УДК 577.21:582.926.2

N. SHCHERBAK, O. KISHCHENKO, L. SAKHNO, I. KOMARNYTSKY, M. KUCHUK

Institute of Cell Biology and Genetic Engineering NAS of Ukraine, Kyiv
E-mail: natasha@iicb.kiev.ua

Lox-DEPENDENT GENE EXPRESSION IN TRANSGENIC PLANTS OBTAINED VIA *AGROBACTERIUM*-MEDIATED TRANSFORMATION

Lox sites of the Cre/lox recombination system from bacteriophage P1 were analyzed for their ability to affect on transgene expression when inserted upstream from a gene coding sequence adjacent to the right border (RB) of T-DNA. Wild and mutated types of lox sites were tested for their effect upon bar gene expression in plants obtained via Agrobacterium-mediated and biolistic transformation methods. Lox-mediated expression of bar gene, recognized by resistance of transgenic plants to PPT, occurred only in plants obtained via Agrobacterium-mediated transformation. RT-PCR analysis confirms that PPT-resistant phenotype of transgenic plants obtained via Agrobacterium-mediated transformation was caused by activation of bar gene. The plasmid with promoterless gus gene together with the lox site adjacent to the RB was constructed and transferred to Nicotiana tabacum as well. Transgenic plants exhibited GUS activity and expression of gus gene was detected in plant leaves. Expression of bar gene from the vectors containing lox site near RB allowed recovery of numerous PPT-resistant transformants of such important crops as Beta vulgaris, Brassica napus, Lactuca sativa and Solanum tuberosum. Our results demonstrate that the lox site sequence adjacent to the RB can be used to control bar gene expression in transgenic plants.

Introduction. Designing plants with a required expression pattern of transgenes remains one of the major problems of plant biotechnology. A variety of regulatory elements is necessary in all areas of plant genetic engineering, from basic researches to development of economically valuable crops. Development efforts ever more have focused on the use of tissue-specific or inducible promoters to control expression of the gene of interest [1, 2]. Nevertheless, the successful selection of transgenic plants requires strong constitutive promoters and traditionally regulatory elements derived from non-plant sources (actually in most cases from plant pathogens) are often used. For a number of vectors used for plant transformation nowadays such as pPZP [3] and pCAMBIA derivatives (http://www.cambia.org/daisy/cambia/materials/vectors.html), as well as the pCGN [4] 35S promoter from cauliflower mosaic virus (CaMV) has been used to drive the selective marker genes. Though, it has been found that expression of transgenes under control of 35S promoter was unstable under field condition because of naturally occurring CaMV infection and homology depended gene silencing resulting from their interaction [5]. CaMV 35S enhancers can influence the expression of nearby genes [6] that could lead to the loss of tissue-specific expression of transgenes controlled by appropriate promoter [7, 8]. In addition, the repetitive use of the same promoter is known to induce transgene inactivation due to homology of the sequences [9–11]. One of the approach to escape these problems is to create a synthetic promoter [12, 13]. Thereby, alternative regulatory sequences with little sequence similarity either to the plant pathogens or to extensively used promoters, are going to be available sources as building blocks for the promoter engineering.

Lox site sequences used in our experiments were initially selected for recombination events in transgenic plant. Since the introduction of the Cre/*lox* system from bacteriophage P1 into plant genome [14] it has become the best characterized and the most widely used recombination system in both commercial plant biotechnology and basic plant research [15]. The Cre/*lox* recombination system has been used to induce chromosomal rearrangements [16, 17], to insert foreign genes precisely into a pre-existing sites [18] and it has also been used as the method for producing marker free transgenic crops [19–23]. Though, to our knowledge, none of recombination sites has been functionally tested in plants for their ability to influence transgene expression. In our research we found that DNA sequence of *lox* site from bacteriophage P1 promoted gene expression when placed adjacent to the right border (RB) in the proximal upstream region of gene coding sequence. Genome DNA sequences which normally do not function as gene regulatory elements and become activated as promoter when

placed in a new context adjacent to gene referred to as cryptic [24–26]. By analogy with cryptic gene regulatory elements *lox* site sequence confers strong constitutive expression of a transgene under certain conditions only, notably, placed adjacent to the RB in the proximal upstream region of gene coding sequence. Previously we described the use of vectors containing *lox* site between the RB of T-DNA and promoterless *bar* gene for obtaining PPT-resistant transgenic plants [27, 28]. After the initial demonstration of the feasibility of *lox*-mediated expression of *bar* gene we used set of vectors for the further investigations. The vectors with promoterless *gus* gene were constructed too. The potential benefit of *lox*-mediated expression for crop species transformation has been studied by example of *Beta vulgaris, Solanum tuberosum, Lactuca sativa* and *Brassica napus*. We have finally demonstrated that *lox*-mediated expression provides an effective alternative for at least *bar* gene expression in transgenic plants from a wide range of plant species.

Materials and methods. *Vector constructions.* Plasmid vectors pICH3737, pCBV19, pICH3744, pICH9393, pICH9414, pICH9702 and pICH3831 were generously donated by «Icon Genetics GmbH» (Germany). Vector pICH3737 includes *bar* gene coding region without promoter near RB between *loxA* and *loxM* sites. Construct pICH9393 is the pICH3737 derived vector that contained wild type of *lox* site *lox*P instead of *lox*A and *nos* terminator between *lox* site and RB. Plasmids pICH3744 and pICH9414 were the same as pICH337 and pICH9393, respectively, except that the additional *lox* site in a direct orientation was introduced in the constructs. All plasmid vectors held *nptII* gene under control of *nos* promoter as well. Constructs pICH9702 and pICH1567 had -*lox*-*bar*- located internally in the T-DNA and differed with the wild type of *lox*P sites in pICH9702 and mutative *lox*A sites in plasmid pICH1567. Also we used the vector pICH3831 containing promoterless *bar* gene adjacent to the RB (without *lox* site).

Plasmid pCB100 contained *loxP* site between RB promoterless *gus* gene coding sequence. The plasmid was derived from pICH9414 by substitution of *bar* gene coding region for *gus* gene (RB-*lox*-*gus*-). The *gus* gene fragment had previously been digested with *SacI* and *PstI* and inserted into the vector pICH9414 which was incurred a treatment of same restriction enzymes.

Plasmid pCB108 was the pICH3737-derived vector and contained *bar* gene coding sequence with a *loxA* site located upstream without *nptII* gene (deletion in *XhoI*/*EheI* fragment). Plasmid pCB148 contained AT rich region between RB and promoterless *bar* gene. The plasmid was derived from pCB108 by substituting *lox*A site (*BamHI*/*Ecl136II* restriction) for the spacer between tobacco plastome genes: *petB* (gene of cytochrome b_6/f complex) and *psbB* (gene of photosystem II). Plasmid pCB164 contained *bar* gene coding region near LB with the *lox*A between *bar* gene and LB (LB-*lox*-*bar*). This vector also contained -*lox*-*gus*- gene located in the middle of T-DNA.

Obtained plasmids were transformed into *Agrobacterim tumefaciens* strain GV3101 and resulting bacteria were used for plant transformation.

Plant transformation and growth condition. For *Agrobacterium*-mediated plant transformation experiments aseptic plant or seedlings were used. Aseptic plants were grown at 25 °C (potato at 23 °C), 16 h photoperiod under 4000 *lx* light intensity. Transformation was accomplished using *Agrobacterim tumefaciens* strain GV3101 containing the indicated construct. *Agrobacterium* cultures were grown 48 h in LB medium with appropriate antibiotics (100 mg/l carbenicillin, 50 mg/l rifampicin, 25 mg/l gentamicin) at 28 °C in dark. The resulting cultures were independently centrifuged at 4000 *g* for 15 min and resuspended in the equal volume of cocultivation media supplemented with 0.2 mM acetosyringone. Obtained bacterial suspensions were cultivated for 1 h on rotary shaker and than used for plant genetic transformation.

Agrobacterium-mediated transformation of *Nicotiana tabacum* (cv. Petit Havanna Ottawa) was carried out using the conventional leaf disc method. Tobacco leaf discs were incubated with *A.tumefaciens* containing binary vector for 24 h at 26 °C in dark. Infected discs were transferred to regeneration medium (MS medium [29] supplemented with 1 mg/l BA, 0.1 mg/l NAA, 7 g/l agar, 25 g/l sucrose) containing 400 mg/l cefotaxime and 100 mg/l kanamycin for selection. Kanamycin-resistant shoots were rooted on selective MS medium with 100 mg/l kanamycin. In the groups of about thirty transformants transgenic plants were tested for PPT-resistance on the selective medium containing 5 mg/l PPT.

For biolistic transformation of tobacco the plasmids were prepared from amplified *E. coli* XL-blue cultures using the QIAGEN Plasmid Maxi Kit.

To absorb DNA to the microprojectiles (Tungsten M-17 Microcarriers, 1.1 μm, BioRad) 10 μl of DNA (1 μg/μl) was added to 50 μl of a suspension of tungsten particles (0.06 mg/ml in 50 % glycerol) in a 1.5-ml Eppendorf tube. After DNA addition of the DNA, 10 μl of PEG/$MgCl_2$ solution (50 % PEG 2000, 5 M $MgCl_2$) was added to the suspension. After 20 min of incubation at room temperature the particles were pelleted by centrifugation in a Microfuge for 30 s, and the supernatant was removed. The final microprojectile preparation was resuspended in 60 μl of absolute ethanol. Leaves were placed abaxial side up on MS medium supplemented with 1 mg/l BA and 0.1 mg/l NAA and bombarded in 14 cm from the end of the barrel of the particle gun. The pressure in the sample chamber was reduced to 0.05 atmospheres prior to bombardment. In all experiments, ten Petri plates were bombarded per construction.

Seeds of diploid O-type sugar beet (*Beta vulgaris* breeding lines KS 3, KS 7, SC 01733, SC 023-2 and SC 03441) were provided by Institute for Sugar Beet Research of the Ukrainian Academy of Agrarian Sciences (Kyiv, Ukraine). Prior to surface sterilization sugar beet seeds were incubated at +4 °C for a week then soaked in water at the room temperature overnight. Seeds were surface sterilized in 40 % (v/v) formalin for 2 min, transferred to 70 % ethanol for 30 s, treated with 30 % (v/v) bleach (1.5 % sodium hypochlorite) for 20 min and washed 3 times for 10 min in autoclaved distilled water. Seeds were germinated on MS medium containing 15 g/l sucrose and 2 mg/l BA at 22 °C in the dark. Genetic transformation of sugar beet was carried out as previously reported [30]. *Agrobacterium* culture resuspended in MS medium supplemented with 20 g/l sucrose, 2 g/l glucose, 2 mg/l BA and 0.2 mM acetosyringone was used for vacuum infiltration of sugar beet etiolated derooted seedlings. After vacuum-infiltration, seedlings were transferred to sterile filter paper and incubated in the dark at 22–24 °C for 3 days. Then the seedlings were cut into 0.7–1.0 cm pieces, incised and placed on MS medium containing 15 g/l sucrose and 2 mg/l BA. Within 6–8 weeks a friable callus has arisen from cotyledons and hypocotyls. Kanamycin-resistant callus was isolated and further cultivated on regeneration medium composed of MS basal salts, 30 g/l sucrose, 29 mM silver thiosulfate, 0.5 g/l polyvinil pirrolidone, 1 mg/l BA, 0.3 mg/l IAA and 0.4 mg/l gibberellic acid, 300 mg/l cefotaxime and 100 mg/l kanamycin. Callus cultures were cultivated at 24 °C under scattered light and 16h photoperiod with 3 weeks subcultivation period. Shoot regeneration occurred within 4–10 weeks. Selected shoots were transferred to MS medium with 100 mg/l kanamycin for root formation.

Aseptic plants of potato (*Solanum tuberosum* L. cv. Lugavskoj) used for genetic transformation were provided by Institute for Potato Research of the Ukrainian Academy of Agrarian Sciences (Nemeshaevo, Ukraine). Internodes of propagated *in vitro* potato plants were cut into about 0.8 cm fragment and pre-cultivated on MS medium supplemented with 40 mg/l adenine hemisulphate, 2 mg/l Dicamba and 0.5 mg/l BA for a week. The explants were vacuum-infiltrated with bacterial suspension. After three-day cocultivation the explants were washed, blotted with sterile filter paper and transferred on MS medium supplemented with 25 g/l sucrose, 29 mM silver thiosulfate, 0.5 g/l polyvinil pirrolidone, 40 mg/l adenine hemisulphate, 200 mg/l, 1 mg/l zeatin, 2 mg/l gibberellic acid 500 mg/l cefotaxime and 100 mg/l kanamycin. Shoots regenerated within 6–8 weeks.

For canola genetic transformation (*Brassica napus* varieties Kalinovskij, VNIS-100) the leaves of 3–4 weeks aseptic plants were used. The explants were placed on MS medium containing 2 mg/l 2,4-D, 1 mg/l NAA, 0.1 mg/l BA, 0.1 mg/l kinetin and 1 g/l sodium thiosulfate as an agent increasing the susceptibility of the plant tissues to *Agrobacterium* and positively affected transformation efficiency [31]. After 3-day pre-cultivation explants were soaked in the *Agrobacterium* culture for 20 min and placed on the same medium for callus formation. The explants with callus were then transferred to MS-basal medium supplemented with 2 mg/l BA, 1 mg/l zeatine, 1 mg/l NAA, 1 mg/l gibberellic acid, 1 mg/l abscisic acid and 5 mg/l PPT for shoot development. Green shoots formed in 3–4 weeks were replaced on a hormone-free MS medium complemented with 5 mg/l PPT. Regenerated plants were grown on MS medium with 10 mg/l PPT and root development occurred under these conditions without any additional initiation factors.

Cotyledon explants of lettuce (*Lactuca sativa* cv. Odeskij kucheriavij) were obtained after seed surface sterilization seeds in 50 % Domestos (com-

mercial bleach ~ 5 % sodium hypochlorite) for 15 min and germination on MS medium under 16 h day length illumination at 25 °C for 6–7 day. Fully-expanded cotyledons were cut at the base of the petiole and placed in *Agrobacterial* solution, which preparing was described above. In 20 min cotyledon explants were moved to sterile filter paper and incubated at room temperature for 2 day in darkness. After that the explants were placed on the B5 based [32] regeneration medium (B5 medium salts with 2.5 % sucrose, 3 mg/l kinetin, 0.5 mg/l NAA) supplemented with 100 mg/l kanamycin for selection. As shoot appeared, each one was tested for PPT resistant by removing and placing on the selective medium with 5 mg/l PPT. Well established plants were transferred into the soil in the greenhouse.

PCR analysis. Genomic DNA was isolated from young leaves as describd [33]. PCR was carried out in a reaction volume of 20 μl containing 50 ng DNA, 200 μM each of forward and reverse primers, 200 μM dNTPs and 1 U Taq DNA polymerase («Fermentas»). Thermal cycling (=amplification) was performed on a Mastercycler® personal («Eppendorf») with an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s; primer annealing at 65 °C for 30 s, and elongation at 72 °C for 30 or 45 s. Sequences of the primers used:

for *bar* gene amplification were

barpr1 5′-ATGAGCCCAGAACGACGCCCGGCC-3′
barpr2 5′-GCATGCGCACGGTCGGGTGTTGG-3′
barpr3 5′-CCGTACCGAGCCGCAGGAAC-3′
barpr4 5′-CAGATCTCGGTGACGGGCAGGAC-3′

for *nptII* gene amplification

kanpr1 5′-CCTGAATGAACTCCAGGACGAGCA-3′
kanpr2 5′-GCTCTAGATCCAGAGTCCCGCTCAG-AAG-3′

for *gus* gene amplification

guspr1 5′-TGGGTGGACGATATCACCGTGGTGA-3′
guspr2 5′-GGCCCCAATCCAGTCCATTAATGCG-3′

The products of the amplification were separated on a 1 % (w/v) agarose gels.

RNA isolation and RT-PCR. For reverse transcription polymerase chain reactions (RT-PCR) 1 μg of total RNA isolated from young leaf tissues of tobacco and beet plants was used. The RNA extraction protocol was followed as Logemann et al. [34]. DNase I (RNase-free) treatment was used to eliminate DNA contamination from RNA samples. First strand cDNA was synthesized from 1 μg total RNA using M-MLV reverse transcriptase and random hexamer primers («Fermentas») at 37 °C for 1 h. Then 2 μl of the reaction mixture was used as a template for PCR with specific primers for transgenes.

Histochemical assay of GUS activity. Histochemical staining for β-glucuronidase (GUS) activity was performed as described by Jefferson [35] with modifications. Tissues were stained for 12 h at 37 °C in 0.1 M phosphate buffer pH 7.0 containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), 10 mM EDTA, 2.5 mM $K_4[Fe(CN)_6]$, 2.5 mM $K_3[Fe(CN)_6]$, 2 mM dithiotreitol, 0.1 % (v/v) Triton X-100 and 20 % (v/v) methanol. After staining, chlorophyll from green tissues was removed by washing in 70 % (v/v) ethanol.

GUS quantification. Protein was extracted from in vitro plant leaves by homogenization in GUS extraction buffer (50 mM NaH_2PO_4, 10 mM EDTA, 0.1 % Triton X-100, 0.1 % Sarcosyl). Aliquots of the extract (50 μl) were added to 250 μl of assay buffer (extraction buffer containing 1 mM MUG) and incubated at 37 °C. After 1 h incubation 50 μl samples were removed and placed in 2.95 ml stop buffer (200 μM sodium carbonate). Specific activity of GUS expression was determined with fluorometric assay using MUG as a substrate and quantified by the PerkinElmer LS 55 Fluorescence spectrometer with 365 nm excitation and 455 nm emission wavelengths. Total soluble protein was determined as described by Bradford method [36] using bovine serum albumin (BSA) as a standard. GUS activity was expressed as picomole 4-MU per minute per milligram protein.

Results. *Genetic transformation experiments with vectors contain lox site and promoterless bar gene sequences.* In our experiments a set of vectors based on *lox* site disposition adjacent to promoterless *bar* gene sequence were used (Fig. 1). Most of plasmid vectors held *nptII* gene under control of *nos* promoter as well. In experiments with the vectors containing *nptII* gene transgenic plants were selected according to their growth capacity on the medium with kanamycin and then tested on the selective medium containing PPT. The presence of *bar* gene in selected tobacco plants was confirmed with PCR analysis (Fig. 2). Herbicide resistance/sensitiv-

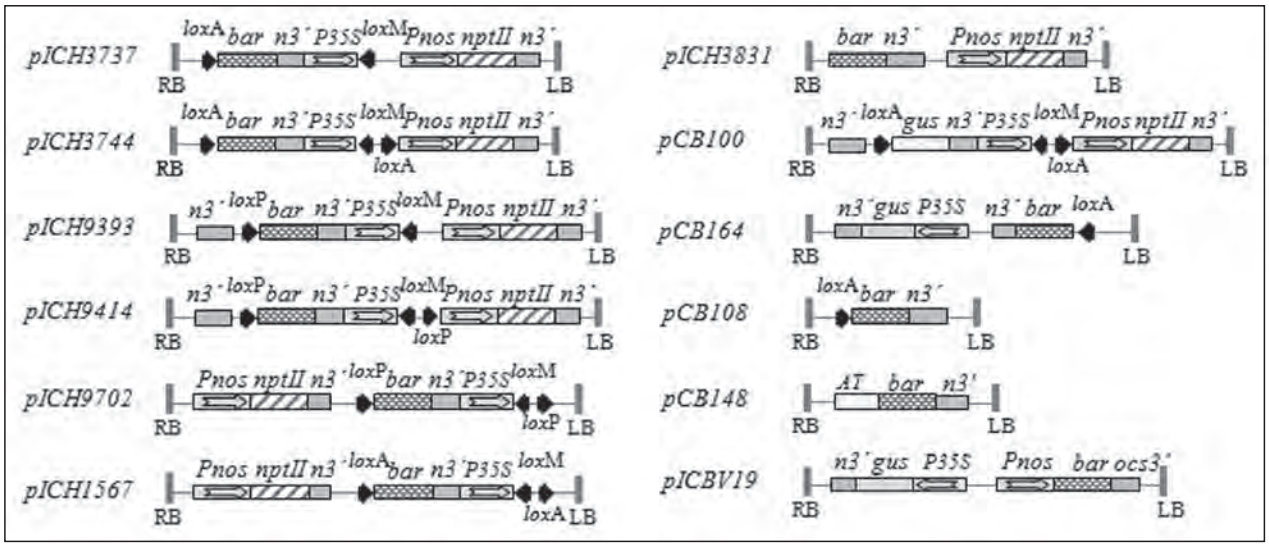

Fig. 1. Schematic representation of the T-DNA region of transformation vectors used in this study: RB – right border, LB – left border, *Pnos* – promoter of the nopaline synthase gene, *n3′* – terminator of the nopaline synthase gene, *ocs3′* – terminator of the octopine synthase gene, *P35* – CaMV 35S promoter, *AT* – AT rich region of intergenic spacer of chloroplast DNA

ity studies of transformants harboring RB-*lox*-*bar* sequence reproducibly resulted in approximately 80 % of PPT-resistant transgenic plants. The results from a series of transformation experiments are represented at Fig. 3.

Neither the localization of *nos* terminator between *lox* site and the RB nor the presence of additional *lox* site within the T-DNA had an effect on *bar* gene expression: the number of PPT-resistant transgenic plants obtained using pICH3737 and pICH3744 vectors was similar to that with the vectors pICH9414 and pICH9393. The frequency of PPT-resistant transgenic plants obtained using these 4 vectors did not differ from each other at >95 % confides using Students T-test. We can conclude that additional *lox* site placed in the direct orientation and therefore can recombine with *lox* near RB (pICH3744, pICH9414) and create favourable conditions for putative recombination had no effect of the *lox*-mediated expression.

In contrast, disposition -*lox*-*bar*- internally in the T-DNA thoroughly changed the result of PPT tests. Transformation experiments with plasmid vectors pICH1567 and pICH9702 resulted without obtaining any PPT-resistant transgenic plants. Another strategy of experiments with direct selection on the regeneration medium with PPT immediately after co-cultivation also was used for

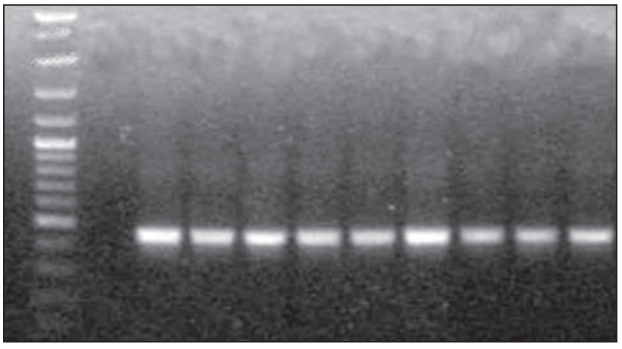

Fig. 2. PCR analysis of DNA isolated from transgenic tobacco plants using primers for bar gene: *1* – negative control with DNA from non-transformed plant; *2* – positive control, plasmid pICH3737 DNA; *3–10* – DNA from different transgenic plants; M – molecular weight marker 1 kb Plus DNA Ladder (Gibco BRL)

these constructs. Invariable result was obtained in the numerous transformation experiments: no PPT-resistant transgenic plants were obtained after co-cultivation with the *Agrobacterium* strain containing pICH1567 and pICH9702. Based on these data we suggest that *lox*-mediated expression occurred only when *lox* site was associated with RB.

The results of biolistic transformation experiments have been estimated using a scheme such

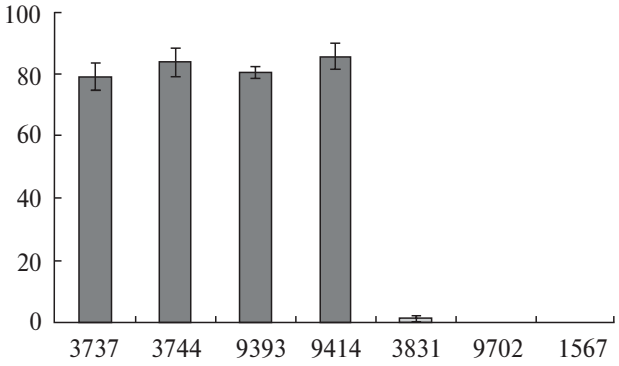

Fig. 3. Quantification of PPT resistant transgenic plants (%). Each bar represents quantity of PPT resistant transgenic tobacco plants obtained in experiments with appropriate constructs. Averages and standard deviations (±SD, $n = 3$) of three independent transformation experiments for each constructs are shown. Approximately thirty kanamycin resistant transgenic lines were analyzed in one experiment for each constructs

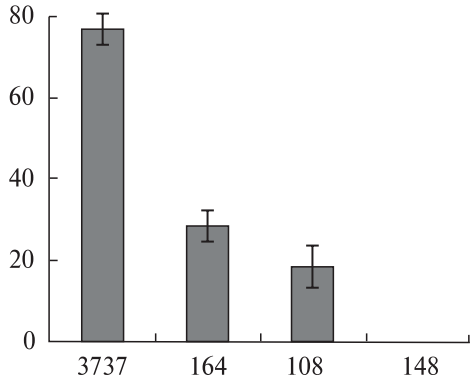

Fig. 4. Efficiency of tobacco plants regeneration (%) on the selective medium with 5 mg/l PPT, after *Agrobacterium*-mediated transformation. Regeneration efficiency was estimated as ratio of explants number producing green shoots on selective medium to total number of explants analyzed in experiment. Regeneration efficiency represents as averages (±SD) of three independent transformation experiments for each constructs

as *Agrobacterium*-mediated transformation: plants were regenerated on the medium with kanamycin and then tested on the selective medium containing PPT. Only 3 % of obtained kanamycin resistant plants were tolerant to PPT. The result of direct transformation led us to conclude that *lox*-mediated expression of *bar* gene depended on the method of DNA delivery and occurred mainly in plants obtained via *Agrobacterium*-mediated transformation.

A set of vectors was also used for transformation experiments with direct selection of transgenic plants on the medium with PPT. Vector pCB108 was constructed to investigate *lox*-mediated expression without presence of additional promoter or selective gene sequence in the construct. In order to determine if combining of *lox* site and LB sequence produces the same result as that seen with RB-*lox*, vector pCB164 was designed. Transgenic tobacco lines were selected immediately after cocultivation on the medium with 5 mg/l PPT. We noted a general decrease of transformation efficiency by comparing these vectors with ones characterized above (Fig. 4). Substitution of the *lox* site by AT rich region of intergenic spacer of chloroplast DNA in construct pCB148 resulted in loss of ability to obtain PPT resistant plants and therefore to indicate *bar* gene expression.

Constructions and activities of RB-lox-gus-fusion. Translational fusion was constructed between *gus* reporter gene and RB-*lox* upstream regions. We used plasmid pICH9414 for designing a new vector – pCB100 which contains promoterless *gus* gene instead of *bar* gene. Transgenic tobacco plants were raised and GUS activity was assayed using standard histochemical analysis (Fig. 5). The GUS activity varied between individual transgenic plants though most of them exhibited the absence of *gus* gene expression. Fluorometric assay of GUS activity was carried out to quantify distinguishes between *gus* gene expression controlled by 35S promoter and RB-*lox*- sequences. GUS activity directed by RB-*lox*- sequences in some transgenic plants obtained with vector pCB100 was fairly high but less that activity of *gus* gene under control of 35S promoter.

Crop species transformation. To investigate the possibilities of *lox*-mediated expression in crop species RB-*lox-bar* vectors were inserted into sugar beet, lettuce, canola and potato plants via *Agrobacterium*-mediated transformation. In general, the data obtained from stable transgenic crop species showed good agreement with the discussed above data of tobacco transformation.

Twenty one transgenic callus clones of sugar beet were selected on the medium supplemented with 100 mg/l kanamycin after *Agrobacterium*-mediated transformation with vector pICH3744. Eight of them were morphogenic and were able to regenerate shoots on selective medium. Herbicide resistance/sensitivity studies of sugar beet were carried out by

cultivation of obtained transgenic plant petioles on regeneration medium supplemented with 10 mg/l PPT (Fig. 6, *i*, *k*).

Lettuce and potato transgenic plants were regenerated on the selective medium with kanamycin and then screened by herbicide selection. Lettuce green shoots were transferred on the selective medium (with 5 mg/l PPT) for rooting and resistant rooted plants were identified for the further analysis. Internodes of transgenic kanamycin resistant potato plants were cultivated on regeneration medium with 5 mg/l PPT and hereby transgenic potato clones tolerant to PPT were selected (Fig. 6, *g*, *h*).

An alternative method with direct selection on the regeneration medium containing 5 mg/l PPT for *Brassica napus* was used. It was shown that kanamycin resistant marker could not be used successfully as reliable marker for transgenic *Brassica napus* plant selection [37, 38]. The same is true for canola selection on the regeneration medium with kanamycin in our experiments. Therefore *Brassica napus* transgenic plants with *lox*-depended *bar* gene expression were obtained using direct selection on the regeneration medium with PPT (Fig. 6, *a*, *b*). Eighty eight independent transgenic lines of *Brasica napus* resistant to PPT were obtained in our experiments.

PPT-resistant primary transformants of all examined species were transferred to soil and treated with BASTA herbicide. The results of herbicide application of transgenic plants in greenhouse entirely agreed with *in vitro* test results for all plant species used in this report (Fig. 6, *l*, *m*). Self-fertilization seeds (R1) from the primary transformants of tobacco and canola were harvested and germinated on the medium containing 5mg/l phosphinotricin. The 3:1 segregation of the selectable marker gene in progeny of tobacco and canola transgenic plants was observed (Fig. 6, *d*).

RT-PCR analysis. The effect of the *lox* site sequence on expression level of *bar* gene in obtained transgenic plants has been further confirmed with reverse transcription PCR (RT-PCR) technique. No expression of *bar* gene was observed in the leaves of wild-type plants. In the case of transgenic plants that hosted RB-*lox*-*bar* and 35S-*bar* gene transcript was detected (Fig. 7). Results of RT-PCR analysis corroborated *lox*-mediated expression of *bar* gene in transgenic plants.

In silico analysis. In silico analysis of RB-*lox* region using Plat CARE and PLACE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) revealed TATA motifs within *lox*A site (vectors pICH3737 and pICH3744) sequences located 94, 82 and 72 bp upstream of the

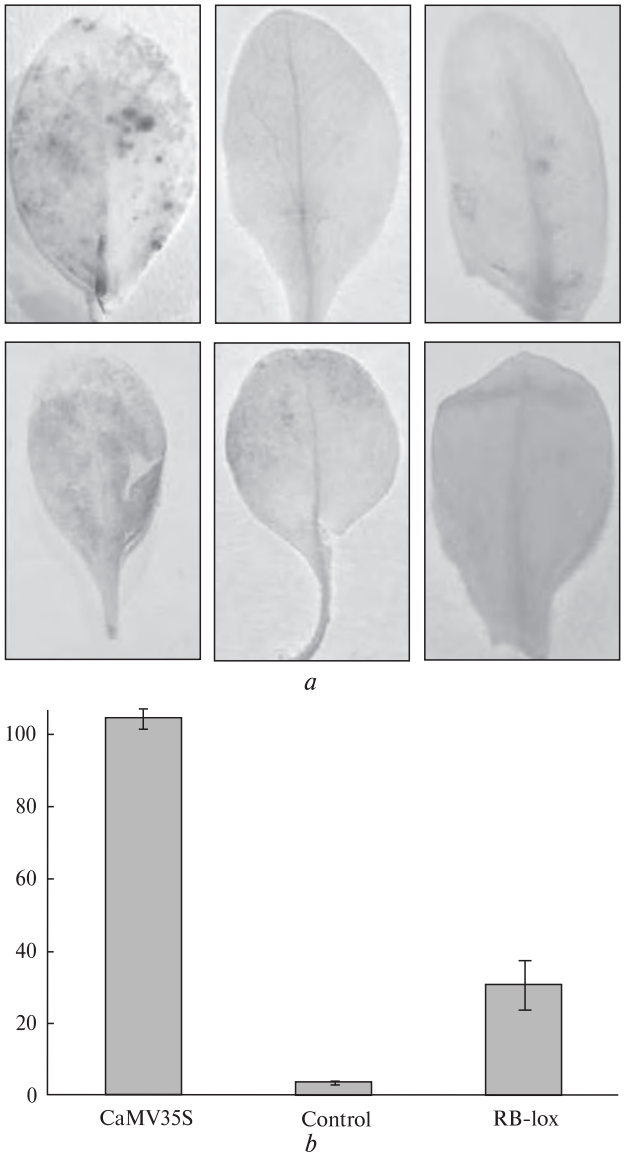

Fig. 5. Expression analysis of *LB-lox-gus* sequence in transgenic tobacco plant: *a* – histochemical staining of GUS activity of transgenic tobacco plants transformed with vector pCB100. GUS activity was detected in primary transformants (*in vitro* plant leaves), *b* – the bar diagram denotes GUS activities of *in vitro* transgenic plant generated for the GUS vectors: pCB100 and pICBV19. The data present average (±SD) of five independent GUS-positive lines

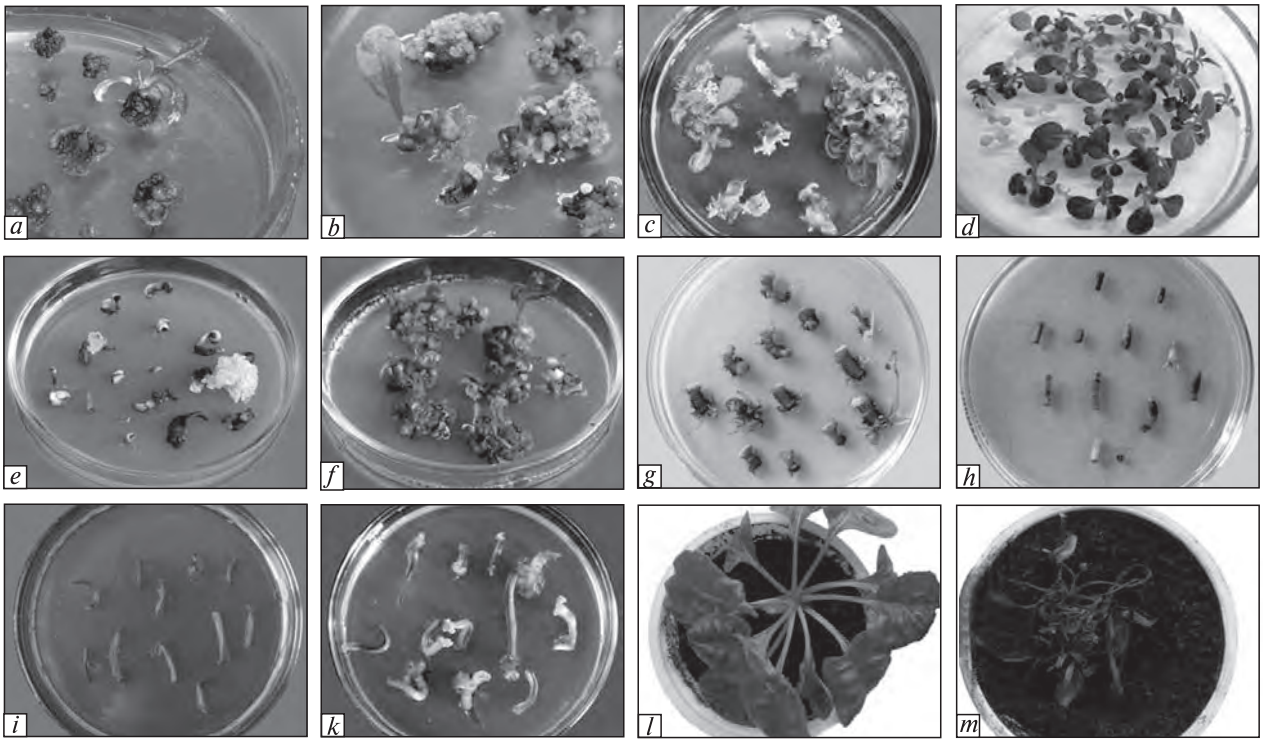

Fig. 6. Regeneration and selection of PPT resistant transgenic plants: *a*, *b* – regeneration of canola plants after cocultivation with *Agrobacterium* containing vector pICH9393 on the selective medium with 5 mg/l PPT; *c* – regeneration of tobacco plants transformed with vector pCB164 on the selective medium with 5 mg/l PPT; *d* – selection of F1 tobacco plants (pCB108) on the medium with 5 mg/l PPT; *e*, *f* – callus formation and regeneration of sugar beet plants transformed with vector pICH3737; *g*, *h* – transgenic (*g*) and non-transgenic control (*h*) potato explants at the stage of regeneration on the selective medium; *k*, *i* – sugar beet transgenic (*k*) and non-transgenic (*i*) explants at the stage of regeneration on the selective medium; *l*, *m* – sugar beet transgenic (*l*) and non-transgenic control (*m*) plants herbicide application in greenhouse

transcription start site (Fig. 8, *b*). The same TATA motif was found in *lox*P sequence (vectors pICH9393 and pICH9414). Several sequences that resemble previously identified plant *cis*-acting regulatory elements were identified in the region adjacent to *lox* site, mainly in RB sequence: CAAT boxes, ATGCAAAT motif, as well as TGA-element and ATCT-motif that have partial overlap with the CAAT box (Fig. 8, *a*).

Discussion. Teeri et al. [39] demonstrated that activation of promoterless *nptII* coding sequence adjacent to the RB of T-DNA is possible after plant transformation. The establishment of this protocol has opened up the possibility to identify new regulatory sequences needed to drive transgene expression. In the studies presented here, we report that *lox* site located near RB and upstream of the transcription start site positively affected expression of *bar* gene coding sequence situated in vectors without control of any known promoter sequence. Whereas *bar* gene coding sequence was placed in close to the RB we compared our result with published data of gene expression in promoter tagging experiments. If the transcriptional activation of introduced *bar* gene in our experiments occurred by plant promoter tagging we would expect 4–5 % of transformants resistant to PPT. Foster et al. [25] reported that one from thousand transgenic tobacco line created by T-DNA tagging demonstrated constitutive *gus* gene expression. According to the published data 4 % is a usual result for strong gene expression in promoter tagging experiments [40, 41]. The frequency of *gus* gene transcription fusion and *gus* gene expression in different plant tissues was higher [42]. Koo et al. [43] revealed 13 % of gene trapping efficiency

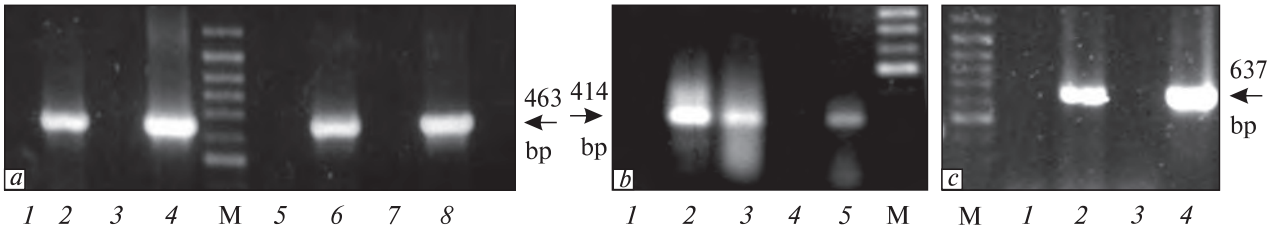

Fig. 7. RT-PCR analysis of PPT-resistant transgenic plants: *a* – PCR analysis of cDNA synthesized with reverse transcriptase from tobacco RNA using primers for *bar* gene (barpr3 and barpr4); *1* – negative control with cDNA from non-transformed plant; *4* – positive control with cDNA of tobacco transformed with plasmid pICBV19 (*bar* gene under35S promoter); *2*, *6*, *8* – cDNA of transgenic tobacco plants (pICH3737); *3*, *5*, *7* – control: the same samples without using of reverse transcriptase; M – molecular weight marker 1 kb Plus DNA Ladder (Gibco BRL); *b* – PCR analyses of cDNA synthesized with reverse transcriptase from RNA isolated from sugar beet plants using primers for *bar* gene (barpr1 and barpr2); *1* – negative control with cDNA from non-transformed plant; *3* – positive control with cDNA of sugar beet transformed with plasmid pICBV19; *2* – cDNA of transgenic sugar beet (pICH3744) with primers for *bar* gene; *4* – control: the same sample without using of reverse transcriptase; *5* – control: DNA of transgenic sugar beet (pICH3744) with primers for *bar* gene; M – molecular weight marker (Promega); *c* – PCR analyses of cDNA synthesized with reverse transcriptase from RNA isolated from sugar beet plants using primers for *nptII* gene (kanpr1 and kanpr2); *1* – negative control with cDNA from non-transformed plant; *2* – cDNA of transgenic sugar beet (pICH3744) with primers for *nptII* gene; *3* – control: the same sample without using of reverse transcriptase; *4* – positive control with DNA of transgenic sugar beet (pICH3744); M – molecular weight marker (Promega)

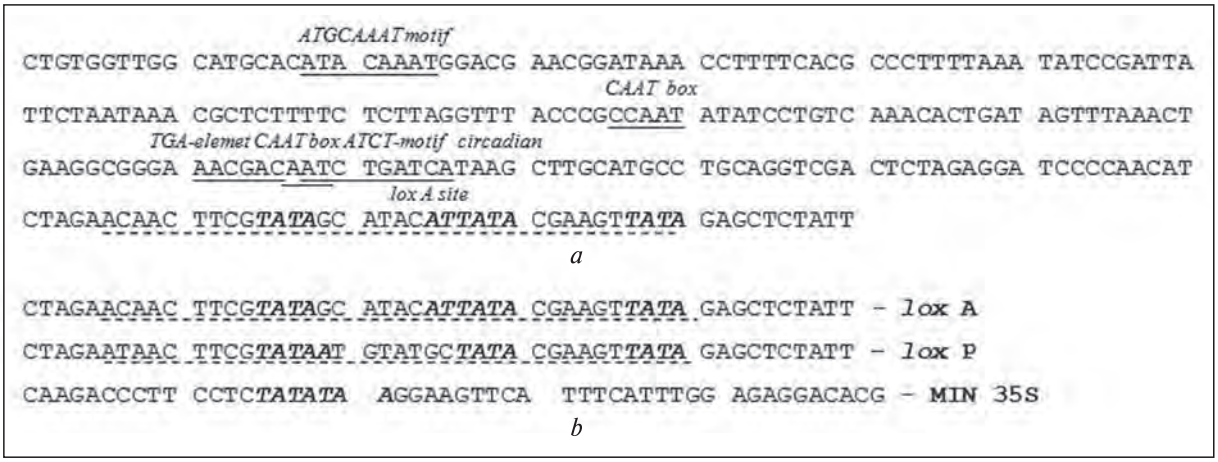

Fig. 8. Sequence of RB-*lox* region: *a* – putative regulated sequences reviled by Plat CARE and PLACE database are underlined, TATA box are in bold, *lox* site sequences are underlined with dashed lines; *b* – sequence comparison of *lox*A and *lox*P sites with the min 35S promoter

and Yamamoto et al. [44] reported the same result for promoterless trapping vectors and 41 % of gene trapping efficiency for IRES-type trapping vector. We note that in our studies, however, enormous for promoter tagging experiments number of transgenic plants was resistant to PPT. The further investigation confirmed that localization of the *lox* site and *bar* gene near RB created a strong gene expression system that is more effective in case of *Agrobacterium*-mediated transformation but for a wide range of plant species, including several important crops.

To gain insight into the mechanisms of *lox*-mediated expression, we studied the sequence of *lox* site and adjacent region in binary vector and compared it with several known promoters. Several TATA motifs within *lox* site sequence were revealed. Furthermore, the minimal 35S promoter

is associated with very low basal transcription in the absence of additional upstream regulatory motifs [45] and it is highly improbable that minimal promoter can support expression level sufficient for strong herbicide tolerance of transgenic plants. In addition, we constructed the vector with arbitrary AT rich sequence (spacer between tobacco plastome genes) placed instead of *lox* site. Transformation experiments with such vector resulted without obtaining any PPT-resistant plant.

Only several regulatory sequences were found in the region adjacent to *lox* site, including RB sequence. Neither G-box nor *as-1* element present in 35S promoter [46] and in other plant, viral and *Agrobacteria* promoter were found in this sequences. *As-1* element associated with expression in roots has been reported to act synergistically with other subdomains to confer expression in different tissues. The elements like *as-1* element are present in other viral and *Agrobacteria* promoter sequences [47, 48]. We can conclude that the DNA context surrounding several regulatory sequences which were found in RB-*lox* region differs markedly from the previously examined promoter elements. We suppose that using the *lox* site in genetic vectors will help to express transgenes with reduced possibility of homology dependent gene silencing as this element have reduced sequence homology with widely used promoters.

We note that gene expression mediated by these two elements (RB-*lox*-) being critically dependent on their spacing in the vector and method of T-DNA delivery. Though separating of the *lox* sequence and the RB by 282 kb of *nos* terminator sequence did not influence *bar* gene expression, no occurrence of *bar* gene expression was observed when *lox-bar* sequence was replaced in the middle of the construct. Experiments with vector pCB164 containing one *lox* site placed near LB upstream of *bar* gene demonstrated possibilities of *lox*-depended expression as well.

Constructs based on *lox*-mediated expression constructs have been used effectively for obtaining herbicide resistant transgenic plant of sugar beet, canola, lettuce and potato. It seems to be completely real to involve new crop species in transformation experiments with vectors containing *lox*-site near the RB instead of conventional promoters.

One of the goals of this research was to elucidate the main principles of *lox*-mediated expression. This goal is far from complete, but our results do confirm that *lox* site provides an interesting alternative to promoters derived from plant pathogens in that *lox* sites are not associated with them and can be used to control gene expression in transgenic plant.

Н.Л. Щербак, Е.М. Кищенко, Л.А. Сахно, И.К. Комарницкий, Н.В. Кучук

Lox-ОПОСРЕДОВАННАЯ ЭКСПРЕССИЯ ГЕНОВ В ТРАНСГЕННЫХ РАСТЕНИЯХ, ПОЛУЧЕННЫХ С ПОМОЩЬЮ АГРОБАКТЕРИАЛЬНОЙ ТРАНСФОРМАЦИИ

Проанализирована способность *lox*-сайтов Cre/*lox* системы рекомбинации бактериофага P1 влиять на экспрессию трансгенов при расположении этой последовательности непосредственно возле правого бордера (RB) перед кодирующей последовательностью гена. Нативная и мутированная последовательность *lox*-сайта были размещены в векторах для трансформации возле гена *bar* и проведена генетическая трансформация растений с помощью агробактерии и биолистическим методом. *Lox*-опосредованная экспрессия гена *bar*, обусловливающая устойчивость растений к фосфинотрицину, наблюдалась только у растений, которые получены с помощью агробактериальной трансформации. Методом РТ-ПЦР анализа подтверждено, что в трансгенных растениях, устойчивых к фосфинотрицину, происходит транскрипция гена *bar*. Сконструирован вектор, в котором ген *gus* и предшествующий ему *lox*-сайт размещены вблизи правого бордера, и проведена трансформация табака этим вектором. Экспрессия гена *gus* задетектирована в листьях трансгенных растений. Векторы, у которых последовательность *lox*-сайта предшествует гену *bar* возле правого бордера (RB-*lox-bar*), успешно использованы для получения устойчивых к фосфинотрицину трансгенных растений таких видов, как *Beta vulgaris, Brassica napus, Lactuca sativa* и *Solanum tuberosum*. Наши результаты подтверждают возможность использования последовательности *lox*-сайта возле правого бордера для контроля экспрессии гена *bar* в трансгенных растениях.

REFERENCES

1. *Potenza K., Aleman L., Sengupta-Gopalan C.* Targeting transgenic expression in research, agricultural, and environmental applications: promoters used in plant transformation // In Vitro Cell Dev. Biol. – 2004. – **40,** № 1. – P. 1–22.
2. *Corrado G., Karali M.* Inducible gene expression systems and plant biotechnology // Biotechnol. Adv. – 2009. – **27,** № 6. – P. 733–743.
3. *Hajdukiewicz P., Svab Z., Maliga P.* The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation // Plant. Mol. Biol. – 1994. – **25**, № 6. – P. 989–994.

4. *McBride K., Summerfelt K.* Improved binary vectors for *Agrobacterium*-mediated plant transformation // Plant. Mol. Biol. – 1990. – **14,** № 2. – P. 269–276.
5. *Al-Kaff N., Kreike M., Covey S., Pitcher R., Page A., Dale P.* Plants rendered herbicide-susceptible by cauliflower mosaic virus-elicited suppression of a 35S promoter-regulated transgene // Nat. Biotechnol. – 2000. – **18**, № 9. – P. 995–999.
6. *Zheng X., Deng W., Luo K., Duan H., Chen Y., McAvoy R., Song S., Pei Y., Li Y.* The cauliflower mosaic virus (CaMV) 35S promoter sequence alters the level and patterns of activity of adjacent tissue- and organ-specific gene promoters // Plant Cell Rep. – 2007. – **26**, № 8. – P. 1195–1203.
7. *Jagannath A., Bandyopadhyay P., Arumugam N., Gupta V., Burma P.K., Pental D.* The use of a Spacer DNA fragment insulates the tissue-specific expression of a cytotoxic gene (barnase) and allows high-frequency generation of transgenic male sterile lines in *Brassica juncea* L. // Mol. Breed. – 2001. – **8**, № 1. – P. 11–23.
8. *Yoo S.Y., Bomblies K., Yoo S.K., Yang J.W., Choi M.S., Lee J.S., Weigel D., Ahn J.H.* The 35S promoter used in a selectable marker gene of a plant transformation vector affects the expression of the transgene // Planta. – 2005. – **221**, № 4. – P. 523–530.
9. *Selker E.* Gene silencing: repeats that count // Cell. – 1999. – **97**, № 2. – P. 157–160.
10. *Vaucheret H., Fagard M.* Transcriptional gene silencing in plants: targets, inducers and regulators// Trends Genet. – 2001. – **17**, № 1. – P. 29–35.
11. *Matzke M.A., Aufsatz W., Kanno T., Mette M., Matzke A.* Homology-dependent gene silencing and host defense in plants // Adv. Genet. – 2002. – **46**, № 1. – P. 235–275.
12. *Kumar D., Patro S., Ranjan R., Sahoo D., Maiti I., Dey N.* Development of useful recombinant promoter and its expression analysis in different plant cells using confocal laser scanning microscopy // PLoS One. – 2011. – **6**, № 9. – e24627.
13. *Mehrotra R., Gupta G., Sethi R., Bhalothia P., Kumar N., Mehrotra S.* Designer promoter: an artwork of cis engineering // Plant. Mol. Biol. – 2011. – **75**, № 6. – P. 527–536.
14. *Dale E., Ow D.* Intra- and intermolecular site-specific recombination in plant cells mediated by bacteriophage P1 recombinase // Gene. – 1990. – **91**, № 1. – P. 79–85.
15. *Gilbertson L.* Cre-*lox* recombination: Cre-active tools for plant biotechnology // Trends Biotechnol. – 2003. – **21,** № 12. – P. 550–555.
16. *Qin M., Bayley C., Stockton T., Ow D.W.* Cre recombinase-mediated site-specific recombination between plant chromosomes // Proc. Nat. Acad. Sci. USA. – 1994. – **91**, № 5. – P. 1706–1710.
17. *Medberry S., Dale E., Qin M., Ow D.W.* Intra-chromosomal rearrangements generated by Cre-*lox* site-specific recombination // Nucl. Acids Res. – 1995. – **23,** № 3. – P. 485–590.
18. *Day C., Lee E., Kobayashi J., Holappa L., Albert H., Ow D.W.* Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced // Genes Dev. – 2000. – **14**, № 22. – P. 2869–2880.
19. *Gleave A., Mitra D., Mudge S., Morris B.* Selectable marker-free transgenic plant without sexual crossing: transient expression of cre recombinase and use of a conditional lethal dominant gene // Plant Mol. Biol. – 1999. – **40**, № 2. – P. 223–235.
20. *Corneille S., Lutz K., Svab Z., Maliga P.* Efficient elimination of selectable marker genes from the plastid genome by the cre-*lox* site-specific recombination system // Plant. J. – 2001. – **27**, № 2. – P. 171–178.
21. *Zhang W., Subbarao S., Addae P., Shen A., Armstrong C., Pechke V., Gilbertson L.* Cre-lox mediated marker gene excision in transgenic maize (*Zea mays* L.) plants // Theor. Appl. Genet. – 2003. – **107**, № 7. – P. 1157–1168.
22. *Wang Y., Chen B., Hu Y., Li J., Lin Z.* Inducible excision of selectable marker gene from transgenic plants by the cre/*lox* site-specific recombination system // Transgenic Res. – 2005. – **14**, № 5. – P. 605–614.
23. *Chakraborti D., Sarkar A., Mondal H., Schuermann D., Hohn B., Sarmah B., Das S.* Cre/lox system to develop selectable marker free transgenic tobacco plants conferring resistance against sap sucking homopteran insect // Plant Cell Rep. – 2008. – **27**, № 10. – P. 1623–1633.
24. *Fobert P., Labbe H., Cosmopolous J., Gottlob-McHugh S., Ouellet T., Hattori J., Iyer V., Miki B.L.* T-DNA tagging of a seed coat-specific cryptic promoter in tobacco // Plant J. – 1994. – **6**, № 4. – P. 567–577.
25. *Foster P., Hattori J., Labbe H., Ouellet T., Fobert P., James L., Iyer V., Miki B.* A tobacco cryptic constitutive promoter tCUP revealed by T-DNA tagging // Plant Mol Biol. – 1999. – **4**, № 1. – P. 45–55.
26. *Malik K., Wu K., Li X.Q., Martin-Heller T., Hu M., Foster E., Tian L., Wang C., Ward K., Jordan M., Brown D., Gleddie S., Simmonds D., Zheng S., Simmonds J., Miki B.* A constitutive gene expression system derived from the tCUP cryptic promoter elements // Theor. Appl. Genet. – 2002. – **105**, № 4. – P. 505–514.
27. *Shcherbak N., Belokurova V., Getsko I., Komarnitskiĭ I., Kuchuk N.* Effect of *lox*-sites of the Cre *lox* recombination system on promoterless *bar* gene expression in transgenic plants // Cytology and Genetics. – 2006. – **40**, № 1. – P. 1–7.

28. *Sakhno L., Gocheva E., Komarnitskiĭ I., Kuchuk N.* Stable expression of the promoterless bar gene in transformed rapeseed plants // Cytology and Genetics. – 2008. – **42**, № 1. – P. 16–22.
29. *Murashige T., Skoog F.* A revised medium for rapid growth and bioassays with tobacco tissue cultures // Physiol. Plant. – 1962. – **15**, № 3. – P. 473–497.
30. *Kishchenko E., Komarnitskii I., Kuchuk N.* Production of transgenic sugar beet *(Beta vulgaris* L) plants resistant to phosphinothricin // Cell. Biol. Int. – 2005. – **29**, № 1. – P. 15–19.
31. *Olhoft P., Lin K., Galbraith J., Nielsen N., Somers D.* The role of thiol compounds in increasing *Agrobacterium*-mediated transformation of soybean cotyledonary-node cells // Plant Cell Rep. – 2001. – **20**, № 8. – P. 731–737.
32. *Gamborg O., Miller R., Ojima K.* Nutrient requirements of suspension cultures of soybean foot cells // Exp. Cell Res. – 1968. – **50**, № 1. – P. 151–158.
33. *Cheung W., Hubert N., Landry B.* A simple and rapid DNA microextraction method for plant, animal and insect suitable for RAPD and other PCR analyses // PCR Meths. Appl. – 1993. – **3**, № 1. – P. 69–70.
34. *Logemann J., Schell J., Willmitzer L.* Improved method for the isolation of RNA from plant tissues // Anal. Biochem. – 1987. – **163**, № 1. – P. 16–20.
35. *Jefferson R., Kavanagh T., Bevan M.* GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants // EMBO J. – 1987. – **6**, № 13. – P. 3901–3907.
36. *Bradford M.* A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding // Anal. Biochem. – 1976. – **72**, № 1. – P. 248–254.
37. *Gupta V., Sita G., Shaila M., Jagannathan V.* Genetic transformation of *Brassica nigra* by Agrobacterium based vector and direct plasmid uptake // Plant Cell Rep. – 1993. – **12**, № 7/8. – P. 418–421.
38. *Poulsen G.* Genetic transformation of *Brassica* // Plant. Breed. – 1996. – **115,** № 4. – P. 209–225.
39. *Teeri T.H., Herrera-Estella L., Depiker A., Montagu M., van Palva E.T.* Identification of plant promoters in situ by T-DNA mediated transcriptional fusion to the *nptII* gene // EMBO J. – 1986. – **5**, № 8. – P. 1755–1760.
40. *Alvarado M.C., Zsigmond L.M., Kovбcs I., Cséplö A., Koncz C., Szabados L.M.* Gene trapping with firefly luciferase in *Arabidopsis.* Tagging of stress-responsive genes // Plant Physiol. – 2004. – **134**, № 1. – P. 18–27.
41. *Ryu C.H., You J.H., Kang H.G., Hur J., Kim Y.H., Han M.J., An K., Chung B.C., Lee C.H., An G.* Generation of T-DNA tagging lines with a bidirectional gene trap vector and the establishment of an insertion-site database // Plant. Mol. Biol. – 2004. – **54**, № 4. – P. 489–502.
42. *Topping J.F., Lindsey K.* Insertional mutagenesis and promoter trapping in plants for the isolation of genes and the study of development // Transgenic Res. – 1995. – **4**, № 5. – P. 291–305.
43. *Koo J., Kim Y., Kim J., Yeom M., Lee I.C., Nam H.G.* A GUS/luciferase fusion reporter for plant gene trapping and for assay of promoter activity with luciferin-dependent control of the reporter protein stability // Plant. Cell. Physiol. – 2007. – **48**, № 8. – P. 1121–1131.
44. *Yamamoto Y.Y., Tsuhara Y., Gohda K., Suzuki K., Matsui M.* Gene trapping of the Arabidopsis genome with a firefly luciferase reporter// Plant J. – 2003. – **35**, № 2. – P. 273–283.
45. *Cazzonelli C.I., Burke J., Velten J.* Functional characterization of the geminiviral conserved late element (CLE) in uninfected tobacco // Plant Mol. Biol. – 2005. – **58**, № 1. – P. 465–481.
46. *Benfey P.N., Chua N.H.* The cauliflower mosaic virus 35s promoter: combinatorial regulation of transcription in plants // Science. – 1990. – **250**, № 1. – P. 959–966.
47. *Bouchez D., Tokuhisa J.G., Llewellyn D.J., Dennis E.S., Ellis J.G.* The ocs-element is a component of the promoters of several T-DNA and plant viral genes // EMBO J. – 1989. – **8**, № 1. – P. 4197–4204.
48. *Dey N., Maiti I.B.* Structure and promoter/leader deletion analysis of mirabilis mosaic virus (MMV) full-length transcript promoter in transgenic plants // Plant Mol. Biol. – 1999. – **40**, № 1. – P. 771–782.

Received 26.11.12

ABBREVIATIONS

RB – T-DNA right border
LB – T-DNA left border
PPT – phosphinotricin
GUS – β-Glucuronidase
NAA – naphthalene-acetic acid
BA – 6-benzylaminopurine
MUG – 4-methylumbelliferyl β-d-glucuronidase
MU – 4-methylumbelliferone