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TRANSFORMATION EFFECTIVENESS FOR *Arabidopsis thaliana* PLANTS BY DNA-CONSTRUCTIONS WITH SITE-SPECIFIC RECOMBINASE SYSTEM CRE/*loxP*

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Using of new approach with site-specific recombinase system Cre/*loxP* under the control of 35S-promoter to generate marker-free genetically modified plants was developed. The analysis of recombinase system was carried out during the next generation of *Arabidopsis thaliana* plants, produced by agrobacterium transformation method. For this purpose two types of DNA-constructions were used for establishing better variant. The histochemical analysis of the plants progeny T_1 transformed by both construct types was described. As a result of our work, it was established that the amount of marker-free transformants was arising during every next transformation offspring independently of the used construct type. The new strategy provides a simple and rapid way to eliminate swelective and marker genes.

Key words: site-specific recombinase system Cre/*loxP*, marker genes excision.

In our days the producing of marker-free transgenic plants are paying big attention because the genetically transformation process requires presence of the antibiotics or herbicides resistance genes in plant genome. The presence of these kind of genes in commercial crops is raising public concerns. Also, releasing of the genetically modified organisms into environment has raised concern about possible leaking of antibiotic or herbicide resistant genes into the wild relatives or weeds genome. Therefore, the development of genetic engineering methods for prevention of drain the selectable marker genes (SMG) into the environment might lead to the changing of public concern about genetically modified organisms (GMO) as well as to simplify the process of commercialization. According to Puchta [1], several strategies have been developed to generate marker-free transgenic plants. Among these strategies, the site-specific recombination technology is the best characterized. During the last decade this systems has been developed either in eukaryotic or prokaryotic cells. The efficiency of this approach in plant biotechnology for the first time was demonstrated for tobacco plants, transformed by site-specific recombinase system Cre/*loxP* [2]. Besides recombinase Cre other site-specific system were used also, like FLP/*frt*-system from *S. cerevisiae* [3], the R-RS system of the pSR1 plasmid of *Z. rouxii* [4], phiC3 system from Streptomyces phage [5, 6], Gin-*gix* system from bacteriophage Mu [7], CinH-RS2 from *Acetinetobacter* [8] and ParA of the parCBA plasmid operon [9].

Site-specific recombination occurs in specific recognition site of DNA and brings to excision or ligation of the sequence ends through the integration event [10, 11]. The X-ray analysis of the crystal structure for some site-specific recombinases with their DNA targets has shown an interaction between their complexes [12]. Recognition sites for these recombinases are palindromes witch flanks 6–8 base pairs (b.p.) and inverting repeats 12–13 b.p. for recombinase binding. The skewness of the inverting repeats is used as a vector for direction of recombinase activity. Currently, Cre/*loxP* has become the best-described site-specific recombination system from the bacteriophage P1. It belongs to the tyrosine integrase family [10]. The system consists of two short DNA recognition sequences known as *lox* (locus of crossingover) and the *cre* gene. In the *E.coli* genome this system performs the transformation of two plasmids from P1 into the monomeric structure by the recombination event between two *lox*-sites. The activity of Cre recombinase

does not require additional proteins or cofactors [13] that simplify the employment of this system in genetic engineering. Since the transient expression of Cre has occurred, it leads to DNA-autoexcision between the *loxP*-limited sequence (for example, selective marker genes) in plant genome. The transient protein expression in transformed plants is initiated by specific inducible or tissue-specific promoters. Hence, the development and improvement of site-specific recombinase system technic for plant transformation provides the chance to integrate T-DNA into genome during the one step [13]. Thus, the site-specific recombinase system under the control of heat-shock promoter was constructed for induction of the recombinase protein expression with further excision of *loxP*-limited DNA sequences. The efficiency of this approach was described for *Arabidopsis* transformation [14], tomatoes [15], maize [16], tobacco [17] rice and aspen [4]. As an alternative to heat-shock promoter that is used for regulation of T-DNA expression is an inducible promoters that have been identified in embryo and other tissues. Activation of the protein expression under their control occurs in certain organs or tissues during the plant growth [18, 19].

We have developed the new approach with using of site-specific recombinase system Cre/*loxP* under the control of 35S promoter of CaMV to produce marker-free genetically modified plants [20]. This new system provides a rapid and efficient way of transformation without any treatment or presence of special conditions during plant growth. Hence, two types of one vector construction was developed for transformation of *Arabidopsis thaliana* plants to examine the better variant.

The aim of our study is to investigate the efficiency of developed DNA-constructions with site-specific recombinase system Cre*loxP* for transformation of *Arabidopsis thaliana* (L.) plants ecotype Columbia. Stability of the T-DNA expression and efficiency of recombinase Cre were analyzed in T_1 transformants progeny.

Materials and Methods

The analysis of transformation stability was obtained on the next transformants generations. For the plant transformation was used the floral dip *Agrobacterium*-mediated method [21]. In this aim we developed two types of DNA-construction — pORE-lox1HGC and pORE-lox2HGC (Cre/*loxP* 1 Cre/*loxP* 2). The difference between these two constructions is in the order of genes on expression cassette. As a positive control was used vector construction without gene *cre* and excision sites. Both DNA-constructions consist of *cre* recombinase gene, reporter gene *gus*, *nptII* gene flanked by *loxP*-excision sites and puts under control of 35S promoter and nopalin-synthase (*nos*) terminated sequence. The hygromycin resistant gene *hptII* in both constructions was carried out of the *loxP* sites and in case of excision event stays in plant genome.

Obtained seeds from each transformed plant were considered as an independent line of transformants. From a pool of GM plants were estimated 60 lines (T_1 progeny) transformed by every construction type to analyzed the transformation stability. Harvested seeds were sterilized by sodium hypochlorite and hydrochloric acid mix as 3:1 for 4 h. After sterilization step the further germination was obtained on selective MS medium [22] with 100 mg/L hygromycin, 1 g/l casein hydrolysat, 690 mg/l L-proline, 5 mg/l thiamine-HCL, 30 g/l maltose, 3,5 g/l gelrite and 2 mg/l 2,4-D, pH 5,6–5,8. Progeny seeds have grown at 24–26 °C under a 16-h-light photoperiod for the next 10–12 days. Thereafter, from a pool of shoots for each line was calculated amount of the resistant and sensitive plants to estimate the effectiveness of the transformation event.

The excision event in plant genome and estimation of the Cre/*loxP* system activity for each type construction was obtained for shoots that grew on hygromycin-containing medium by histochemical analysis via GUS-activity. Whereas the *gus* gene was put in *loxP*-limited sequence, the negative GUS staining indicates the recombinase excision event. Detection of *gus* gene expression was obtained according to the standard protocol [23]. Eight antibiotic resistant samples from each line were selected with further transferring to the buffer: 50 mM $NaPO_4$, pH 7,0, 10 mM Na_2EDTA, 0,1% triton X-100 and 1 mg/l 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). The incubation was carried out at 37 °C for 4 h. After incubation step samples were decoloured with 50% ethanol with further conserving in 70% ethanol at 4 °C. Thereafter, GUS-negative or chimeric plants were transplanted in soil. Eight plants from selected lines have grown in soil at greenhouse conditions for the next 21–28 days. During their growth histochemical analysis of plant tissues was obtained for estimation of GUS-activity. In aim to examine GUS-test results was carried out PCR-analysis according to the protocol, described in [21].

The statistical data analysis was obtained by Microsoft Exel program. All studies were carried out in three repeats. The difference between two average values was reliable at $P < 0,05$ (*).

Results and Discussion

One of the problems to generate transformated plants is in maintaining of the stable expression for foreign genes during the further generations. The expression of foreign genes may partially or completely disappear from the progeny genome. Hence, the stability of transformation through the generations depends on many factors such as a suitable design of DNA-construction. Thereby we used 35S promoter to provide suitable expression level of T-DNA in transformants genome. Previously, in a number of works has been described the using of site specific recombinase system Cre/*loxP* under the control of tissue-specific or heat-shock promoters. Particularly, the regulation of Cre recombinase gene expression has been accomplished either using an embryo tissue promoter [24] or inducible heat-shock promoter HSP81-1 [25]. The limitation such of sequences however, requires to provide specific conditions for transformation event or to attract the additional agents to initiate the expression of recombinase protein. Thus, the using of 35S promoter would simplify the design of developed constructions. In addition, 35S promoter and *nos*-terminator sequences are reliable constitutive regulation system that uses to control the expression of T-DNA in plant genome. In the work, presented by Kim et al. [26] was described the vector construction with site-specific recombination system Cre/*loxP* under control of 35S promoter and *nos*-terminator to generate transformed *Arabidopsis* plants. According to the vector construction, sequence of *gus* gene was separated and carried out of the *loxP* sites. Aim of this approach is in the junction of *gus* sequence after the excision event completed with the next GUS-expression. Thereby, expression of *gus* gene suggests about expression of recombinase protein. However, after this approach for the development of marker-free plants is obtained, the main problem is in further presence of the selective marker gene (like *gus* gene) in the plant genome. Moreover, the separation of gene sequence in this way would lead to the gene silence that is one of the issues for plant transformation technology. Here, in present approach all selective marker genes are removed completely by site-specific recombination system Cre/*loxP*.

In present work as a gene of interest that carried out from *loxP*-excision sites is hygromycin resistant gene *hptII*. Harvested seeds from *Arabidopsis* T_1 progeny were grown hygromycin-containing medium with further selection of transformed plants from non-transformed. The calculation of sensitive and resistant shoots for each line has been accounted on 10–12 day after their germination (Fig. 1). *Arabidopsis* plants resistant to antibiotic showed no visible phenotype in comparison with wild-type plants (Fig. 2).

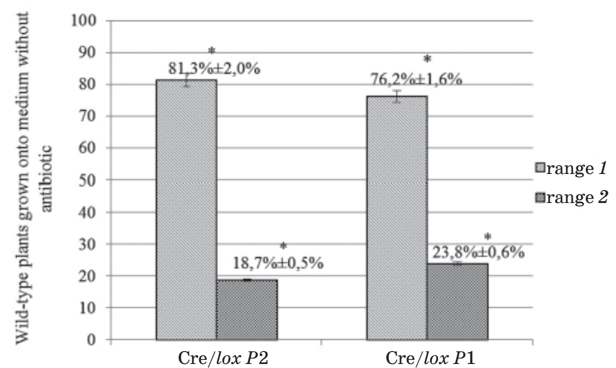
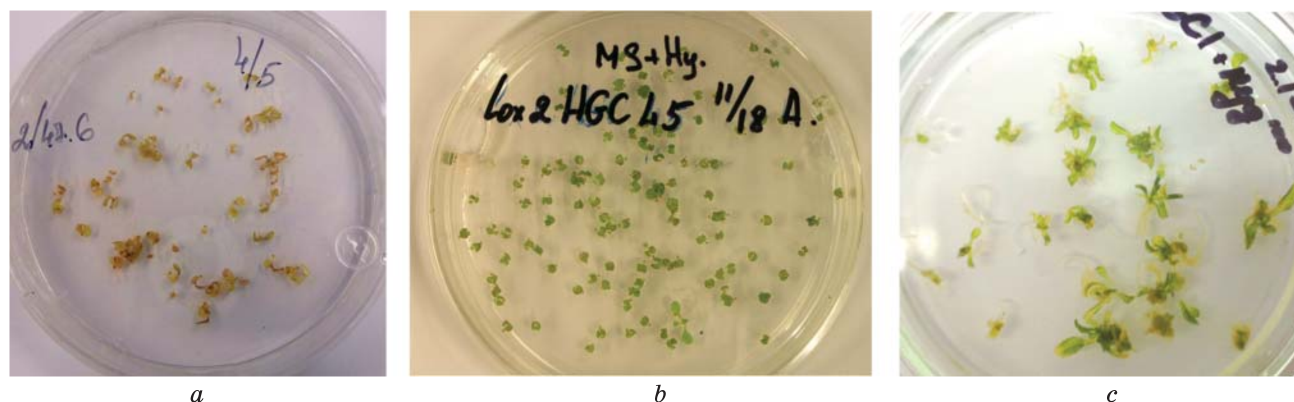


Fig.1. **The average ratio of resistant and sensitive to the antibiotic plants (vertical,%) for every T_1 generation line, harboring the vector constructs Cre/*loxP* 1 and Cre/*loxP* 2:**
range *1* — the rate of antibiotic resistant plants;
range *2* — the rate of antibiotic sensitive plants.
As a control were used wild-type plants that grown on non-selective medium (100%).
Here and below: * $P < 0,05$

Seeds germination on selective medium has shown a high frequency of transgenic lines T_1 progeny transformed by both types of DNA-construction. Ratio of plants harboring the Cre/*loxP*1 construct and resistant to the selective agent was higher in comparison with plants, transformed by Cre/*loxP*2 construct. Total ratio of the antibiotic resistant plants harboring Cre/*loxP*1 is 81,3% and plants with Cre/*loxP*2 is 76,2%. The total ratio between resistant and sensitive to selective agent plants for each transformants line has been counted separately (total amount is 60–70 seedlings per line).

Hystochemical analysis of GUS-activity was carried out for each line on 8 samples of T_1 transformed *Arabidopsis* plants that were



a *b* *c*

Fig. 2. ***Arabidopsis thaliana*** **transformed plants T_1 generation that grown on selective medium MS with hygromycine:**
a — non-transformed plants, wild type; *b* — sensitive to the selective agent plants; *c* — antibiotic resistant plants

grown on selective medium. The results of GUS-test for transformed plants were split on three groups: the first group — GUS-positive stained lines (completely stained); the second group — positive and negative GUS-stained samples per line; third – lines with negative stained samples (Fig. 3). According to the results, lines that exhibited a positive GUS-staining pattern indicated the presence of particular stained samples (Fig. 3, *b*). Thus results denote on chimeric plants. Hence, excision event of *loxP*-limited sequences with *gus* gene from genome was obtained completely in certain locations or specific plant tissues.

According to this analysis data we can assume that the using of both developed DNA-constructions with site-specific recombination Cre/*loxP* for plant transformation is successful. Moreover, it is indicated that the using of 35S promoter provides stable expression of marker gene on high level. Since marker gene sequences put in the *loxP*-limited location as well as an enzyme sequence we might suggest about the equal expression level for *gus* and *cre* genes. According to the results of GUS-test, 25% of lines transformed by DNA-construction Cre/*loxP* 1 indicating that they most likely were marker-free, and 12% lines showed GUS-positive and GUS-negative stained samples. The ratio of lines harboring vector construction Cre/*loxP* 2 with auto-excision was estimated in 15%. Lines, transformed by Cre/*loxP* 2 construct have shown the ratio of GUS-positive and GUS-negative samples in 13% (Fig. 4). Previously obtained histo-chemical analysis for T_0 transgenic plants indicated the increasing of negative GUS stained lines in about 5–7% transformed by both types of DNA-constructuion [21]. Chimeric staining of transformed tissues detects the excision event of marker genes eather.

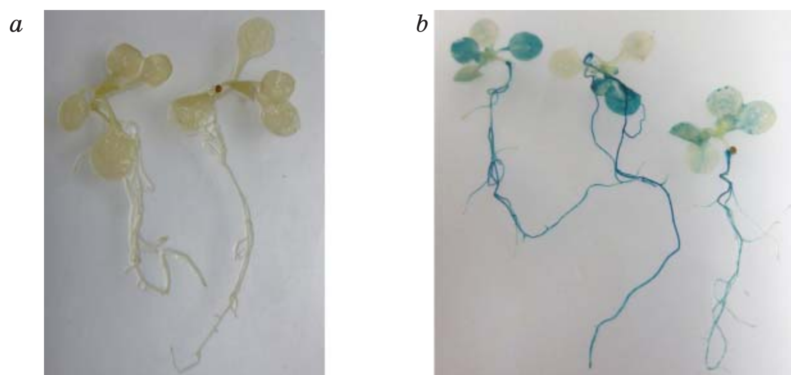


Fig. 3. **The expression of gus gene in** ***Arabidopsis*** **shoots of T_1 progeny transformed by vector constructions Cry/*loxP*1 and Cry/*loxP*2:**
a — GUS-negative stained samples; *b* — chimeric plant with particular GUS-stained result

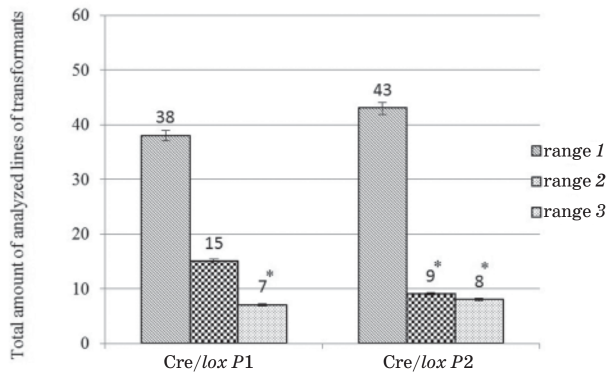


Fig. 4. **The results of histochemical analysis for the expression of gus gene in *A. thaliana* lines T_1 progeny that were transformed by vector constructions Cry/*loxP*1 and Cre/*loxP*2:**

range *1* — lines with GUS-positive results (control);

range *2* — lines with positive and negative GUS-stained samples;

range *3* — lines with GUS-negative results

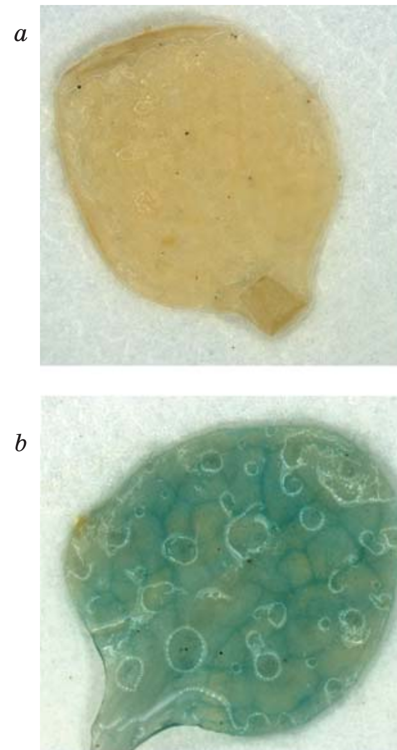


Fig. 5. **Histochemical analysis of gene gus expression in transformated *A.thaliana* plants during their growing in the soil:**

a — absence of gene *gus* expression in leaves;

b — *gus* gene expression

The plant growth might lead to the gene silencing. In general, this phenomenon occurs during the nuclear DNA transformation and might be explained by multi-copying insertion of T-DNA into genome, methylation of the introduced DNA, duplexes of repeated genes, etc. [27]. One of the features of this phenomenon is the low expression level of integrated genes, weak development and growth of plant. However, during our experiment the germination of plants has been admitted at a high level. The expression of marker gene was estimated by GUS staining. Moreover, the Cre recombinase activity and the number of excision events may be associated with germination of transformants onto selective medium. Therefore, plants were transferred to the soil in order to avoid the gene silencing for further studying. In this aim were selected lines that showed the partially or completely negative GUS staining results after germination onto selective medium. After a period of plants cultivation in soil was performed analysis to estimate *gus* expression. Histochemical analysis was accomplished using plant leaves (Fig. 5). According to the results, appearance of new lines with completely negative GUS-test was not detected. Only individual plants within some lines have shown the absence of *gus* expression (Fig. 5, *a*) or weak expression of β-glucuronidase on low level. To avoid the gene silencing event and confirm the presence of T-DNA in the plant genome of GUS-negative stained samples we performed molecular-genetic analysis using PCR [21].

Thereby, it has been shown the effective using of the new approach to obtain marker-free plants and the stable expression of the T-DNA to the next generation of transformants. For transformation event were used several types of DNA-construction developed for a new approach with aim to produce genetically modified marker genes plants. Along with determining of the effectiveness of developed approach was performed analysis for recombinase system activity within each type of DNA-constructions. The stable expression of T-DNA in the plant genome for both types of constructions was estimated using histochemical analysis for T_1 transformants. Some transformants with GUS-positive result have shown the inhomogeneous expression of *gus* gene in different plant tissues that indicating their chimeric. Some lines showed completely negative GUS staining result that suggesting about Cre recombinase activity and excision of the marker gene within the *loxP* sites. It is determine that the number of marker-free *Arabidopsis* lines is increasing with each new generation of plants. Hence, developed approach for the using of site-specific recombinase Cre is effective to produce marker-free plants.

Moreover, the using of DNA-constructions without tissue-specific promoters as well as their expression does not depend on the environmental conditions, greatly simplifies the process of transformation and increases obtaining of the regenerants. This approach is simple in using and allows the obtaining of transformed marker-free plants.

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ЕФЕКТИВНІСТЬ ТРАНСФОРМАЦІЇ *Arabidopsis thaliana* ДНК-КОНСТРУКЦІЯМИ З РЕКОМБІНАЗНОЮ СИСТЕМОЮ CRE/*loxP*

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Розроблено новий підхід у застосуванні сайт-специфічної рекомбіназної системи Cre/*loxP* під контролем 35S-промотору для одержання генетично модифікованих рослин без маркерних та селективних генів. Аналіз рекомбіназної системи здійснювали впродовж наступного покоління рослин *Arabidopsis thaliana*, виділених за допомогою агробактеріальної трансформації. Для генетичної трансформації було використано два типи ДНК-конструкцій з метою визначити більш доцільний варіант. Наведено результати гістохімічного аналізу рослин покоління T_1 трансформованих обома варіантами конструктів. Встановлено, що кількість трансформантів, які не містять маркерних генів, збільшується з кожним наступним поколінням незалежно від варіанта трансформувальної конструкції. Запропонований підхід є простим у застосуванні та потребує мінімальних затрат часу.

Ключові слова: сайт-специфічна рекомбіназна система Cre/*loxP*, видалення маркерних генів.

ЭФФЕКТИВНОСТЬ ТРАНСФОРМАЦИИ *Arabidopsis thaliana* ДНК-КОНСТРУКЦИЯМИ С РЕКОМБИНАЗНОЙ СИСТЕМОЙ CRE/*loxP*

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Разработан новый подход в применении сайт-специфической рекомбиназной системы Cre/*loxP* под контролем 35S-промотора для получения генетически модифицированных растений без маркерных и селективных генов. Анализ рекомбиназной системы осуществляли в течение последующего поколения растений *Arabidopsis thaliana*, полученных с помощью агробактериальной трансформации. Для генетической трансформации были использованы два типа ДНК-конструкций с целью определения более целесообразного варианта. Приведены результаты гистохимического анализа трансформантов поколения T_1, трансформированных обоими вариантами конструктов. Установлено, что количество трансформантов, не содержащих маркерных генов, возрастает с каждым последующим поколением независимо от варианта трансформирующей конструкции. Предложенный подход прост в использовании и требует минимальных затрат времени.

Ключевые слова: сайт-специфическая рекомбиназная система Cre/*loxP*, удаление маркерных генов.