsive genes, *RD29A* and *ADH1* were fused to Firefly luciferase (*FFLuc*) reporter gene were transformed by *Agrobacterium*–mediated *in planta* transformation by "vacuum infiltration" [3].

Inflorescence of *Arabidopsis* transgenic plants was immersed into a solution of *Agrobacterium*. A vacuum was applied and then released, causing air trapped within the plant to bubble off and be replaced with *Agrobacterium* solution. Seeds from infiltrated plants were germinated on selective MSAR seed medium containing 15 mgL^{-1} hygromycin [6].

Three-week-old hygromycin resistant seedlings were transferred into Petri dishes and sprayed with 1mM D-luciferin solution (Biosynth AGStaad, Switzerland), and assayed for luciferase activity using a low light imaging with a CCD (charged coupled device) camera system (Visilux Imager, Visitron Systems GmbH, Puchheim, Germany). Bioluminescence images were processed using the MediaView 4,5r6 software (Universal Imaging Corporation, Downingtown, PA).

Measurement and Analysis of Kinetics of Luciferase Activation

Temporal activation of the *luc* reporter was characterized by sequential recording of bioluminescence images. Two–week-old seedlings were sprayed with and 2μM 17^{β} - estradiol solution and then subjected to luminescence imaging as described above. Images were analyzed using the Metaview 4,5r6 software and processed in Microsoft Excel Worksheets (Microsoft, Redmond, WA). Each experiment was repeated three times.

RNA gel blot analysis

Total RNAs were isolated, and gel blot analyses were carried out. An aliquot of 20 μg of total RNA was fractionated by electrophoresis on 1% agarose gel containing formaldehyde and blotted onto a nitrocellulose membrane. The membrane blocked with denaturated salmon sperm DNA was hybridized with 32P-labelled RD29A cDNA specific probe at 42°C overnight. The membrane was washed with 0,1xSSC, 0,1% SDS at 60°C for 230 min. Ubiquitin was used to as control constitutive hybridization probe.

3. RESULTS

Construction and testing of reporter gene constructs

It was demonstrated [4] that the *RD29A::Luc* in transgenic *Arabidopsis* plants is strongly induced by low temperature, exogenous ABA or osmotic stress. The *ADH::Luc* reporter gene was previously constructed by *L. Szabados* and introduced into Arabidopsis (Col-0) by *Agrobacetrium*-mediated transformation.

Luciferase activity in several transgenic lines was highly responsive to salt, sugar, or ABA. To evaluate the activity of the test construct the *RD29A::Luc* and *ADH::Luc* seeds were germinated on MSAR seed medium and the two-weeks-old plants were tested for luciferase activity in the presence of exogenous ABA (fig.1).

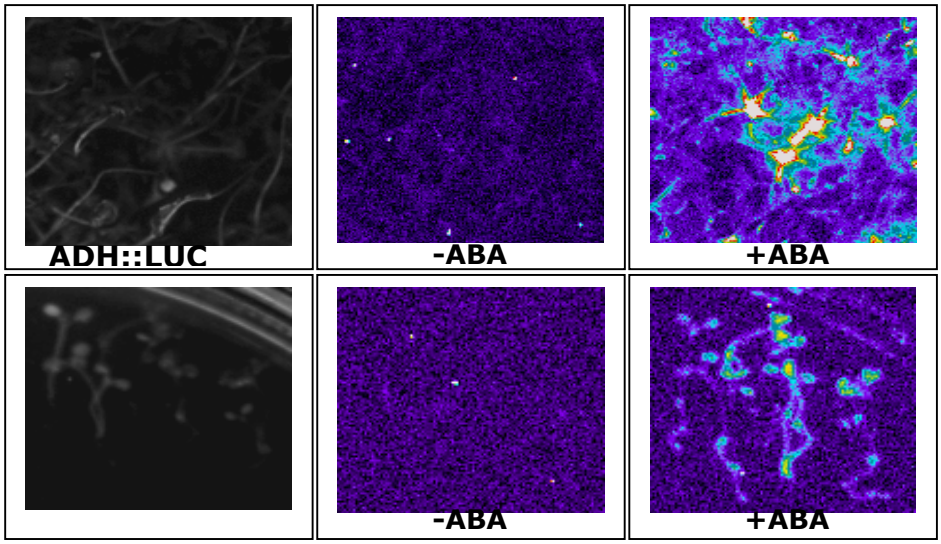

FIG.1. THE ACTIVITY OF THE TEST CONSTRUCTS IN THE PRESENCE OF EXOGENOUS ABA.

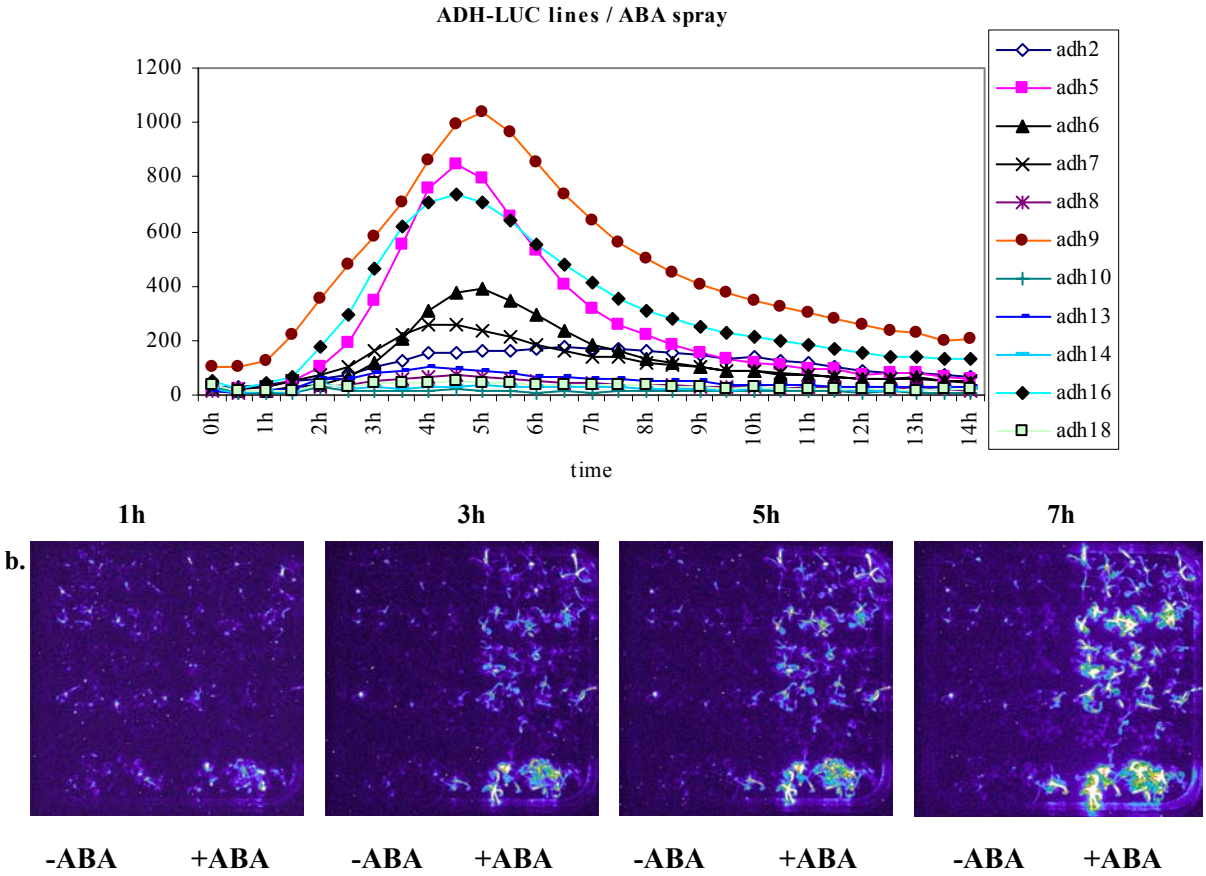

FIG.2. ABA-RESPONSIVE LUCIFERASE ACTIVITY IN 11 *ADH::LUC* LINES
a. The kinetics of ABA-responsive luciferase expression in *ADH::Luc* transgenic seedlings
b. The three-weeks-old seedlings were transferred to germinating media and sprayed with ABA. Luminescence was recorded in multiple time points immediately after spraying. The change in the luminescence is shown after 1h, 3h, 5h and 7h after treatment.

The ADH::LUC lines were also tested for ABA response by recording the luminescence every 30 min., after spaying with 50 μM ABA, for 14 h.

Several lines had increased luminescence activity upon ABA treatment. In response to ABA the luciferase activity was induced after 2 h and reached maximum after 4-5 hours. (fig. 2)

Screening for In Situ Luciferase Gene Fusion

Luciferase Activity of the **RD29A:Ac:Luc** transformants

Using *Agrobacterium*-mediated transformation several thousand transgenic plants were created by floral dipping. All hygromycin resistant T1 seedlings were screened for luciferase expression using bioluminescence imaging.

The seeds were germinated in 50 mgL^{-1} hygromycin MSAR liquid selective media for four days and then plated on solid hygromycin (15 mgL^{-1}) selective media.

Around 5000 hygromycin resistant two-weeks-old seedlings were tested for luciferase activity after spraying with 1mM D-Luciferin. The luminescence was recorded with during 10 min. exposure.

The plants were then sprayed with 2μM 17^{β} - estradiol solution and then subjected to luminescence imaging after 12 h. From 5000 T1 resistant several plants showed luminescence and one plant (named as **RD::Ac::Luc160** line) showed enhanced luminescence in the presence of estradiol (fig.2). These plants were selected and T2 generation was obtained.

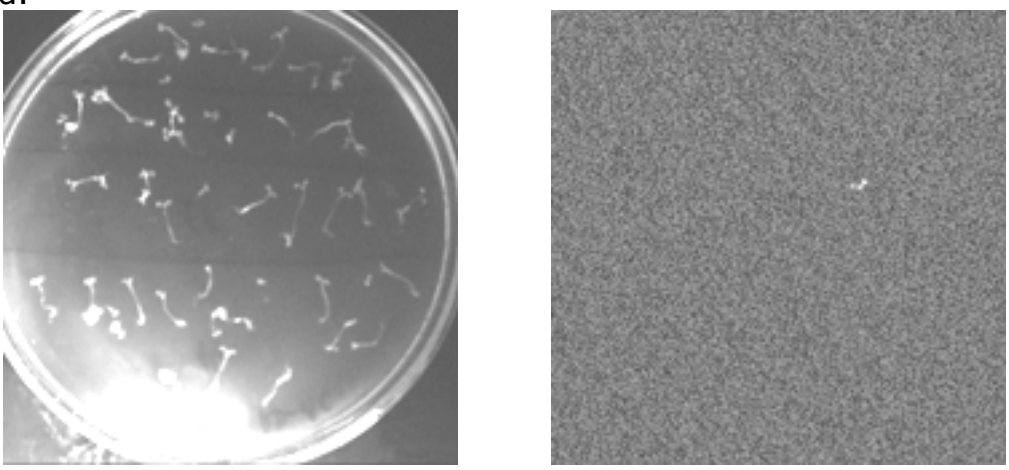

FIG.3. BIOLUMINESCENCE IMAGING OF THE T1 *RD::AC::LUC* SEEDLING AFTER 12H OF ESTRADIOL TREATMENT (BLACK ARROW POINTS THE SELECTED PLANT)

Large scale transformation

We performed a large scale *Agrobacterium*-mediated transformation of transgenic *Arabidopsis* line expressing the *RD29A::Luc* construct using a previously constructed Arabidopsis *cDNA* expression library which was introduced into *Agrobacterium.* We transformed almost 10.000 early flowering plants. These experiments are in progress and few putative lines were selected for further analysis.

Testing for In Situ Luciferase Gene Fusion of T2 lines

T1 plants were transferred into the greenhouse and T2 seeds were collected for further evaluation. In order to test luminescence in T2 plants,

the seeds were sterilized and germinated on MSAR medium, which contained 15 mgL^{-1} hygromycin and 200 mgL^{-1}claforan. 11 days-old seedlings were sprayed with estradiol and immediately the plants were tested for luciferase activity. Constitutive expression of the reporter gene was observed in the absence of the inducer. In the presence of estradiol we couldn't see any change in the lines tested (fig.4).

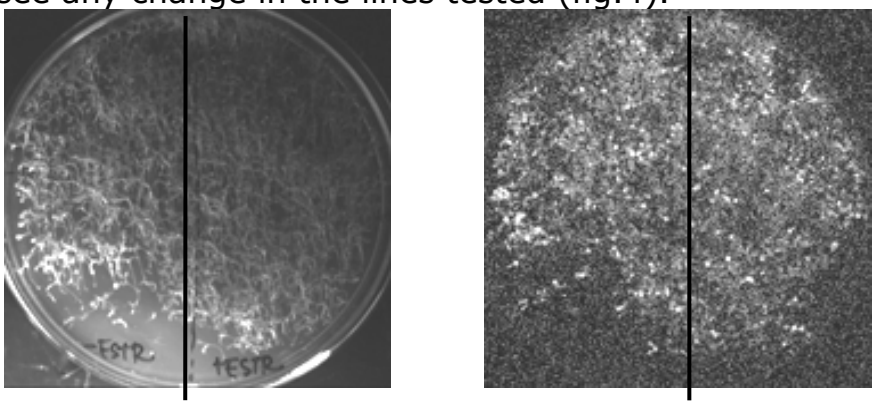

FIG.4. THE CONSTITUTIVE EXPRESSION OF *RD:AC:LUC 160* T2 SEEDLINGS IN THE PRESENCE OF ESTRADIOL AFTER 5 H OF TREATMENT. THE BIOLUMINESCENCE WAS RECORDED EVERY 30 MIN. FOR 15 H

Two *Arabidopsis* lines, expressing the *RD29A::Luc* construct, and transformed with the pTAc17 activation vector was analyzed in more detail. Both lines expressed the reporter gene construct in the absence or in the presence of stress or ABA. In order to test the transcription of the endogenous *RD29A* gene in these and several other putative mutants, Northern analysis was performed. Total RNAs was extracted and Northern blot performed with using radiolabelled *rd29a* specific probe. The results showed moderate increase of RD29A transcription in several transgenic lines (fig. 4).

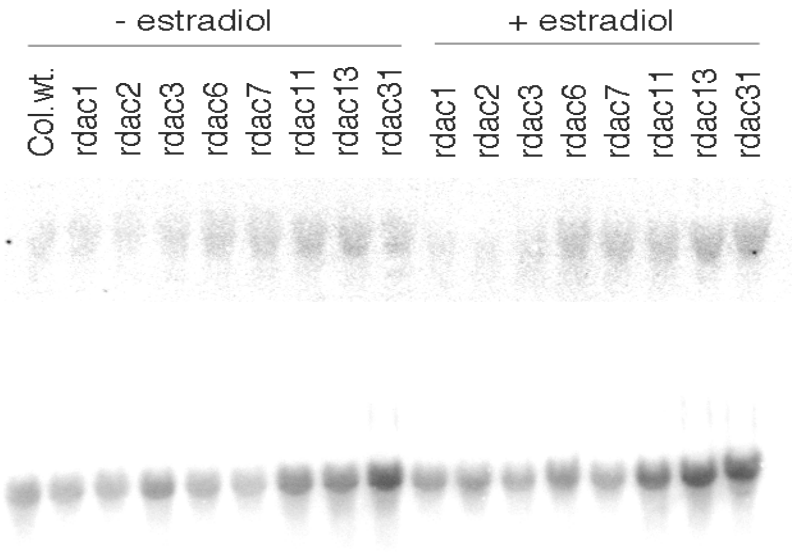

FIG. 4. RD29A TRANSCRIPTION IN SEVERAL TRANSGENIC LINES

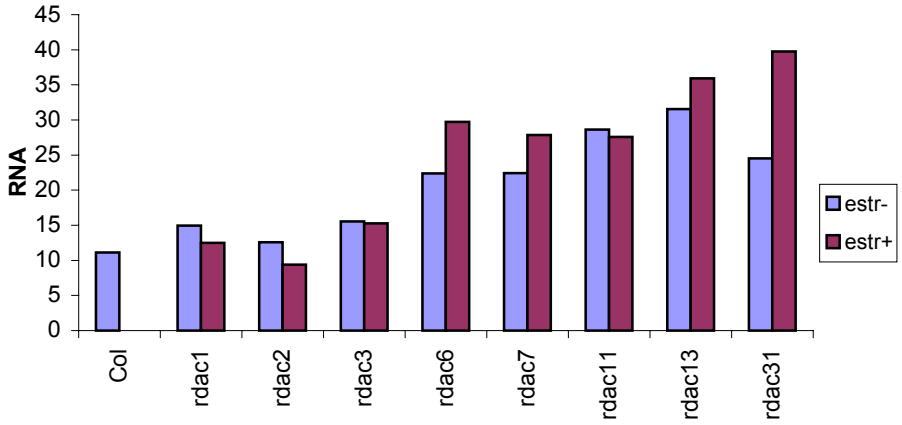

FIG. 5. THE QUANTITATIVE DATA ANALYSIS AFTER NORTHERN BLOT FOR *RD::AC::LUC* LINES

4. DISCUSSIONS

T-DNA has been broadly utilized for generating insertional mutant pools in *Arabidopsis*. For example, the *Arabidopsis* knockout facility at the University of Wisconsin has established a population of 60.480 T-DNA-taggeg lines. Session et al., [8] have reported the generation of 100,000 T-DNA transformed lines and Szabados et al., [9] have analysed the distribution of 1,000 T-DNA sequence tags isolated from their T-DNA insertion lines.

Activation tagging aproach is one method that complenents the tehnologies needed for studing genes whose function cannot be resolved by insetional mutagenesis becouse of gene redundancy, a lethal phenotype in loss of function mutants or phenotype expression only under specific conditions. The activation tagging system has several advanteges, first, because it gives dominant phenotype making possible to analyse function of duplication genes and, secondly, its mutant spectrum differes from that of loss-of-function mutants, often generating beneficial traits for crop improvement.

Using a T-DNA-based *luc* gene fusion vectors we generated several thousand transgenic plants to monitor the transient expression of stress-responsive genes. To date, LUC is the best reporter gene that is suitable for the analysis of temporal changes in gene expression. In most cases the system was used for the analyses of stress responses or circadian rhythm.

The advantage of LUC reporter is the possibility of real time monitoring of expression within the same plant, using non-destructive bioluminescence imaging [12]. The plant material is not necessary restricted to seedlings, but, detached flowers, roots, leaf or cultured cells can be assayed as well. In this way environmental responses can be monitored including those of light, salt, drought, pathogens or hormones [1] Using activation tagging 10.000 T1 plants were tested, and 4 lines selected for further analysis. The tests on *RD:Ac:LUC 160* are in progress.

Our analyses using different types of vectors, which carry the *FFluc* reporter gene, demonstrated the applicability of the system. Using the *FFluc* reporter gene one has the ability to screen in the T1 generation without killing the plants. An important application of luciferase system is the identification of conditionally regulated plant promoters and creation of molecular markers for the analysis of specific environmental responses.

Our results confirmed that the luciferase reporter system can be exploited for the identification and tagging of genes, the expression of which is positively or negatively regulated by ABA, salt or osmotic stress.

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