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Agrobacterium-Mediated Transformation of Maize with Antisense Suppression of the Proline Dehydrogenase Gene by an *In Planta* Method

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Authors' contributions

This work was carried out in collaboration between all authors. Authors YMM and VAV wrote the protocol and performed the PCR analysis, author YSG conducted statistical analysis and literature searches, author OSY prepared DNA for PCR analysis, author IVV conducted inoculation and microscopic experiments and author MIC designed the study and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The aim of this study was to estimate the efficiency of T-DNA transfer during *Agrobacterium*-mediated transformation of maize (*Zea mays* L.) at different temperatures. In addition, the way of T-DNA transfer was studied after application of an *Agrobacterium* suspension at maize pistil filaments.
Study Design: Transgenic maize plants were obtained with an antisense suppressor of the proline dehydrogenase gene (ASPG) by using the binary vector pBi2E. Temperatures of 28-35ºC were used to establish suitable conditions for transformation *in planta*.
Place and Duration of Study: Laboratory of Bioengineering, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences; between May 2008 and May 2013.
Methodology: *A. tumefaciens* strain LBA4404 (pBi2E), containing the marker gene and the ASPG from *Arabidopsis thaliana* was used for maize transformation. The presence of T-DNA in the maize genome was detected by PCR. The proline concentration in

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transgenic hybrids of maize lines was determined colorimetrically.
Results: T-DNA carrying the marker genes (*nptII*, *gus*) and the ASPG construct was detected in the maize genomes after *Agrobacterium*-mediated transformation. PCR analysis of total DNA isolated from 409 kanamycin-resistant diploid F_1 seedlings revealed T-DNA insertions in the genomes of 30 plants. Expression of the ASPG in the maize genome led to a 4.5-fold increase (P=0.05) in free proline content in the transformed plants. Temperatures above 3ºC blocked the T-DNA transfer.
Conclusion: The transfer of the ASPG by *Agrobacterium* T-DNA into the maize genome was achieved with a frequency of 0.3-2.3% at temperatures not higher than 31ºC. The PCR-positive maize plants had a statistically significant increase in the proline concentration in leaf tissues as compared with the non-transformed control. T-DNA may be transported into the maize egg cell by the growing pollen tube after the pistil filaments are inoculated with an *Agrobacterium* suspension.

Keywords: Zea mays L.*; proline dehydrogenase; antisense suppressor; free proline content.*

1. INTRODUCTION

Transformation using the *Agrobacterium tumefaciens* T-DNA transfer system is still a prevalent technology for generating transgenic plants. Most methods of *Agrobacterium*-mediated transformation are based on the co-incubation of plant leaves, roots and stem fragments *in vitro* with bacterial-cell suspensions. A simple, “floral-dip” method has been suggested for transformation of plant germ cells, in which inflorescences are dipped into a suspension of *Agrobacterium* carrying activated *vir* genes [1]. The *Agrobacterium*-mediated T-DNA transfer methods under *in planta* and *ex planta* conditions, including methods that require *in vitro* cultivation of transgenic plant cells and tissues, have been described [2,3]. Production of transformants *in planta* seems especially promising for monocotyledonous plants, including maize [4], since plant regeneration from suspension and callus cultures is difficult. However, T-DNA insertions have not been detected in some experiments on *Agrobacterium*-mediated transformation of maize pollen [5]. Cells of the embryo sac and sperm cell nuclei are considered to be the main targets for *Agrobacterium* infection *in planta* [6]. Nevertheless, the optimal conditions and precise mechanism of T-DNA transfer to germ cells by *in planta* methods have not been described.

It has been suggested that increasing the content of proline may improve plant resistance to drought and salt stress [7,8]. After the insertion of the *Escherichia coli proA* and *proBosm* genes, involved in proline biosynthesis, into tobacco plants, the overproduction of proline in the transgenic plants enhanced tolerance to salt stress [9]. Another approach is the blocking of translation of the enzymes implicated in proline degradation. Several authors have reported positive results from inserting genetic constructs containing the antisense sequence of the proline dehydrogenase gene into the genome of tobacco [8,10,11]. Proline dehydrogenase catalyzes the first step in proline conversion to glutamic acid. RNA products resulting from transcription of the transferred antisense sequence in transgenic plants associate with the complementary mRNA of proline dehydrogenase; thereafter, the duplex is destroyed by cell enzymes. This reduces the translation of the gene, and as a result, the amount of free proline may increase in the cells. Transgenic tobacco plants expressing the proline dehydrogenese gene in antisense orientation contained 1.5–7-fold more free proline and showed higher stress resistance in the laboratory than the control plants [8]. Simultaneous manipulations of proline biosynthesis and degradation resulted in a more than

50-fold increase in proline content compared to that of the wild-type tobacco plants [11]. The antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana* [12]. Increased proline content correlates with drought and salt tolerance in various plants (petunia, rice, soybean) [7]; however, no such data are available for maize.

The aims of this study were (1) to estimate the efficiency of *Agrobacterium*-mediated transformation of maize by using T-DNA as a carrier at different temperatures, (2) to obtain transgenic maize plants with high proline content, and (3) to study how T-DNA transfer happens *in planta*.

2. MATERIALS AND METHODS

2.1 Transformation Procedure

For transformation of maize, we used *A. tumefaciens* strain LBA4404, carrying the binary vector pBi2E (courtesy of A.V. Kochetov), containing the marker gene of neomycin phosphotransferase under the nopaline synthase promoter and a duplicate fragment of the proline dehydrogenase gene from *Arabidopsis thaliana* (GeneBank: AB028614, U59508) as an inverted repeat under the 35S promoter of cauliflower mosaic virus RNA [10]. An *Agrobacterium* cell suspension (60 µl) with acetosyringone-activated *vir* genes was applied onto the pistil filaments of the mother maize line according to Mamontova et al. [4]. Transformation *in planta* was performed at 28–29ºC, 30–31ºC, and 33–35ºC. Thirty minutes after inoculation, the maize plants were artificially pollinated and the ears were left until complete grains developed. The following lines were used in the ASPG experiment: AT-3 and Zarodyshevyi marker saratovskii purpurnyi (ZMSP) (provided by V. Tyrnov) and RNIISK and “Sweet” (provided by V. Zhuzhukin). For obtaining matroclinal haploids, we used maternal plants from the line AT-3 and pollinators from the haploid inducer line ZMSP. The experimental and control maize seeds were incubated with 10% sodium hypochlorite for 15 min, washed twice and germinated for 7–8 days on MS medium with kanamycin (100–200 µg ml^{-1}) and 150 mM NaCl as selective agents. After that, the seeds were planted in soil.

A. tumefaciens LBA4404 with the binary vector pA2069, containing the *npt*II and *gus* genes (courtesy of G. Raldugina), was used for maize pollen transformation. Maize pollen was collected from the line ZMSP during plant flowering, placed on a medium of 17% sucrose solution with 1% bacteriological agar and incubated with 100 µl of an *Agrobacterium* suspension for 12 h. Then, pollen grains were washed and placed on a medium with X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide, Sigma, USA). Coloration was observed on the next day.

2.2 PCR Analysis

The presence of T-DNA in the genome of maize seedlings grown from the obtained grains was revealed by PCR. We used the primers 5'-GAGGCTATTCGGCTATGACTG-3' and 5'-CGAGATCATCGCCGTCGGGC-3' (Syntol Co., Russia), which are specific for the 500-bp fragment of the *npt*II gene. The amplification profile was as follows: a preliminary denaturation step of 94ºC for 5 min and 35 cycles of 94ºC for 1 min, 60ºC for 1 min, and 72ºC for 1 min. Primers for the ASPG genetic construct were forward 5'-AACAAACTGGATCCGGCGATCTTAC-3’ and reverse 5'-

GAGATGTTGGTCTAGATTTGGCAGC-3' (Syntol Co., Russia). Preliminary DNA denaturation was at 95ºC for 3 min, and 35 cycles of 95ºC for 1 min, 58ºC for 1 min, and 72°C for 1 min. The size of the expected PCR product was calculated to be 550 bp. Amplification was conducted by using a Mastercycler personal apparatus (Eppendorf, Germany). The PCR results were visualized by gel electrophoresis in a 1% agarose gel.

To rule out the possibility of a false-positive PCR result arising from contamination during pistil filament treatment from *Agrobacterium* possibly present in plant tissues, we performed control PCR for the *A. tumefaciens*-specific *virC1* and *virC2* genes, located outside the T-DNA region of the Ti plasmid [13]. Primers were forward VCF 5'-ATCATTTGTAGCGACT-3' and reverse VCR 5'-AGCTCAAACCTGCTTC-3'. DNA amplification conditions were as follows: preliminary denaturation at 95ºC for 3 min; 35 cycles at 95ºC for 1 min, 55°C for 1 min, and 72ºC for 1 min. The DNA isolated from *A. tumefaciens* served as a positive control.

2.3 Microscopy

Samples were viewed under a Leica Microsystems DM2500 microscope (Swiss), and the results were photographed with a Leica Microsystems DFC 420 C digital photo camera.

2.4 Proline Assay

Grains of the hybrid lines AT-3 and ZMSP, obtained by processing the mother plants, were germinated at 28–29ºC in a medium with kanamycin and NaCl. The proline concentration was determined in transgenic hybrids of the maize lines AT-3 and ZMSP according to [14]. Briefly, 0.3 g of maize leaves sampled from flowering plants was homogenized, and 3 ml of a 3% sulfosalicylic acid solution was added. After centrifugation at 5000 *g* for 10 min, 1.5 ml of the supernatant liquid was mixed with 1.5 ml of acidic ninhydrin (Aldrich, USA) and 1.5 ml of glacial acetic acid (Reakhim Co., Russia). The mixture was incubated in a water bath at 100ºC for 1 h. The reaction was stopped by cooling on ice, after which 3 ml of toluene (Reakhim Co., Russia) was added to each test tube and the test tubes were vigorously shaken for 15–20 s. The absorbance of the upper colored layer, after settling for 15 min, was measured at 490 nm. The content of free proline in the samples was determined by a calibration curve (1, 5, 10, 15, and 20 μg ml^{-1}) by using a commercially available proline standard (Aldrich, USA).

3. RESULTS AND DISCUSSION

3.1 Application of Transformation *in planta* for the Transfer of the ASPG to the Maize Genome

Inoculation of pistil filaments with a suspension of *Agrobacterium* cells carrying pBi2E was performed at 28–29ºC, 30–31ºC and 33–35ºC. The seed set after transformation was in the range 1 to 200 grains per ear (Fig. 1). The average seed set for the 83 ears obtained was 66 grains per ear.

At 28–29ºC, 20 plants of the line AT-3 were inoculated with *Agrobacterium* and pollinated by the ZMSP line, from which we obtained 16 ears with grains (on the average, 118 grains per ear).

At 30–31ºC, 20 plants of the line AT-3 were inoculated with *Agrobacterium* and were pollinated by the ZMSP line, 40 plants of the line RNIISK were inoculated with *Agrobacterium* and self-pollinated, and 12 plants of the "Sweet" line were inoculated and

self-pollinated. From the treated ZMSP, RNIISK, and “Sweet” plants, we obtained 15 ears (on the average, 94 grains per ear), 35 ears (on the average, 29 grains per ear), and 10 ears (on the average, 37 grains per ear), respectively.
At 33–35ºC, 12 plants of the line RNIISK were inoculated with *Agrobacterium* and self-pollinated, from which we obtained 7 ears with grains (on the average, 63 grains per ear).

Fig. 1. RNIISK line ears obtained after *Agrobacterium*-mediated pistil transformation *in planta* and artificial pollination

In sum, 3734 maize seedlings were incubated in kanamycin and salt-containing medium, out of which 409 proved to be kanamycin resistant and salt tolerant. These were planted into the soil. Analysis of the total DNA isolated from the 409 seedlings showed that 30 seedlings (0.8% of the total number of seedlings analyzed) contained a 0.55-kbp PCR product of the ASPG (Fig. 2) or a 0.5-kbp PCR product of the *npt*II gene. Half of the randomly selected PCR-positive samples did not contain nucleotide sequences of the *virC1* and *virC2* genes.

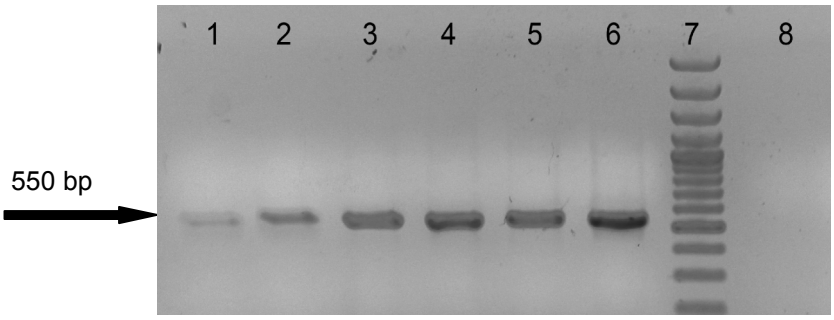

Fig. 2. PCR analysis of the total DNA isolated from maize leaves for the presence of the ASPG

Lanes: 1-5 – ASPG-containing hybrid maize plants (AT-3xZMSP); 6 – plasmid DNA from A. tumefaciens LBA4404 containing the binary vector pBi2E (positive control); 7 – molecular weight marker (SM0321/2/3, Fermentas, Lithuania); 8 – non-transformed hybrid maize plants (AT-3xZMSP; negative control)

The efficiencies of different methods of transformation of *Arabidopsis*, wheat, rice and maize *in planta* are listed in [3] and range from a few hundredths of percent to a few percent. The

relatively low efficiency of transformation in our experiments might be explained by the apparently high temperatures during transformation *in planta*.

3.2 Factors Influencing the Effectiveness of *Agrobacterium* Transformation *in planta*

Temperature is the most important factor in *Agrobacterium*-mediated plant transformation [15]. Temperatures of 22–25ºC during silk treatment with an *Agrobacterium* suspension were less favorable for *Agrobacterium*-mediated transformation compared to 18–20ºC [16]. These findings seem related to the data on the temperature effects on T-DNA transfer machinery inside the *Agrobacterium* cells. The temperature range of 19 to 22ºC is optimal for the expression of *vir* genes in *Agrobacterium* [17], whereas 28ºC is already critical for the excretion and assemblage of *vir*-dependent *Agrobacterium* T-pili, essential for successful T-DNA transfer [18]. Transfer of T-DNA into tobacco plantlets under laboratory conditions is completely suppressed at 31ºC [19]. Our findings are in line with the previous observations, as the percentage of PCR-positive seedlings was 2.3% when the transformation was performed at 28–29ºC and 0.3% when it was performed at 30–31°C and we did not obtain any transgenic line at 33–35ºC (Table 1). The difference between the transformation frequencies was significant, as found by Fisher's test (P=0.05).

Table 1. Frequency of ASPG insertion into the maize genome at different temperatures

Assessed index	Temperature (°C)		
	28–29[1]	**30–31[2]**	**33–35[3]**
N_0 of ears / N_0 of ears with transformed grains	16/7	60/3	7/0
Seedlings from T_0-generation grains	1425	1919	390
Kanamycin-resistant seedlings	178	223	8
N_0 of PCR-positive seedlings	26	4	0
Percent of PCR-positive seedlings per ear, %[4], $\bar{x} \pm SE$[5]	2.3±1	0.3±0.2	0

1 – 1425 hybrid seedlings of AT-3 x ZMSP lines
2 – 984 hybrid seedlings of AT-3 x ZMSP lines, plus 609 seedlings of the RNIISK line, plus 326 seedlings of the "Sweet" line
3 – 390 seedlings of the RNIISK line
4 – Sample comprised 83 ears, each containing 1 to 200 caryopses
5 – Mean ± standard error

3.3 Proline Content in Transgenic Plants

Increasing the proline content improves plant resilience to drought and salt stress [7,8]. Previously, it was shown that transgenic maize plants expressing the ASPG construct have a 4.6-fold increase in proline content and can grow well in a medium containing 150 mM NaCl [20]. We selected transgenic plants in the presence of 150 mM NaCl in the medium and measured the proline content of six ASPG-carrying maize plants. An average proline concentration of 247±18 μg g^{-1} fresh weight was found in leaves of transgenic plants, a value 4.5-fold higher than that in the control plants (54±11 μg g^{-1} fresh weight). These data attest to a significant increase in free proline content in the leaf tissues of F1 seedlings, indicating that the expression of the proline dehydrogenase gene is suppressed in the transgenic plants. The messenger RNA genes of maize proline oxidase (LOC100281186) and of *Arabidopsis* proline dehydrogenase have a homology site which is 146 nucleotides in size (E

value 7e-10). Probably, the suppression of the maize proline dehydrogenase gene could have been caused by this homology.

3.4 Traveling of T-DNA to the Maize Egg Cell

During *Agrobacterium*-mediated transformation of *Arabidopsis* by the “floral dip” method, the productive events of T-DNA transfer occur on the female floral structures and do not occur in the early stages of anther or pollen/microspore development prior to pollen release [6]. The key factor for *Arabidopsis* “floral dip” transformation is an opportunity for *Agrobacterium* to reach close to the ovary and penetrate it early in flower development. The female gametophyte of maize is not available for the bacteria deposited on the pistil thread. We speculate that the genetic transformation in our experimental system proceeds by the pollen-tube pathway. It is known that the pollen tube moves toward the micropyle (the germ pore). This movement is guided by an attractor protein produced by the micropyle cells [21]. In theory, there are two possible ways of how the T-DNA can be delivered into the nucleus of maize during *in planta* transformation. In the process of germination of pollen, *Agrobacterium* may interact with the germinating pollen tube and may transform the pollen nucleus. In addition, the T-complex may be delivered together with the pollen tube to the embryo sac, where the transformation of the egg or zygote occurs.

Previously [4] and in this study, we have shown that transformation of the female gametophytes of maize *in planta* is possible. To determine whether the egg cells underwent transformation in our experiments, we used a haploid inducer maize line. Under certain conditions, a haploid maize egg cell may start differentiating. Haploids rarely form spontaneously, usually accounting for no more than 0.1% of the offspring [22,23]. If maternal forms are pollinated by pollen of haploid inducer maize lines, the number of matroclinal haploid plants (originating from egg cells) increases by one or two orders of magnitude [22,23]. The high proportion of haploids is determined by the haploid-inducing capacity of the ZMSP line with defected sperm cells [23]. As a result, part of the sperm nucleus can fertilize the central cell, but the egg remains unfertilized and gives a haploid embryo. Specific markers of the embryo and seedling colors make it easier to perform a preliminary selection in the offspring of haploid forms among 4- to 10-day-old seedlings on the basis of morphological characters. When the line ZMSP is used as a pollinator in the diploid offspring, plants acquire a purple color and matroclinal haploid plants remain green like the maternal line [23].

Since the matroclinal haploids (12 of the 16 analyzed) obtained with the use of the ZMSP haploid inducer line contained T-DNA insertions (data not shown), we propose that T-DNA may be transported into the egg cell by the growing pollen tube after the pistil filaments are treated with an *Agrobacterium* suspension [4]. However, it cannot be excluded that along with the transformation of the female gametophyte in diploid forms of maize, the male gametophyte could also be transformed.

For determination of *Agrobacterium*-mediated transformation of the male gametophytes (pollen grains), the pollen of the ZMSP line was incubated with a suspension of *Agrobacterium* LBA4404 (pA2069) carrying the *npt*II and *gus* genes under *in vitro* conditions. The expression of the marker gene was observed with a frequency of 22.3±3.6% only in the pollen grains treated with *A. tumefaciens* (Fig. 3).

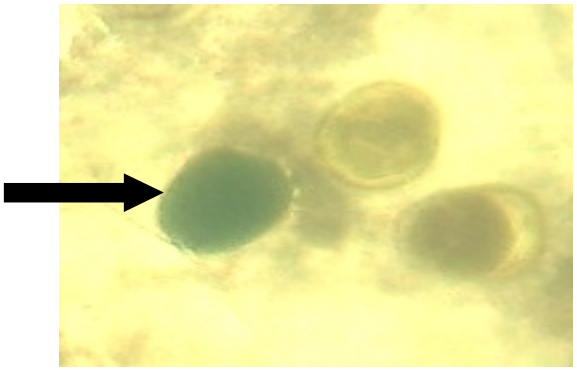

Fig. 3. *Gus* gene expression in ZMSP pollen grains after co-incubation with a cell suspension of *A. tumefaciens* LBA4404 containing the binary vector pA2069

4. CONCLUSION

We have established the effectiveness of ASPG transfer by *Agrobacterium* T-DNA into the maize genome at a frequency of 0.3–2.3 % at a temperature not higher than 31ºC. The PCR-positive maize plants had a statistically significant (4.5-fold) increase in the proline concentration in leaf tissues (P=0.05) as compared with the control, indicating that the transgene suppressed the expression of the proline dehydrogenase gene in the transformants. Furthermore, we demonstrated that the T-DNA can be transported into the egg cell by the growing pollen tube after the pistil filaments are treated with an *Agrobacterium* suspension.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 1998;16(6):735-43. DOI: 10.1046/j.1365-313x.1998.00343.x.
2. Chumakov MI. *Agrobacterium*-mediated plant transformation under *in planta* conditions. Transgenic Plant J. 2007;1(1):60-65.
3. Chumakov MI, Moiseeva EM. *In planta Agrobacterium*-mediated plant transformation technologies (review). Biotechnologiya, Russian. 2012;1:8-20.
4. Mamontova EM, Velikov VA, Volokhina IV, Chumakov MI. *Agrobacterium*-mediated *in planta* transformation of maize germ cells. Russian J. Genet. 2010;46(4):501-04. DOI: 10.1134/S1022795410040186.

5. Langridge P, Brettschneider R, Lazzeri P, Lorz H. Transformation of cereals via *Agrobacterium* and the pollen pathway: a critical assessment. Plant J. 1992;2(4):631-38. DOI: 10.1111/j.1365-313X.1992.00631.x.
6. Desfeux C, Clough SJ, Bent AF. Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the *Arabidopsis* floral-dip method. Plant Physiol. 2000;123(3):895-04. DOI: 10.1104/pp.123.3.895.
7. Valliyodan B, Nguyen HT. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Curr Opin. Plant Biol. 2006;9(2):189-95.
8. Kolodyazhnaya YaS, Titov SE, Kochetov AV, Trifonova YuA, Romanova AV, Komarova ML, Koval, VS, Shumny VK. Evaluation of salt tolerance in *Nicotiana tabacum* plants bearing an antisense suppressor of the proline dehydrogenase gene. Russian J. Genet. 2006;42(2):212-14. DOI: 10.1134/S1022795406020153.
9. Sokhansanzh A, Neumyvaikin LV, Moseiko NA, Piruzyan ES. Transfer of bacterial genes participating in proline biosynthesis into plants and their expression under the control of various promoters. Russian J. Genet. 1997;33(7):763–69.
10. Kochetov AV, Titov SE, Kolodyazhnaya YaS, Trifonov EA, Romanova AV, Komarova ML, Koval ML, Shumny VK. Tobacco transformants bearing antisense suppressor of proline dehydrogenase gene are characterized by higher proline content and cytoplasm osmotic pressure. Russian J. Genet. 2004;40(2):216-18. DOI: 10.1023/B:RUGE.0000016999.53466.e1.
11. Stein H, Honig A, Miller G, Erster O, Eilenberg H, Csonka LN, Szabados L, Koncz C, Zilberstein A. Elevation of free proline and proline-rich protein levels by simultaneous manipulations of proline biosynthesis and degradation in plants. Plant Science. 2011; 181(2):140-50. DOI: 10.1016/j.plantsci.2011.04.013.
12. Nanjo T, Kobayashi M, Yoshiba Y, Kakubary Y, Yamaguchi-Shinosaki K, Shinosaki K. Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. FEBS Letters. 1999; 461(3):205-10.
13. Sawada H, Ieki H, Matsuda I. PCR detection of Ti and Ri plasmids from phytopathogenic *Agrobacterium* strains. Appl. Environ. Microbiol. 1995;61(2):828-31.
14. Bates LE, Waldren RP, Teare ID. Rapid determination of free proline for water stress studies. J. Plant and Soil. 1973;39(1):205-07.
15. Tempe J, Petit A, Holsters M, Montagu M, Shell J. Thermosensitive step associated with transfer of the Ti plasmid during conjugation: Possible relation to transformation in crown gall. Proc. Nat. Acad. Sc. USA. 1977;74(7):2848-49.
16. Chumakov MI, Rozhok NA, Velikov VA, Tyrnov VS, Volokhina I. *Agrobacterium* mediated *in planta* transformation of maize via pistil filaments. Russian J. Genet. 2006;42(8):893-97. DOI: 10.1134/S1022795406080072.
17. Fullner KJ, Nester EW. Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens*. J. Bacteriol. 1996;178(6):1498-04.
18. Fullner KJ. Role of *Agrobacterium vir*B genes in transfer of T complexes and RSF1010. J. Bacteriol. 1998;180(2):430-34.
19. Chumakov MI, Kurbanova IV, Solovova GK. *Agrobacterium*-mediated transformation of undamaged plants. Russian J. Plant Physiol. 2002;49(6):799-03. DOI: 10.1023/A:1020921814222.
20. Moiseeva EM, Agafonov DA, Velikov VA, Volokhina IV, Chumakov MI. Elevated proline content in maize plants expressing a fragment of the proline dehydrogenase gene in antisense orientation. Russian J. Plant Physiol. 2012;59(3):419–22. DOI: 10.1134/S1021443712020070.
21. Marton ML, Cordts S, Broadhvest J, Dresselhaus T. Micropylar pollen tube guidance by egg apparatus 1 of maize. Science. 2005;307(5709):573-76. DOI:10.1126/science.1104954.

22. Tyrnov VS, Enaleeva NK. Independent development of the embryo and endosperm in maize. Doklady Akademii Nauk USSR, Russian. 1983;272(3):722-25.
23. Tyrnov VS, Zavalishina AN. Induction of a high frequency of emergence of matroclinal maize haploids. Doklady Akademii Nauk USSR. Russian. 1984;276(3):735-38.

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