

Optimization of Biological and Physical Parameters for Biolistic Genetic Transformation of Common Wheat (*Triticum aestivum* L.) Using a Particle Inflow Gun

V. S. Fadeev, O. V. Blinkova, and A. K. Gaponenko

Bioengineering Center, Russian Academy of Sciences, Moscow, Russia;
fax: (495)135-05-71; e-mail: alexg@biengi.ac.ru, lis_vit@rambler.ru

Received May 30, 2005

Abstract—The parameters for delivery of expression cassettes to cells of wheat morphogenic callus induced from immature embryos were optimized. Three systems (gradation, delayed, and regeneration) for in vitro selection of transgenic wheat tissue using the *bar* gene, providing resistance to the herbicide phosphinothricin (PPT), were compared. The efficiency of gene delivery to the cells competent for plant regeneration was assessed by comparing the number of spots transiently expressing *uidA* gene (encoding β -glucuronidase) per unit surface of the morphogenic calluses treated under various conditions. The selection systems in question were evaluated by comparing the transformation efficiency frequencies. The optimal parameters for wheat biolistic transformation using a particle inflow gun were determined, namely, the distance between the particle source and the target tissue (12 cm) and helium pressure during the shot (6 atm). The optimal time of callus tissue development on the medium inducing callus formation was determined (10–14 days). Comparison of the three selection variants demonstrated that the regeneration system was the most efficient for producing true transgenic plants of common wheat.

DOI: 10.1134/S1022795406040077

INTRODUCTION

Common wheat (*Triticum aestivum* L.) is among the major agricultural plants used in the human diet as well as according to its cultivation area. An increase in wheat resistance to abiotic (low and high temperatures, drought, and salinization) and biotic (phytopathogenic microorganisms and insect pests) factors is of great commercial value. Genetic engineering is an essential method for improving agronomic characteristics of plants, supplementing the traditional breeding approaches. Although a number of laboratories in the world are involved in producing and improving transgenic wheat, the transformation efficiencies they achieved are mainly low. The transformation efficiency may be elevated via optimization of the transformation parameters and various methods for selection of transgenic cells and regenerants. For the practical purposes, it is necessary to produce a large number of independent transgenic events in widely cultivated wheat cultivars.

The technique of biolistic tissue transformation was developed by Sanford and Klein [1]. The general principle of this transformation approach is bombardment of the target tissue in a vacuum chamber with the metal microparticles carrying DNA. So far, transgenic plants belonging to various families of the plant kingdom have been obtained. A variety of ballistic devices are available, including gas, gunpowder, and electric ones [1–5]. Different operation modes of such devices and specific features of their design require selection of the condi-

tions for biolistic transformation for each particular device and each plant species to be transformed. Among the commercial devices of this type, most widespread are PDS1000He (BIORAD) and helium-utilizing Particle Inflow Gun (PIG), constructed in 1992 by Finer et al. [2]; the latter is simple and inexpensive in operation.

The factors influencing the biolistic efficiency may be conditionally divided into the following three groups.

(1) Physical parameters: the helium pressure necessary for a sufficient acceleration of DNA-carrying microparticles, the value of negative vacuum pressure in the chamber with target cells, and the distance of the target tissue to the particle source and sieve plate. This group of parameters includes also the size of microparticles, their number per one shot, and the number of bombardments of the target tissue.

(2) Chemical parameters: types and concentrations of the reagents used for DNA precipitation onto metal particles, namely, inorganic salts (CaCl_2 , MgCl_2 , and $\text{Ca}(\text{NO}_3)_2$) and organic components (PEG, glycerin, ethanol, and spermidine). This group of parameters includes also pH of the medium, concentration of DNA to be delivered, and the nature of the metal for producing microparticles (tungsten, gold, or platinum).

(3) Biological parameters: the degree of competence of the cells to be transformed for regeneration of morphogenic structures and plants; the degree of plasmoly-

Table 1. Compositions of the media for cultivation of common wheat somatic cells and in vitro plant regeneration

Medium	Medium components
(A) Medium for callus induction	MS macro- and microsalts, B5 vitamins, 2,4-D (2 mg/l), sucrose (30 g/l), and agar (8 g/l)
(B) Medium for osmotic impact	MS macro- and microsalts, B5 vitamins, 2,4-D (2 mg/l), sucrose (142 g/l), and agar (8 g/l)
(C) Medium for shoot regeneration	MS macro- and microsalts, B5 vitamins, kinetin (1 mg/l), BAP (1 mg/l), NAA (0.5 mg/l), sucrose (30 g/l), and agar (8 g/l)
(D) Medium for rooting	1/2 MS macro- and microsalts, 1/2 B5 vitamins, NAA (0.5 mg/l), sucrose (15 g/l), and agar (8 g/l)

sis of the explant cells induced by pre- and post-bombardment osmotic impact (cultivation on the medium with increased osmotic pressure); and the nature of introduced DNA, its structure (circular double-stranded, circular single-stranded, linear double-stranded, or linear single-stranded). The most important factor of this group influencing the transformation efficiency is the state of the cell population at the moment of bombardment event, i.e., the ratio of the cells at the stage of DNA synthesis to the dividing cells.

The method used for selection of transgenic plants is among the key factors influencing the efficiency of wheat transformation. The gene *bar*, which determines the resistance to phosphinothricin (PPT) is a widespread gene encoding selective markers. PPT is the main component of the herbicide bialaphos (commercial names of the herbicide, BASTA, LIBERTY).

The goal of this work was to optimize the parameters for biolistic transformation of common wheat with a helium device, particle inflow gun, constructed by Finer et al. and to choose the optimal technique for selection of transformed tissues of commercial common wheat cultivars using the herbicide phosphinothricin as a selective agent.

MATERIALS AND METHODS

Plant material. Russian spring wheat cultivars *Lada*, *Ester*, and *Mis*, introduced in the Volga region and the Republic of Tatarstan (the authors are N.V. Davydova et al., Research Institute of Agriculture of the Central Nonchernozem Zone) were used in the work.

The plants for experiments were grown in a greenhouse at a photoperiod of 16 h, day temperature of 25°C, and night temperature of 15°C. Cut immature spikes were decorticated to place the immature caryopses into a refrigerated chamber at a temperature of 4°C for 48 h.

Sterilization of material. The immature caryopses were sterilized with 70% ethyl alcohol for 6 min followed by washing with sterile distilled water three times for 5 min.

In vitro induction of wheat somatic cells. Immature wheat embryos on days 10–12 after pollination were used as explants for producing morphogenic calluses. The size of the embryos used in experiment varied from

0.5 to 1.5 mm (Fig. 1a). The embryos were isolated and placed onto the medium for inducing callus formation, A [6]. The compositions of the media used for cultivation of wheat somatic cells and tissues are listed in Table 1.

Regeneration and rooting of shoots. To regenerate shoots, the morphogenic callus was placed onto regeneration medium, C (Fig. 1b). Calluses were cultivated on the regeneration medium for 2–4 weeks with one–two passages depending on the development rate of explants at an illumination of 5000 lx. The morphogenic calluses that developed shoots were transferred into the tubes with medium D for rooting. The shoots were cultivated on the rooting medium (D) for 4–6 weeks until development of primary roots (Fig. 1c). The rooted regenerated plantlets were replanted into perlite and placed into a humid chamber. As the plants developed, they were transferred into soil and raised until complete maturation under greenhouse conditions.

Genetic constructs. The plasmids psGFP-BAR, pUbi1-GUS, and pGST-BAR were used. The genetic construct psGFP-BAR contains *bar* gene, determining the resistance to herbicide phosphinothricin (PPT), and the marker gene *gfp*, encoding a green fluorescent protein [7]. This vector, constructed specially for transformation of cereals, contains cereal-specific promoters—rice *Act1* and corn *Ubi1*, which provide a constitutive gene expression in cereal plants [8]. The genes inserted (*bar* and *gfp*) contain introns in order to boost their expression. The plasmid pGST-BAR was constructed from psGFP-BAR. The gene *gst* (encoding glutathione-S-transferase), isolated from *Arabidopsis thaliana*, was cloned instead of *gfp* gene at the sites of *NcoI* and *NotI* restriction endonucleases. The plasmid pUbi1GUS was constructed using psGFP-BAR and p035 to provide expression of the marker gene *uidA* (GUS), encoding the enzyme β -glucuronidase, in the transformed tissue. These genetic constructs were produced at the Laboratory of N.V. Ravin.

Isolation of plasmid DNA. Plasmid DNA from an overnight *Escherichia coli* strain DH10 culture was isolated by the alkaline lysis technique followed by purification with phenol–chloroform.

Sterilization of microparticles. A weighed sample of M10 microparticles (50 mg) was sterilized with alcohol for 15 min. The particles were sedimented using an

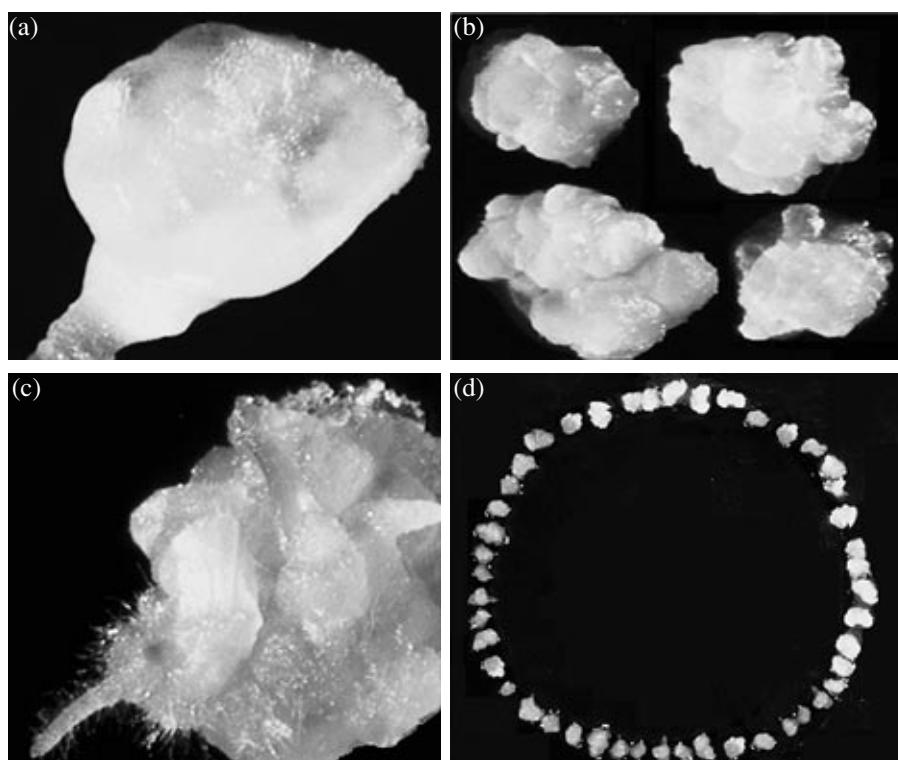


Fig. 1. Cultivation of common wheat (*Triticum aestivum* L.) tissues: (a) an immature embryo after 72-h cultivation; (b) a morphogenic callus; (c) shoot induction and rooting; and (d) morphogenic calluses prepared for biolistic transformation.

Eppendorf 5415 centrifuge at 14 000 rpm for 6–8 min and washed three times with sterile water. The final volume of particle suspension in water amounted to 500 μ l.

The expression vectors were precipitated onto the particles by calcium–permidine method [2].

Osmotic impact on the tissue. On days 4, 7, 10, and 14, the explants used in the experiment on parameter optimization were transferred onto osmotic medium B, containing 0.4 M sucrose as an osmotic agent. In the experiment on optimization of biolistic transformation parameters, 20 explants with a size of $\sim 3\text{--}4\text{ mm}^2$ were placed on one petri dish and kept on osmotic medium B for 4–6 h before biolistic transformation and 20–24 h after the transformation.

In the experiment on comparison of selection systems, 50 morphogenic calluses on day 10 of cultivation with a size of $3\text{--}4\text{ mm}^2$ were placed on one petri dish (Fig. 1d) and kept on osmotic medium B for 4–6 h before biolistic transformation and 20–24 h after the transformation.

Initial parameters of biolistic transformation. Biolistic transformation was performed using a particle inflow gun. The parameters developed for soybean transformation were used as initial, namely, gas volume for the shot, 6 cm^3 ; the distances from source particles (nozzle of filter holder) to target tissue, 17 cm and from target tissue to sieve plate, 9 cm; helium pressure during the shot, 7 atm; cross-section of sieve plate mesh,

500 μm ; and negative vacuum pressure in the chamber during the shot, 30 mm Hg; each petri dish was bombarded twice [2].

In the experiment on comparison of selection systems, the following parameters were used in addition to those listed above: helium pressure during the shot, 6 atm and a distance of target tissue (callus) from the particle source (filter holder), 12 cm.

Determination of the uidA (GUS) gene expression. The plant explants were stained histochemically according to Jefferson's technique [9]. The preparations were examined using a Wild Leica (Heerbrugg, Switzerland) stereomicroscope.

Determination of gfp gene expression. Both transient and stable *gfp* expressions were assessed using a stereomicroscope equipped with a fluorescent module with a 100-W mercury lamp and GFP2 filter with a Leica 480/40-nm absorbent filter and 510-nm cutoff filter (Heerbrugg, Switzerland). The expression was evaluated on days 2, 7, and 35 after biolistic transformation.

The variants of wheat transformant selection using PPT as a selective agent are listed in Table 2.

PCR assay. All the phosphinothricin-resistant plants produced in vitro were assessed for the presence of *bar* gene and *nos3'* terminator sequences. For the PCR assay, wheat genomic DNA was isolated from juvenile leaves [10]. PCR was performed using the preparation of genomic DNA and two pairs of primers specific for

Table 2. Variants for selection of common wheat transformants using PPT as a selective agent

Selection variants	Callus formation (PPT, mg/l)	Regeneration (PPT, mg/l)	Rooting (PPT, mg/l)	Selection period, days
Gradation: test 1	One week (1) Two weeks (3) Two weeks (5)	5	5	~98
Delayed: test 2	One week without PPT Two weeks (5) Two weeks (5)	5	3	~92
Regeneration: test 3	Two week without PPT	5	3	~70

the *bar* gene and the *nos3'* terminator. Amplification was conducted in an Eppendorf Mastercycler gradient (Germany) in a volume of 25 μ l of a mixture containing 1 \times buffer for BioTaq polymerase (2 mM MgCl₂, 17 mM (NH₄)₂SO₄, and 6 mM Tris-HCl, pH 8.8), 5 nmol of dNTP-mix, 20 ng of DNA template, 6.25 pmol of forward and reverse primers each, and 1.5 AU of BioTaq DNA polymerase (Dialat LTD, Russia).

PCR was run in the following conditions: (1) 2 min at 95°C; (2) 40 cycles of 30 s at 95°C, 30 s at 65°C, and 40 s at 72°C; (3) 7 min at 72°C.

Statistical analysis of the data. The effect of each pair of parameters at a constant volume of the gas used (6 cm³) on the delivery of expression vectors to target tissues was studied in eight replicates. For each experiment, 20 explants of Lada or Mis cultivar were used. The cultivar Ester was studied in six replicates, also 20 explants per experiment. The mean values were calculated from eight experiments for each pair of parameters. Standard deviation (SD) was calculated as

$$\sqrt{\frac{n \sum x^2 - (\sum x)^2}{n^2}}$$
, where n is the general population and x , the value estimated. Confidence interval was calculated as $\chi \pm 1.96 \left(\frac{\sigma}{\sqrt{n}} \right)$, where n is the general population and σ , standard deviation.

RESULTS AND DISCUSSION

The majority of studies on optimizing the parameters for biolistic transformation of common wheat use PDS1000He (BIORAD) device [11]. We used a particle inflow gun, for which the experimental data on the optimal parameters for common wheat biolistic transformation are yet absent. PIG was applied successfully to transform soybean (*Glycine max* Merrill.) and corn (*Zea mays* L.) [2]. The parameters that we obtained—12-cm distance from the particle source to the target tissue and 6-atm helium pressure—are optimal for transformation of common wheat. These values coincide with the parameters optimized for transformation of sunflower (*Helianthus annuus* L.) [12]. Note that the

optimized parameters are species- and cultivar-specific. For example, transformation of cassava (*Manihot esculenta* Crantz.) using PIG is performed at a distance of 10.5 cm and helium pressure of 6–8 atm [13].

Optimization of the Parameters for Common Wheat Biolistic Transformation

Two physical parameters influencing the delivery of expression cassettes to the cells obtained from immature embryos and competent for shoot regeneration were compared to optimize the biolistic transformation parameters for common wheat cultivars: the excess helium pressure during the shot (5, 6, 7, and 8 atm) and the distance from the particle source (nozzle of filter holder) to the target tissue (10, 12, and 14 cm).

The effect of the time of plant explant cultivation on the medium A, inducing callus formation, on transformation was evaluated as an important biological parameter influencing the rate of expression vector delivery. The visually selected morphogenic calluses and immature embryos were bombarded on days 4, 7, 10, and 14 after planting embryos on medium A in the case of Lada and Mis cultivars. The biolistic transformation of the cultivar Ester was performed on days 4, 7, and 10 after planting on medium A.

The efficiency of gene delivery was assessed via detection and calculation of the blue spots, displaying a transient expression of *uidA* gene. The results are listed in Tables 3–5.

The highest expression level of *uidA* gene was detectable 48 h after the biolistic treatment. A high transient expression level of the marker gene was observed when using three pairs of biolistic parameters: 5 atm and 10 cm, 6 atm and 12 cm, and 8 atm and 14 cm (Tables 3–5).

These results indicate that the optimal parameters for the delivery of expression cassettes are helium pressure of 6 atm, 12-cm distance to the target tissue, and the morphogenic calluses on day 10 or 14 of cultivation on medium A, inducing callus formation (Fig. 2). For example, over 500 events of expression cassette delivery per ~3–4 mm² area were recorded in experiment with the cultivar Lada; 223.3 and 366.3 events, respec-

Table 3. Assessment of the efficiency of *uidA* gene delivery to wheat cultivar Lada cells depending of physical and biological parameters

<i>L</i> , cm	5 atm		6 atm		7 atm		8 atm	
	mean	SD	mean	SD	mean	SD	mean	SD
Day 4 of cultivation								
10	161.7	±14.62	136.6	±16.25	65.9	±14.76	51.9	±11.61
12	163.6	±10.53	190.9	±16.23	155.7	±14.02	107.4	±14.61
14	62.5	±21.46	73.3	±23.35	101.1	±17.12	165.6	±21.27
Day 7 of cultivation								
10	245.3	±18.16	±214.5	±27.65	167.6	±19.67	100.1	±15.26
12	198.7	±31.00	±286.6	±23.14	165.4	±23.52	138.1	±24.08
14	109.2	±26.36	±152.3	±26.42	232.4	±30.31	275.7	±30.16
Day 10 of cultivation								
10	340.3	±27.38	283.4	±15.39	231.6	±16.70	147.1	±16.88
12	278.9	±10.58	533.0	±35.25	353.5	±16.35	256.8	±12.46
14	167.5	±18.46	227.6	±22.19	303.7	±17.73	376.8	±16.37
Day 14 of cultivation								
10	266.0	±26.87	255.3	±19.49	221.3	±16.93	126.5	±18.80
12	277.4	±35.76	377.4	±39.04	274.2	±21.66	220.5	±21.06
14	190.9	±21.07	229.0	±21.88	292.8	±20.02	307.5	±31.85

Notes for Tables 3–5: *L*, distance from the particle source to the target, cm. The mean efficiency was obtained from eight experiments; SD, standard deviation. The highest expression level is bold-faced.

tively, for the cultivars Mis and Ester. The maximal number of spots displaying transient expression were obtained in the experiment with the cultivar Lada; this value was minimal for the cultivar Mis, while the cultivar Ester appeared intermediate in this respect (Fig. 2).

According to our studies, the transformation efficiency depends on the genotype, which determines the frequencies of morphogenic callus induction and shoot regeneration as well as the survival rate of the plant tissue during bombardment and selection. The background for this conclusion was that the cultivars used displayed different frequencies of the foci with a transient expression of *uidA* (GUS) gene at the same biolistic parameters. Different regeneration and transformation efficiencies (varying from 0 to 76%) were observed when assessing the effect of 129 hybrid genotypes produced via crosses with the commonly used wheat cultivar *Bobwhite* on the regeneration and transformation competences [14].

Under conditions of our experiment, the combination of 10-cm distance and 6 atm appeared inefficient for biolistic transformation of common wheat. Thus, we pioneered in demonstrating with the use of the GUS reporter system that a 12-cm distance from the particle source to the target tissue and helium pressure of 6 atm were the optimal physical parameters for PIG-assisted wheat biolistic transformation. The optimal time point for transformation was days 10–14 from the initiation of common wheat tissue culture.

Transient and Stable Expressions of gfp Marker Gene

Note that the GUS reporter system allows for in situ analysis; however, it is inappropriate for dynamic monitoring of reporter gene expression. Thus, we used the GFP (green fluorescent protein) reporter system to monitor the dynamics of transient and stable expressions in common wheat cells. The main advantage of this system is the possibility of intravital assessment of reporter gene expression. For this purpose, we used here the vector psGFP-BAR.

The transient expression of the *gfp* marker gene was recorded already 4 h after biolistic transformation. The transient expression of *gfp* gene reaches its maximum 6.5 h after transformation to disappear completely after 17 days [15]. The *gfp* expression observed after this moment is regarded as stable.

The presence/absence of expression of the *gfp* marker gene was assessed on days 2, 7, and 35 after biolistic transformation. On day 2, the frequency of *gfp* transient expression amounted to 100% of the evaluated explants for all the selection variants used (Fig. 3). The frequency of transient expression detectable on day 7 was 30 to 70% (Table 6). The stable *gfp* expression, detected on day 35 when the calluses were replanted from callus-inducing/selection medium to regeneration/selection medium, amounted to 9–13%; in test 3, the stable expression was assessed in the explants cultivated on the medium inducing shoot formation and amounted to 22.9% (Table 6).

Table 4. Assessment of the efficiency of *uidA* gene delivery to wheat cultivar Mis cells depending of physical and biological parameters

L, cm	5 atm		6 atm		7 atm		8 atm	
	mean	SD	mean	SD	mean	SD	mean	SD
Day 4 of cultivation								
10	123.0	±21.73	113.5	±17.97	84.3	±16.64	61.3	±19.45
12	116.4	±17.25	136.5	±13.20	94.3	±20.62	90.6	±13.48
14	56.6	±20.52	91.3	±10.05	114.0	±12.02	139.9	±14.69
Day 7 of cultivation								
10	152.7	±20.46	131.6	±16.11	95.8	±7.43	77.7	±17.43
12	142.1	±19.34	167.5	±16.70	140.0	±10.62	124.7	±12.59
14	79.0	±15.92	73.8	±16.51	138.9	±19.46	159.3	±19.40
Day 10 of cultivation								
10	189.4	±14.51	166.0	±17.94	135.4	±16.58	106.1	±12.06
12	158.4	±11.64	223.3	±31.84	166.0	±17.95	151.4	±13.11
14	74.3	±15.00	126.8	±10.49	161.0	±13.53	192.1	±17.19
Day 14 of cultivation								
10	172.9	±16.83	140.1	±19.74	117.3	±30.57	105.4	±12.35
12	156.9	±13.33	225.6	±25.02	170.3	±19.75	131.9	±18.92
14	89.8	±13.65	138.5	±7.83	167.0	±11.64	229.1	±31.01

Table 5. Assessment of the efficiency of *uidA* gene delivery to wheat cultivar Ester cells depending of physical and biological parameters

L, cm	5 atm		6 atm		7 atm		8 atm	
	mean	SD	mean	SD	mean	SD	mean	SD
Day 4 of cultivation								
10	170.2	±25.69	143.6	±18.42	146.2	±17.28	89.5	±14.52
12	159.3	±12.75	183.5	±23.94	163.5	±30.12	142.3	±14.65
14	51.5	±21.91	82.8	±19.32	148.3	±29.83	189.2	±20.92
Day 7 of cultivation								
10	218.7	±25.87	187.7	±20.51	157.6	±20.72	119.5	±20.76
12	204.3	±22.71	244.3	±22.76	190.6	±26.05	169.8	±23.75
14	119.8	±25.84	162.2	±20.19	189.7	±10.76	228.8	±26.68
Day 10 of cultivation								
10	336.6	±24.85	270.8	±12.59	161.2	±25.16	139.3	±12.10
12	282.4	±12.53	366.3	±38.70	263.5	±24.21	163.0	±14.97
14	153.2	±15.76	195.0	±11.61	224.4	±34.96	331.8	±39.02

Comparison of Techniques for Selection of Transgenic Wheat Tissues

The selection systems were evaluated via comparison of the three most efficient techniques involving PPT selective agent. The selection variants used in the work are shown in Table 2. Initially, the frequency of primary transformants was determined according to the number of plants obtained upon an in vitro selection on the selective medium. Then the transformation effi-

ciency for common wheat plants was calculated from the data of PCR assay of the genomic DNA isolated from all the primary transformants produced. Electrophoretic patterns of PCR assay of the transformants with respect to the *bar* gene and the *nos3'* terminator are shown in Figs. 4b and 4c. These results show that the lengths of PCR fragments corresponding to the *bar* gene and the *nos3'* terminator, amounting to 340 and 185 bp, and fit the theoretically expected values.

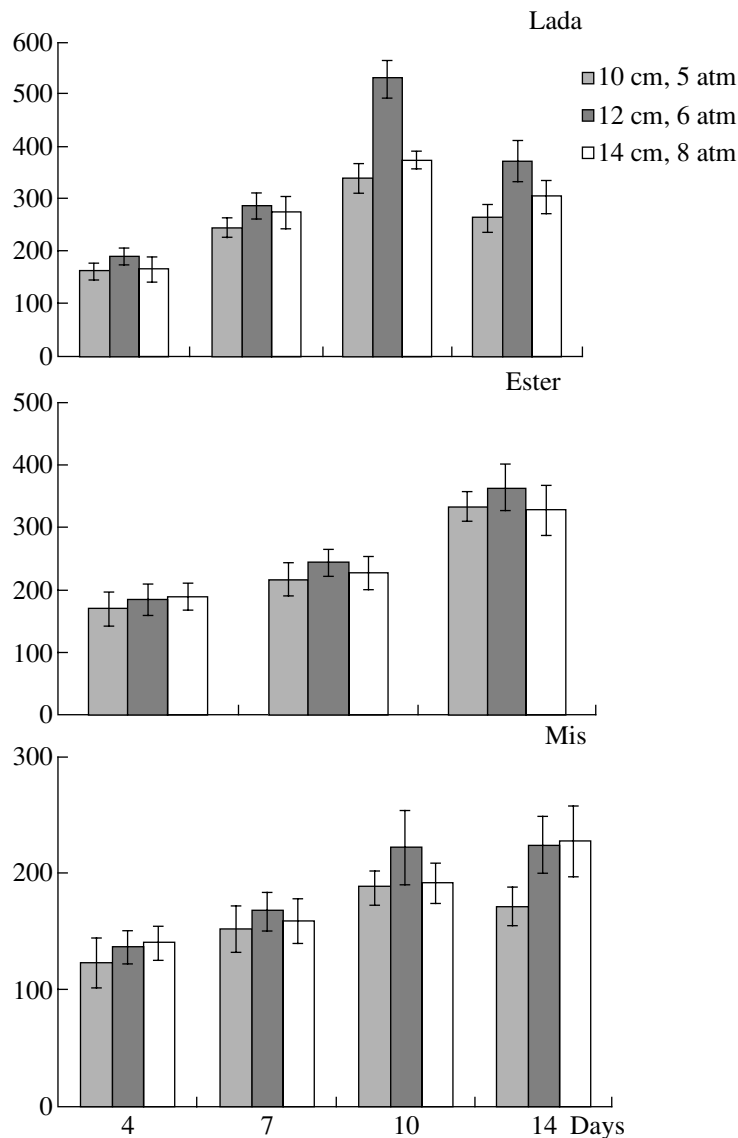


Fig. 2. The maximal value of transient *uidA* gene expression for three pairs of physical parameters depending on cultivation time and common wheat cultivars used (ordinate, the number of expression spots).

The gradation technique for selecting tissues upon transformation involves a gradual (stepwise) increase in the concentration of selective agent (phosphinothricin) in the culture medium. The selection impact on the explant commenced on day 2 after biolistic transformation. The tissue was transferred from osmotic medium to the medium inducing callus formation containing 1 mg/l PPT followed by increase in PPT concentration to 5 mg/l at the stage of callus formation. Overall, the selective treatment covered 98 days. The gradation technique allows for stringent selection of the true transformants. The transformation efficiency for wheat plants thus determined (test 1) amounted to 0.85% for the cultivar Lada and 2.12% for the cultivar Mis (Table 6).

Selection of plant tissues according to delayed selection technique (test 2) commenced 7 days after the

biolistic transformation. The treated callus tissue was transferred from osmotic medium to medium A for callus induction. A delay in selective action on the tissue is intended to increase the number of cells that successfully integrated the DNA introduced into their genome. The division rate of meristematic cells (the callus tissue used in the experiments is of this type) amounts to 20–24 h; thus, the number of cells potentially containing the transgene within their genome will grow from 1 to 64–128 during 7 days of tissue growth on the callus-inducing medium. An increase in the total mass of transgenic tissue elevates the probability of transgenic cells to survive under selective pressure. The total period of selection using the delayed technique was 92 days (Table 2); the transformation efficiency thus calculated amounted to 0.65% versus the transforma-

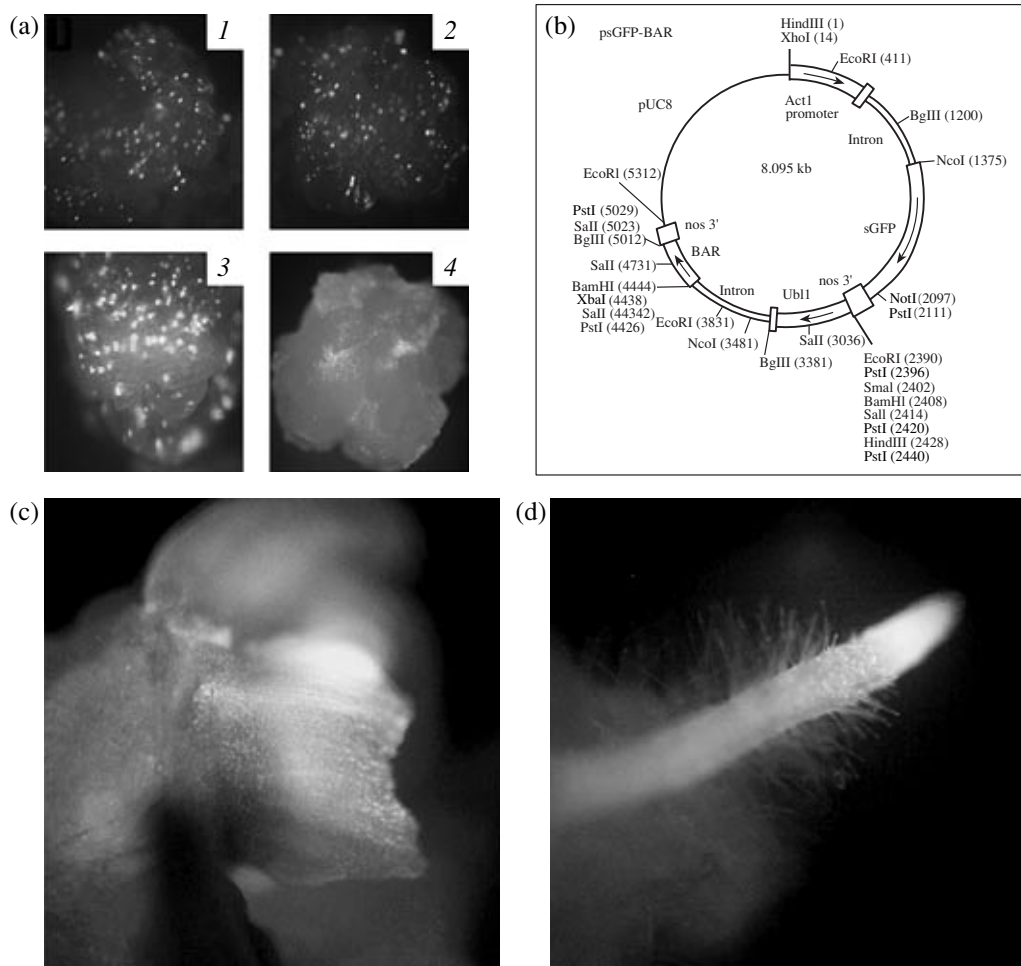


Fig. 3. The transient and stable expressions of marker *gfp* gene. (a) Transient expression: (1 and 2) 24 h after biolistic transformation; (3) 2 days after biolistics; and (4) control (morphogenic callus without biolistic treatment). (b) Schematic map of the plasmid psGFP-BAR. (c) Stable *gfp* expression in a wheat shoot on day 35 after biolistic transformation. (d) A root of transgenic wheat with a high *gfp* expression on day 35 after biolistic transformation. The number of nucleotide pairs is given in parentheses.

tion frequency calculated according to the number of primary transformants equal to 1.57% (Table 6).

The distinction of the regeneration selection system (test 3) is that the selection of transformed tissues is commenced at the stage of regeneration. After the

biolistic transformation, the tissue was kept for 10–12 days on medium A for induction of callus formation. This long cultivation on the medium for induction of morphogenic calluses assists in excluding the cases of transient expression, which disappears 14 days after

Table 6. Efficiency of common wheat transformation using various selection systems

Cultivar	Number of explants per experiment	Number (%) of GFP + calluses		Number of primary transformants (transformation frequency, %)	Total number of transgenic plants (transformation efficiency, %)
		7 days after biolistics	35 days after biolistics		
Lada, test 1	466	186 (40)	51 (10.9)	4 (0.85)	4 (0.85)
Lada, test 2	446	214 (48)	57 (12.8)	7 (1.57)	3 (0.65)
Lada, test 3	140	98 (70)	32 (22.9)	9 (6.43)	7 (5)
Lada, test 3, <i>gst</i>	350	–	–	23 (6.57)	17 (4.86)
Mis, test 1	106	34 (32)	9 (8.9)	2 (2.12)	2 (2.12)
Mis, test 2	104	44 (42)	10 (9.6)	1 (0.96)	–

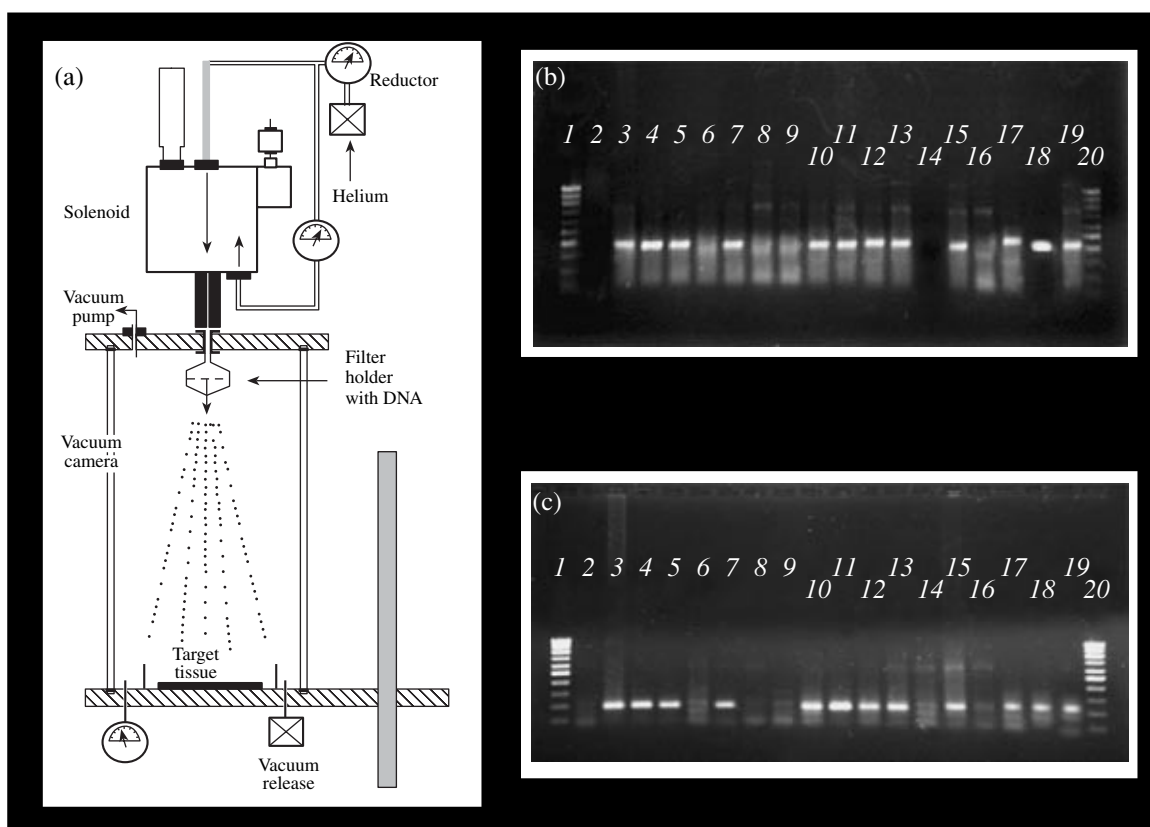


Fig. 4. (a) Scheme of particle inflow gun (PIG) and PCR assay of a part the putative transformants produced using the primers specific to (b) *bar* and (c) *nos3'*. Lanes in the electrophoretic pattern: (1 and 20) MWMarker 1 kb; (2) negative control, wheat DNA; (3–17) DNA of primary transformants; (18) positive control, potato DNA; and (19) psGFP-BAR.

transformation. Replanting of calluses onto regeneration medium was the start of selection (Table 2). It is known that the herbicide PPT does not inhibit the chloroplast isoform of wheat (*T. aestivum* L.) glutamine synthetase; however, the root isoform is sensitive to this herbicide [16, 17]. Thus, the determinative impact of the herbicide on the plant tissue takes place during regeneration and rooting, i.e., during 2–4 weeks at the stage of regeneration (depending on the shoot development rate) and 4–6 weeks at the stage of rooting. Development of a vigorous root system is the main qualitative indicator of tissue resistance to the herbicide. The efficiency of transformation with the genetic construct psGFP-BAR assessed using the regeneration selection system amounted to 5%. Similar results were obtained with the pGST-BAR genetic construct: its efficiency was 4.86% versus the transformation efficiency calculated according to the number of primary transformants amounting to 6.43% (Table 6).

The regeneration selection system recorded the maximum transformation efficiency of 4.86–5%, depending on the genetic constructs used. The graduation and delayed selection systems gave an insignificant difference in transformation efficiencies and a wide scattering of data in the general population, which made the results obtained using these systems for selec-

tion of common wheat transgenic tissues statistically nonsignificant.

The earliest work on transformation of common wheat (*T. aestivum* L.) dates back to 1992. Numerous studies aimed at optimization of parameters for biolistic transformation, selection, and optimization of cultivation techniques have been performed since that time [18–25]. The efficiency of biolistic wheat transformation reported in these papers amounts to 1–5%. Our results demonstrate that the used approach consisting in optimization of physical and biological parameters for common wheat biolistic transformation, on the one hand, and choosing of the appropriate selection system, on the other, allowed us to obtain high transformation efficiency (Table 6). Unlike the other works on wheat transformation [18–25], this efficiency was achieved by use of a particle inflow gun.

ACKNOWLEDGMENTS

The authors thank John Finer (Ohio State University) for generously providing a particle inflow gun; B.V. Conger (Department of Plant and Soil Science, University of Tennessee, Knoxville, United States) and P. Quail for providing genetic construct psGFP-BAR; and N.V. Ravin for developing the genetic constructs

pUbi1-GUS and pGST-BAR. The authors are grateful to the colleagues from the Bioengineering Center, Russian Academy of Sciences, B.B. Kuznetsov and N. Slobodova, for their assistance in PCR assay of transgenic wheat plants and to N.V. Davydova (Research Institute of Agriculture of the Central Nonchernozem Zone) for the seeds of wheat cultivars used in the work.

The work was supported by the Ministry of Science and Technologies of the Russian Federation.

REFERENCES

- Sanford, J.C., Klein, T.M., Wolf, E.D., and Allen, N., Delivery of Substances into Cells and Tissues Using a Particle Bombardment Process, *J. Particulate Sci. Technol.*, 1987, no. 5, pp. 27–37.
- Finer, J.J., Vain, P., Jones, M.W., and McMullen, M.D., Development of the Particle Inflow Gun for DNA Delivery to the Plant Cells, *Plant Cell Rep.*, 1992, no. 11, pp. 323–328.
- Takeuchi, Y., Dotson, M., and Keen, N.T., Plant Transformation: A Simple Particle Bombardment Device Based on Flowing Helium, *Plant. Mol. Biol.*, 1992, vol. 18, no. 4, pp. 835–839.
- Sautter, C., Waldner, H., Neuhaus-Url, G., et al., Micro-Targeting: High Efficiency Gene Transfer Using a Novel Approach for the Acceleration of Microprojectiles, *Bio/Technology*, 1991, no. 9, pp. 1080–1085.
- Christou, P., McCabe, D.E., and Swain, W.F., Stable Transformation of Soybean Callus by DNA-Coated Gold Particles, *Plant Physiol.*, 1988, no. 87, pp. 671–674.
- Gaponenko, A.K., Muntyan, M.A., Malikova, N.I., and Sozinov, A.A., In Vitro Regeneration of Wheat *Triticum aestivum* L. Plants of Various Genotypes, *Dokl. Akad. Nauk SSSR*, 1984, vol. 278, no. 5, pp. 1231–1235.
- Chalfie, M., Tu, Y., Euskirchen, G., et al., Green Fluorescent Protein As a Marker for Gene Expression, *Science*, 1994, no. 263, pp. 802–805.
- Richards, H.A., Rudas, V.A., Sun, H., et al., Construction of a GFP-BAR Plasmid and Its Use for Switch Grass Transformation, *Plant Cell Rep.*, 2001, no. 20, pp. 48–54.
- Jefferson, R.A., Assaying Chimeric Genes in Plants: The *gus* Gene Fusion System, *Plant Mol. Biol.*, 1987, rep. 5, pp. 387–405.
- Hess, D., Dressler, K., and Nimmrichter, R., Transformation Experiments by Pipetting *Agrobacterium* into the Spikelets of Wheat (*Triticum aestivum* L.), *Plant Sci.*, 1990, no. 72, pp. 233–244.
- Rasco-Gaunt, S., Riley, A., Barcelo, P., and Lazzeri, P.A., Analysis of Particle Bombardment Parameters to Optimized DNA Delivery into Wheat Tissues, *Plant Cell Rep.*, 1999, no. 19, pp. 118–127.
- Gaponenko, A.K., RF Patent no. 2193066 (2002).
- Zhang, P. and Puonti-Kaerlas, J., PIG-Mediated Cassava Transformation Using Positive and Negative Selection, *Plant Cell Rep.*, 2000, no. 19, pp. 939–945.
- Schenk, P.M., Elliott, A.R., and Manners, J.M., Assessment of Transient Gene Expression in Plant Tissues Using the Green Fluorescent Protein, *Ref. Plant Mol. Biol.*, 1998, rep. 16, pp. 313–322.
- Chen, W.P., Gu, X., Liang, G.H., et al., Introduction and Constitutive Expression of a Rice Chitinase Gene in Bread Wheat Using Ballistic Bombardment and the *bar* Gene As a Selectable Marker, *Theor. Appl. Genet.*, 1998, vol. 97, pp. 1296–1306.
- Wild, A., Sauer, H., and Ruhle, W., The Effect of Phosphinothricin (Glufosinate) on Photosynthesis I Inhibition of Photosynthesis and Accumulation of Ammonia, *Z. Naturforsch., A: Phys. Sci.*, 1987, vol. 42, pp. 263–269.
- Vasil, V., Castilo, A.M., Fromm, M.E., and Vasil, I.K., Herbicide Resistant Fertile Transgenic Wheat Plants Obtained by Microprojectile Bombardment of Regenerable Embryogenic Callus, *Biotechnology*, 1992, vol. 10, pp. 667–673.
- Uze, M., Potrykus, I., and Sautter, C., Single-Stranded DNA in the Genetic Transformation of Wheat (*Triticum aestivum* L.): Transformation Frequency and Integration Pattern, *Theor. Appl. Genet.*, 1999, vol. 99, pp. 487–495.
- Wild, A. and Manderscheid, R., The Effect of Phosphinothricin on the Assimilation of Ammonia in Plants, *Z. Naturforsch., A: Phys. Sci.*, 1984, vol. 39, pp. 500–504.
- Weeks, J.T., Anderson, O.D., and Blechl, A.E., Rapid Production of Multiple Independent Lines of Fertile Transgenic Wheat (*Triticum aestivum* L.), *Plant Physiol.*, 1993, vol. 102, pp. 1077–1084.
- Altpeter, F., Vasil, V., Srivastava, V., et al., Accelerated Production of Transgenic Wheat (*Triticum aestivum* L.) Plants, *Plant Cell Rep.*, 1996, vol. 16, pp. 12–17.
- Becker, D., Brettschneider, R., and Lörz, H., Fertile Transgenic Wheat from Microprojectile Bombardment of Scutellar Tissue, *Plant J.*, 1994, vol. 5, pp. 299–307.
- Wright, M., Dawson, J., Dunder, E., et al., Efficient Ballistic Transformation of Maize (*Zea mays* L.) and Wheat (*Triticum aestivum* L.) Using the Phosphomannose Isomerase Gene, *pmi*, As the Selectable Marker, *Plant Cell Rep.*, 2001, vol. 20, pp. 429–436.
- Huber, M., Hahn, R., and Hess, D., High Transformation Frequencies Obtained from a Commercial Wheat (*Triticum aestivum* L. cv. “Combi”) by Microbombardment of Immature Embryos Followed by GFP Screening Combined with PPT Selection, *Mol. Breeding*, 2002, vol. 10, pp. 19–30.
- Jakson, S.A., Zhang, P., Chen, W.P., et al., High-Resolution Structural Analysis of Ballistic Transgene Integration into Genome of Wheat, *Theor. Appl. Genet.*, 2001, vol. 103, pp. 56–62.