Transformation of immature wheat germ (Triticum aestivum L.) by particle bombardment

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Abstract. The aim of the research was to create an effective method for producing transgenic wheat plants suitable for a wide range of promising varieties, both spring and winter crops. The plant material was cultivated at temperatures ranging from 4 to 25°C, either in the dark or in the light, with a 16-hour photoperiod (16/8 - day / night). Osram L36/77 FLUORA and F36W/33 Cool White lamps were used for lighting. The composition of all nutrient media included macro- and micro-salts, vitamins B5, phytohormones and carbohydrates. The pH of the medium was adjusted to 5.8 before autoclaving. The medium was sterilized in an autoclave at pressure of 1.2 atmospheres for 15 minutes. An effective method of regeneration of transgenic wheat plants for ballistic transformation has been developed. Plants obtained by this method are phenotypically normal and fully fertile. The transgenic insertion of the target gene is transmitted to the offspring in accordance with Mendel's laws. The transformation efficiency was high for all the studied varieties and ranged from 1.4 to 7.8%.

1. Introduction
Wheat (Triticum aestivum L.) is the most important crop of all cereals for most of the world's population. It is a staple food for two billion people (36% of the world's population). Wheat yield losses in various environmental conditions occur due to water scarcity (drought), salinity, frost, heat, fungal and viral diseases, and insects. Each of these environmental factors can reduce the yield, on average, by 15%. To increase crop yields, in a rapidly changing climate, and reduce natural resources, a new generation of varieties that are widely adaptive and effectively use the resources of the environment is needed.

Over the past thirty years, various transgenic techniques have been developed to transfer genes from a wide range of organisms to cereals. However, the availability of an effective transformation method for introducing foreign DNA into the genome is a significant barrier for most monocotyledonous species, including maize, rice, oats, barley, and, in particular, wheat (due to the complex polyploid genome, the large number of repetitive DNA sequences, the low ability of plants to regenerate, and difficulties with genetic transformation).

The first bioballistic transformation of wheat was carried out in 1992 [1]. Currently, the process of creating transgenic wheat plants by transferring DNA through particle bombardment is considered quite routine. However, this is true only for some varieties that are responsive to transformation, such as
Bobwhite, Florida, Fielder, Cadenza, etc., but even in these cases, the transformation efficiency does not exceed 1-6% [2, 3, 4, 5, 6].

The transfer of genes of improved traits to "model" wheat requires the subsequent transfer of embedded transgenes to food varieties through conventional crossing, and can make it difficult to select transgenic traits from those linked to undesirable traits [7, 8, 9].

Thus, it would be more preferable if the transformation technology could be applied directly to highly productive production varieties. But to use wheat transformation in the breeding process, it is necessary to obtain a number of transgenic lines for each gene or trait that needs to be changed. It is necessary that the line was acceptable as a commercial varieties and transgenic line must meet certain criteria, such as ease of the transgene integration, the level and stability of the integrated gene expression, stable inheritance of the new phenotype transgene and acceptability, as well as preserving the characteristics of the original line. This led to the need to switch from working with model genotypes to using productive commercial varieties, most of which, as it turned out, transform much worse than laboratory ones. Wheat varieties of the Russian selection are not an exception in this sense.

Recently, a positive effect of picloram (an auxin-like substance) on the induction of wheat callus formation was shown [10]. We assume that a positive effect will be obtained from low-temperature treatment of wheat plants before manipulating the embryos and increasing their morphogenetic potential by reducing the action of intracellular lipoxygenases and peroxidases, which lead to a violation of the cell membranes integrity.

Taking into account all the above, when developing this method, the task was to create an effective method for obtaining transgenic wheat plants, suitable for a wide range of commercially promising domestic varieties, both spring and winter. The expected result was an increase in the efficiency and unification of the regeneration procedure for transgenic wheat plants for particle bombardment.

2. Materials and methods

2.1. Plant material

Spring and winter seeds of soft wheat varieties of Russian selection-Zlata, Amir, Agata, Deya and L-1 were obtained from breeders Davydova Natalia Vladimirovna from the Federal Research Center "Nemchinovka", Bespalova Lyudmila Andreevna from the Krasnodar Research Institute of Agriculture named after P. P. Lukyanenko. Wheat plants were grown in the field conditions of the Department of Remote Hybridization of the Main Botanical Garden named after N. V. Tsitsin of the Russian Academy of Sciences.

2.2. The selection of embryos and training material

Immature wheat germ seeds are harvested 10 days after flowering. Immature embryos with the length of 0.7-1.5 mm are optimal for transformation. The time it takes for the embryo to reach the best stage depends on the genotype and season.

2.3. Composition of media and cultivation conditions

The plant material is cultivated at temperatures ranging from 4 to 25 ºC, either in the dark or in the light, with a 16-hour photoperiod (16/8-day/night). Osram L36/77 FLUORA and F36W/33 Cool White lamps were used for lighting. The composition of all nutrient media included macro-and micro-salts [11], vitamins B5 [12], phytohormones and carbohydrates (Table 1). The pH of the medium was adjusted to 5.8 before autoclaving. The medium was sterilized in an autoclave at pressure of 1.2 atmospheres for 15 minutes.

2.4. Cold shock

Low-temperature treatment of donor plants occurs before sterilizing the seeds. Unripe wheat ears are harvested on 10-14 days after pollination. The cut ears are placed with the cut ends in water in the refrigerator at 4 ± 2 ºC for 48 hours.
Table 1. Composition of wheat transformation media additional components.

| Composition                               | Variant A | Variant B | Variant C | Variant G | Variant D |
|-------------------------------------------|-----------|-----------|-----------|-----------|-----------|
| 2.4-D, mg                                 | 0.5       | 0.5       | 0.1       | -         | -         |
| Picloram, mg                              | 2         | 2         | -         | -         | -         |
| Naphthyl acetic acid, mg                  | -         | -         | -         | -         | 0.5       |
| Maltose, g                                | 40        | 120       | 30        | 30        | 15        |
| Glutamine, g                              | 0.5       | 0.5       | 0.5       | -         | -         |
| Hydrolysate of casein, g                  | 0.1       | 0.1       | 0.1       | -         | -         |
| MES, g                                    | 1         | 1         | 1         | 0.5       | 0.5       |
| Agar-agar, g                              | 8         | 8         | 8         | 8         | 8         |
| Selective agent phosphinotricin (after autoclaving) | - | - | - | + | + |

2.5. Sterilization and cultivation of explants on the medium for the induction of callus formation
Unripe grains are exfoliated from the spikelets and sterilized with 70% ethyl alcohol for 6 minutes. Wash with sterile distilled water 3 times for 5 minutes.

The embryos of 0.7-1.5 mm in size are isolated and placed on medium A to induce callus formation with the shield up, 50 pieces per Petri dish (9 cm), and cultured for 14-18 days in the dark 23 ± 2 °C until the callus is formed.

2.6. Osmotic treatment of explants before transformation
Before transformation, the embryos with the callus are laid out on an osmotic medium (B) containing maltose (120 g/l) as an osmotic agent. Explants are placed in the center of the Petri dish, in the form of a ring with a diameter of 40 mm of 30-40 pieces/cup and are kept for 4-6 hours before ballistics and 20-24 hours after.

2.7. Plasmid DNA and bioballistic transformation of explants
For the selection of transformants, a double-marker psGFP-BAR plasmid containing the gfp-reporter gene (green fluorescent protein) and the selective bar gene (tolerance to the herbicide Basta) was used.

Microparticle sterilization: M10 tungsten microparticles (Sylvania Chemical/Metals) are used for bombardment, the particle size in the mixture varies 0.1-7 microns, the largest number of particles 80% has a size of 0.4-2 microns. The suspension of microparticles weighing 50 mg is sterilized with alcohol (500 µl 96% alcohol) for 15 minutes. The particles are deposited in a centrifuge at 14,000 rpm. for 6-8 minutes. Washing of the particles is carried out three times with sterile water. The final volume of the mixture of particles in water is 500 µl.

The precipitation of DNA is as follows:
1. Place 25 µl of the particle mixture in a sterile Eppendorf, then resuspend the mixture and remove it from the bottom of the Eppendorf.
2. Add 5 µl of DNA, resuspend.
3. Add 25 µl 2.5 M CaCl2 resuspend.
4. Immediately add 5-10 ml of 0.1 M spermidine, resuspend until the mixture of particles precipitates;
5. Place Eppendorf on ice for 5 minutes.
6. Use a pipette to remove 50 ml of the mixture from the nozzle without affecting the particles.
7. For the shot, use 2.5 µl of the mixture (the prepared mixture of 15 µl should be enough for 4-5 shots of 2.5 µl).
   Initial parameters of the ballistic transformation are:
   1. The volume of gas used when firing - 6 cm³.
   2. Cross-section of the dissecting filter cell - 500 microns.
   3. Negative vacuum pressure in the chamber when fired - 30 mm Hg.
   4. The distance from the particle source to the target tissue is 12-17 cm.
   5. Helium pressure: 0.6-0.7 MPa = 6-7 atm (1 megapascal [MPa] = 9.869 physical atmosphere [atm])
   6. Each cup is fired twice.
   After the first shot, the callus is turned over and again laid out in the circle with the diameter of 40 mm.
   The genetic construct for ballistic transformation must contain a selective DNA sequence that allows selective selection of transformed shoots.

2.8. Proliferation of transformed cells
   After transformation (after 20-24 hours), the callus is transferred to the medium (B) for callus proliferation for 3 weeks, in the dark at 23 ± 2 °C. If during these three weeks, the beginning of shoot formation is observed, then the embryos with the callus are transferred to a medium for regeneration with a selective agent before the expiration of the proliferation period.

2.9. Regeneration and selective selection of transgenic shoots
   Embryos with callus (without separating) are placed on a selective medium G for regeneration and selection of shoots for 3 weeks, at a light of 5000 lux and a temperature of 23 ± 2 °C.

2.10. Rooting of shoots on a medium with a selective agent at low temperatures
   The green shoots formed during the selection process are placed in test tubes with medium D for rooting at a light of 5000-10000 lux at a temperature of 20 ± 2 °C. The rooted shoots are transplanted into perlite in a moist environment for adaptation for 1-2 weeks at 15-17 °C. Adapted plants are transferred to the soil until they are fully mature in a greenhouse.
   Shoots of winter varieties should go through the stage of vernalization, after their transfer into the perlite. For this purpose, the plants are kept at a temperature of 3 ± 2 °C for 6 weeks under 5000 lux illumination.

2.11. Selection of transgenic plants

2.11.1. Isolation of DNA. Thoroughly rub the plant tissue (an amount equal to 2-3 leaf discs squeezed out by the Eppendorf lid) in liquid nitrogen. Suspending in 250 µl buffer [0.2 M Tris-HCl pH 9.0; 0.4 M LiCl; 25 mM EDTA; 1% SDS]. Centrifuge for 10 minutes in a microcentrifuge at maximum speed.
   Take 175 µl of the supernatant into a new tube and add 175 µl of isopropanol. Mix the contents by turning the test tube 4-5 times. Centrifuge at maximum speed for 15 minutes. Dry the precipitate by removing the supernatant and leaving the test tube upside down on filter paper for 30 minutes. Add 110 ml of sterile water and dissolve at room temperature for 30 minutes on a shaker. Precipitate the remaining cell debris by centrifugation for 5 minutes. Transfer 100 ml of genomic DNA solution to a new test tube and add 70 ml of phenol saturated with tris buffer pH - 8.0, place in a shaker, and then leave for 5 minutes on the table. Centrifuge at maximum speed for 10 minutes. Transfer 90 µl of the upper phase to a new test tube and add 9 µl of 3M sodium acetate (pH - 6.0) and 250 µl of ethanol, mix by turning 10 times. Place in the freezer for at least 20 minutes at -20°C. Centrifuge at maximum speed for 10 minutes, then remove the residue with a pipette. Add 125 µl of 70% ethanol, put in the freezer for at least 10 minutes at -20 °C. Centrifuge at maximum speed for 10 minutes, then use a pipette to select the liquid as much as possible, dry the precipitate for 5-10 minutes. The precipitate is dissolved in 50 µl of sterile water.
2.11.2. PCR analysis of DNA samples. For PCR, a DNA amplifier (Bio-Rad T100 Thermocycler) is used. PCR conditions: denaturation 95 °C - 4 min, 35 cycles (94 °C - 30 sec, 55 °C - 30 sec, 72 °C - 45 sec) and final completion - 7 min. Selected genomic DNA samples are used as a matrix, and bar1 (5’-TCAACCACATTACAGACAAAG-3’) and bar2 (5’-TGAAGTCCAGCTGCCAGAAACC-3’) are used as primers.

For centrifugation, a desktop microcentrifuge (Eppendorf 5415C) with the maximum rotor speed of 14000 rpm is used. The temperature control of the samples is carried out in a dry thermostat (Eppendorf Termomixer).

The analysis of PCR products is carried out by electrophoresis in agarose gel, followed by staining the gels with ethidium bromide (the concentration of the coloring solution is 1 microgram/ml in water, the staining time is 20 minutes at room temperature) and photographing the resulting picture in ultraviolet light (wavelength - 260-280 nm) with a digital camera. The digitized information is processed using the Adobe Photoshop 7.0 package.

The conclusion about the embedding of a foreign gene in the plant genome is made on the basis of the appearance of a PCR product with the expected size (depending on the specific primers used).

3. Results and discussion

The use of high-quality donor plants is a prerequisite for obtaining effective regeneration of shoots in vitro. In addition to the mandatory protection of plants from diseases such as powdery mildew, dwarf rust, root rot and insects (spider mites, aphids, thrips, etc.), optimal vegetation conditions must be created (in an artificial climate) – high-quality lighting, water supply, soil substrates, fertilizers, humidity and air quality.

After flowering and pollination on day 11, wheat shoots 40 cm long were cut and ears in the amount of 50 pcs (each grade), for 2 days they were kept in a refrigerator at the temperature of 4 °C. This method provides effective regeneration of transgenic shoots if immature embryos with a morphogenic callus obtained from donor plants after a cold shock are used as a target for transformation. As the result of short-term treatment (48) hours at a low temperature of +4 °C, a high frequency of MC induction was obtained for the studied soft wheat varieties up to 97.2%, and the frequency of shoot formation and their subsequent rooting on a selective medium was equal to 62.76-83.98%. After the cold shock, unripe grains were exfoliated from the ears. The seeds were sterilized with 70% ethanol, stirring continuously for 6 minutes and rinsed with sterile water three times for 5 minutes. Immature embryos were isolated using a scalpel, under a stereomicroscope at 10X magnification. The isolated embryos with a size of 0.8 to 1.7 mm are transferred to Petri dishes on medium A, with the flap up, in an amount of 50 pieces (Figure 1 a).

The embryo cups are placed in a climacamber and cultured for 14 days in the dark at the temperature of 24 ± 2 °C until the callus is formed. In the process of callus formation, two types of callus are formed - morphogenic callus (MC) and non-morphogenic callus (NMC). NMC is a loose, white, heavily watered mass of cells, characterized by a high rate of watering and weight gain and the absence of morphogenesis, MK is a dense, yellowish, slowly increasing in mass callus, represented by tightly interconnected meristem globules. On the 7th and 14th day after cultivation, a visual assessment of the resulting callus is performed, the MC is left together with the embryo and used for transformation, and the NMC is cut off and removed from the culture in vitro (it is important not to separate the embryo from the morphogenic callus, but to leave them together). The NMC was cut off on days 6 and 12, the MC was left with the embryo and used for transformation.

On day 13, the explants used in the experiment were transferred to an osmotic medium of 40 pieces per Petri dish and fired at the callus with a ballistic cannon. The degree of negative impact of ballistic transformation decreases with increasing age of the explant from the moment of initiation of tissue culture in vitro [13]. The Zlata variety is the most resistant to ballistic impact on the tissue, the maximum reduction in the formation of morphogenic callus is shown for the Amir. L-1 variety, Agata and Deya occupy an intermediate position in terms of the degree of susceptibility to ballistic impact. The optimal
period for the ballistic transformation of soft wheat is 10-14 days from the moment of initiation of the culture of wheat tissues of all the studied varieties.

![Figure 1. Stages of wheat ballistic transformation: a - cultivation of explants on the medium for the induction of callus formation; b - transient expression of the gfp-gene on the medium B for the proliferation of callus; c - the beginning of shoot formation; d - regeneration and selective selection of transgenic shoots; e - rooting of shoots on the medium with a selective agent; f - formed green shoots in test tubes with the medium D.](image)

Osmotic treatment of explants during bioballistic transformation increased the survival rate of explants and, consequently, the efficiency of transformation. Since the size of the meristem cells of the wheat germ is about 10 microns, and the size of the tungsten particles used as carriers of DNA molecules is 0.4-2 microns, the lesions of the protoplast are large. When exposed to high osmotic pressure, a process called plasmolysis occurs - the separation of the protoplast from the cell wall in a hypertonic solution. This reduces the damage to the protoplast. Plasmolysis is possible in cells that have a dense cell wall - in plants. Explants were laid out on the medium in a circle with a diameter of 40 mm, after 6 hours plasmolysis was observed and the callus was fired twice from both sides. As a result of this work, a collection of T<sub>0</sub> transgenic plants was obtained using a ballistic transformation on day 14 (Table 2).
Table 2. Collection of transgenic plants T0.

| Variety | Number of explants (pcs)/morphogenic callus (%) | The number of regenerants that have been selected (pcs/%) | PCR+ (pcs) | Frequency of transformation (%) |
|---------|-----------------------------------------------|-------------------------------------------------------|------------|-------------------------------|
| Zlata   | 486/97.2                                      | 38/7.8                                                | 38         | 7.8                           |
| Amir    | 256/51.2                                      | 5/1.9                                                 | 5          | 1.9                           |
| Agata   | 367/73.4                                      | 22/5.9                                                | 22         | 5.9                           |
| Deya    | 428/77.1                                      | 17/3.9                                                | 17         | 3.9                           |
| L-1     | 363/73.0                                      | 5/1.4                                                 | 5          | 1.4                           |

After transformation, after 20 hours, the callus was transferred to a medium for callus proliferation in the dark. A day after firing the particles with the psGFP-BAR plasmid applied to them, we observed transient expression of the gfp-gene on all embryogenic callus cells (Figure 1b). Further cultivation of explants on selective media containing picloram showed the active formation of embryo-like structures expressing the gfp-gene. Regenerating plants were selected on a selective medium containing phosphinotricin at a concentration of 5 mg / l at the stage of regeneration of shoots. Rooting of the shoots was carried out at low temperatures on media with a selective agent (3 mg/l). The rooted shoots were transplanted into perlite in a moist environment for adaptation for 1-2 weeks at 15-17°C. All primary transgenic plants were cultivated in a greenhouse to observe morphological changes and produce seeds. The vast majority of shoots selected after gfp visual selection grew actively and took root easily in the presence of ppt (Figure 1b), and then quickly adapted to the greenhouse conditions (Figure 1f-e). None of the transferred seedlings died during acclimatization. A total of 87 suspected transgenic plants were grown in the greenhouse. Plants of the T0 generation that showed resistance to the selective ppt agent were used to analyze the presence of the bar gene (Figure 2). Using the method of the regenerative selection system, the highest frequency of transgenic plants was obtained, which was 1.4-7.8%.

Figure 2. Electrophoretic analysis of PCR products for the presence of the bar-gene in wheat shoots of the Zlata variety transformed by DNA with the bar gene. Tracks: 1-17-PCR on DNA preparations from transformed lines; #2bar-positive control, PCR product on plasmid DNA (0.1 ng/reaction) used for plant transformation; UnP-negative PCR control on DNA preparations from untransformed plants; 100bp-size marker (GeneRuler 100bp, ThermoScientific).
4. Conclusion
The presented system for obtaining transgenic wheat plants takes from 2 to 3 months. One of the most important factors for achieving a highly efficient wheat transformation is the quality of the immature germ. The use of immature embryos at the right stage of development and the size of the embryos is a critical factor. Short-term treatment (48 h) by low temperature +4 °C increases the frequency of induction of morphogenic callus. The positive effect of picloram (an auxin-like substance) on the induction of wheat callus formation was confirmed. In our method, picloram was used to increase the formation of morphogenic callus in immature wheat germ varieties of domestic varieties. The transformed cells were propagated on a medium for proliferation without selective selection, which allowed to increase the yield of transgenic shoots. Selective selection was started at the stage of regeneration of transgenic shoots and continued during rooting, which allowed to reduce the selection time. The method was used on spring and winter varieties of soft wheat of domestic selection-Zlata, Amir, Agata, Deya and L-1. The transformation efficiency was high for all the studied varieties, regardless of the genotype, ranging from 1.4 to 7.8%. This index indicates the high efficiency of the proposed method and is genotype-independent, applicable to a large number of different genotypes of soft wheat. The method can be applied in the creation and economic use of innovative GM varieties (more productive and more marginal) in the wheat market and cause accelerated rates of variety replacement and a radical increase in the profitability of agricultural production and the competitiveness of manufactured products.

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