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Electroporation and morphogenic potential of *Gentiana kurroo* (Royle) embryogenic cell suspension protoplasts

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Abstract

This article presents our further *in vitro* studies into the morphogenic potential of gentian cells, organs, and tissues after modification of their genome. The objective was to study the effect of electroporation and the introduction of foreign genes on the morphogenic potential of *Gentiana kurroo* embryogenic cell suspension protoplasts. Protoplasts were electroporated with DNA plasmids carrying *nptII* and *bar* genes. The stability of cell membranes, the contents of electroporation buffer, the length of electric pulse, the number of pulses and the strength of the electric field were studied. We determined the highest electroporation efficiency by evaluating the highest protoplast survival rate under specific physical conditions. The best results were achieved in the presence of EB1 electroporation buffer where the viability of protoplasts was 70.1%. Protoplast survival at this higher level required culture temperatures near 0 °C, and a 20 μs electric pulse with an electric field of 1.0 kV/cm. After seven days of agarose embedded protoplast culture, a selective agent – kanamycin – was introduced to the medium. The cell transformation effect was improved by a long term culture of callus, regenerated somatic embryos and transformants in the presence of 50 mg/l kanamycin.

Key words: electroporation buffer, electric field, electric pulse, kanamycin resistance protoplast viability, plating efficiency, transformant regeneration

Abbreviations

AS	– adenine sulfate	Kin	– kinetin
BAP	– benzylaminopurine	kV/cm	– kilowatt/centimeter
Dicamba	– 3,6-dichloro-2-methoxybenzoic acid	MS medium	– Murashige and Skoog medium (1962)
DNA	– desoxyribonucleic acid	NAA	– naphtaleneacetic acid
EB1	– electroporation buffer no. 1	*nptII* gen	– gene coding neomycin phosphotransferase
EB2	– electroporation buffer no. 2	PCR	– polymerase chain reaction
GA_3	– gibberelic acid	μs	– millisecond
kan	– kanamycin	*uidA* (*gus*)	– gene coding glucuronidase
		2,4-D	– dichlorophenoxy acetic acid

Introduction

The alternatives to *Agrobacterium* mediated plant cell transformation are biolistic particle bombardment or protoplast chemical and physical treatments. Electroporation consists of the treatment of plant cells with short high voltage electric pulses. The electric pulse shock causes brief permeability of the plasmalemma for high molecular particles, such as DNA (Bates, 1989). The DNA movement is *via* pores formed after electric pulses in the cytoplasmic membrane (Sowers, 1992). The pores are of temporal character and they are related to the increased dipole moment of hydrophilic heads building cell membrane lipids. The dipole heads of phospholipids dislocate in the direction of the electric field, which causes breaks in the continuity of the cell membrane (Kinosita and Tsong, 1977; Neuman et al., 1982, 1996). In addition to various biological parameters connected with the selection of the proper source of protoplasts and their culture conditions, the pivotal requirement for successful cell transformation are the physical conditions of electroporation, specifically the voltage of the electric field, the number of pulses and length of pulse duration (Sauders et al., 1995).

The specific effect of the electric field on tissues cultured *in vitro* was determined by analyzing the growth of isolated protoplasts as well as with protoplast-derived

calli of Colt cherry (*Prunus avium* × *pseudocerasu*). The plant regeneration capacity of electroporated tissues was also investigated. The callus obtained from the protoplasts and subjected to three successive exponential pulses at 250 V or 500 V showed the largest fresh weight increases between subcultures. It also exhibited the highest morphogenic potential, as manifested by increased frequency of plant regeneration calculated based on the number of shoots per callus. These shoots, in turn, expressed (once more) a higher potential for producing a more prolific root system when compared to those derived from non-electropulsed protoplasts of Colt cherry (Ochatt et al., 1988).

There are numerous examples of the use of electroporation in transformation experiments with organized plant structures such as immature embryo or somatic embryos. Embryo and somatic embryos at the torpedo stage of coffee have been electroporated with DNA containing *gus* and *bar* genes, and plant regeneration through secondary somatic embryogenesis has been obtained (Barton et al., 1991). The presence of *gus* and *bar* genes has been confirmed by PCR reaction performed on DNA isolated from regenerated plants (Fernandez-Da Silva and Menendez-Yuffa, 2003). Similar experiments are needed concerning representative species of the Gentianaceae family.

The Gentianaceae family consists of about 1700 species but only 28 are used for plant tissue culture and biotechnology. Among these, *Gentiana kurroo* (2n = 26) (Behera, 2011) is a critically endangered species endemic to the northwestern Himalayas (Khuroo, 2005). This is a small perennial herb with a stout rhizome bearing a decumbent flowering stem. Due to its multiple uses (Behera, 2011), it is a species that is being over exploited in its natural habitat. Hence, it qualifies as an important endangered medicinal plant and is protected by Indian Law (Khuroo, 2005). Its secondary metabolite spectrum is the richest of all gentian species, even comparable to *G. lutea*. These secondary compounds – gentiopicrine, gentiamarin and the alkaloid gentianinare – are used for the treatment of inflammation, pain, fever, and hepatitis (Latif, 2006).

G. kurroo's reproductive biology is connected with its flowers being dichogamous due to protardy. Stigmatic lobes remain adpressed until almost complete anther dehiscence. The stigma becomes receptive to pollen germination about one week after initiation of anther dehiscence. The flowers are cross-pollinated (Raina et al., 2003). The plant is characterized by being mostly wild and not domesticated due to its poor seedling establishment in nature. However, these plants produce abundant seeds. Seed germination under laboratory conditions commenced from six to twenty eight days with a total record of about 70% (Raina et al., 2011, 2003; Tomar et al., 2012). Macroproliferation by rhizomes appears to be an alternative, easy and effective technique for the multiplication and conservation of this herb (Tomar, 2011).

The first papers summarizing the achievements of gentian plant tissue cultures were published in 1988 and in 1991 (Barešová, 1988; Miura, 1991). Since then, considerable progress in the biochemistry and biotechnology of gentians has been achieved. The establishment of a green leaf mesophyll cell cultures and cell suspension protoplast culture procedures have provided a significant basis for progress in gentian somatic cell genetic manipulation (Jomori et al., 1995; Meng et al., 1996; Fiuk and Rybczyński, 2007; Tomiczak and Rybczyński, 2015). Initially, possibilities were presented for establishing plant multiplication of *G. kurroo* by culturing the shoot tips and nodal segments on MS medium (Murashige and Skoog, 1962) supplemented with BAP (benzylaminopurine) and NAA (naphthaleneacetic acid). For the most effective rooting, individual shoots with 3-4 nodes were implanted on semisolid hormone-free MS medium supplemented with 6% sucrose (Sharma, 1993). The MS medium supplemented with 0.5 mg/l IAA and 0.8 mg/l BAP stimulated more than 80% of apical meristems for rich proliferation (Kaushal et al., 2014). Randomly amplified polymorphic DNA (RAPD) and karyotypic analysis showed the lack of variation, and hence the genetic stability of regenerants, and confirmed the efficacy of the protocol for micropropagating plants of *G. kurroo* over a ten year time period (Kaur et al., 2009). Significant progress in vegetative plant cell manipulation occurred when the somatic embryogenesis of primary explants was described for a few gentian species (Mikuła, 1996a, 1996b). Among the studied gentians, *G. kurroo* appeared to be a highly embryogenic species with tremendous morphogenic potential. Both seedling and leaf explants have been used for culture initiation and the establishment of cell suspensions (Fiuk and Rybczyński, 2008). The derived calli appear to be an excellent source of embryogenic cells and their protoplasts. A very high yield of regenerants *via* somatic embryogenesis from proto-

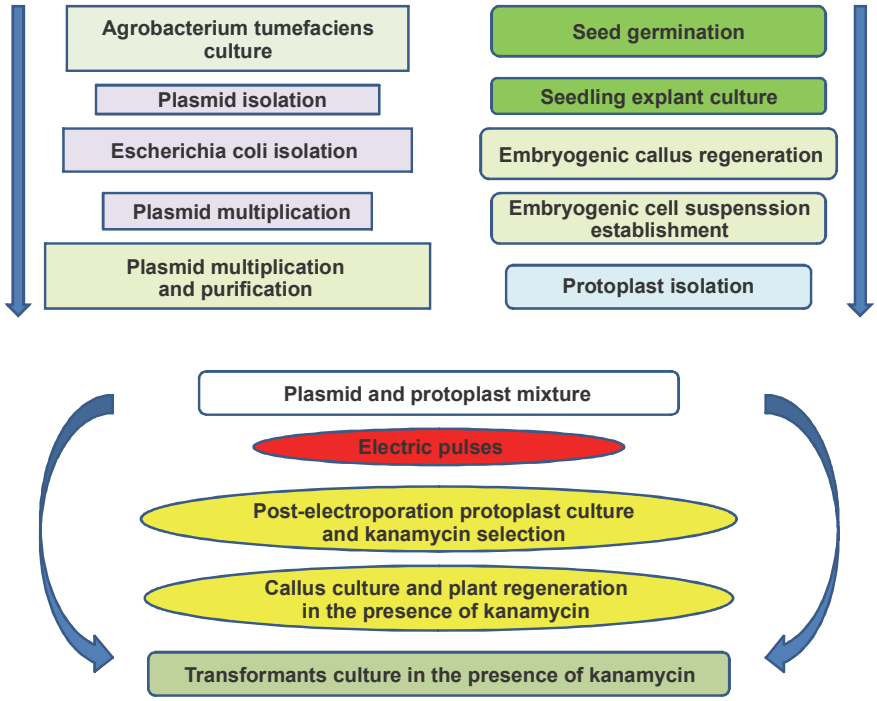

Fig. 1. Diagram of procedures involved in electroporation experiments of *G. kurroo* (Royle) embryogenic cell suspension protoplasts

plast bead culture has confirmed the usefulness of this type of protoplast culture for gentian cell manipulation (Fiuk and Rybczyński, 2007). The cell suspension cultures also produce embryogenic aggregates and somatic embryos which could be used for proteomic studies (Fiuk and Rybczyński, 2007; Niedziela and Rybczyński, 2014). Somatic embryogenesis is a complex process that allows the performance of multidisciplinary studies into the mechanism of embryo induction and its development. There are a huge number of indications that this phenomenon also has great potential for plant propagation (Rybczyński et al., 2007).

This research describes the effect of electroporation and the introduction of foreign genes (*gus* and *ntp*II) on the morphogenic potential of *G. kurroo* embryogenic cell suspension protoplasts (Fig. 1).

Methods

Cell suspension initiation and culture

Seedling explants of *G. kurroo* (cotyledons, hypocotyles and roots) were implanted on an MS medium supplemented with 1.0 mg/l Kin (kinetin) and 0.5 mg/l 2,4-D (2,4-dichlorophenoxy acid) to induce an embryogenic callus culture. A hypocotyl-derived embryogenic callus was used for cell suspension culture establishment on an MS medium supplemented with BAP (benzylaminopurine), NAA (naphthaleneacetic acid), Dicamba (3,6-dichloro-2-methoxybenzoic acid) and AS (adenine sulfate) (Fiuk and Rybczyński, 2007). Cell suspensions were initiated by transferring 1.0 g of embryogenic tissue to 20 ml of liquid medium in 100 ml conical flasks. Once established, the cell suspension cultures were maintained as 80 ml aliquots in large 200 ml conical flasks, with agitation on a rotary shaker at 120 rpm, under diffused light 20 μM $m^{-2}s^{-1}$ at 22 °C. Subcultures of the cell suspension were made every seven days.

For the evaluation of the embryogenic suspension, the production of embryoids was measured by implanting cell suspension fractions (500, 500-300, 300-150 and 150 μm) on MS medium supplemented with 0.5 mg/l GA_3 (gibberellic acid), 1.0 mg/l Kin + 80 mg/l AS.

Protoplast isolation and culture

Protoplasts were isolated from embryogenic cell suspensions which were subcultured every 7 days. One gram of cell suspension was transferred to deep Petri plates and immersed in 20 ml of enzyme mixture. Powdered enzymes – Cellulase RS (YakultHonsha Co., LTD) – 1.5%; Driselase (Fluka AG) – 0.5%; Hemicellulase (Sig-

ma) – 0.25%; Macerozyme R10 (YakultHonsha Co., LTD) – 1.5%; Pectolyase (Seishin Pharmaceutical Co., LTD) – 0.04% – were dissolved in a protoplast washing solution (Frearson et al., 1973). Non-soluble debris were removed by centrifugation. The supernatant was supplemented with 9% of mannitol and 5.0 mM MES (2-[N-Morpholino] ethanesulfonic acid). pH was adjusted to 5.8 (NaOH). The mixture was filter-sterilized using a 0.2 μm membrane filter of Sartorius sterilization system in the cold.

The plant material was incubated in the enzyme mixture for 12 hrs at 28°C in the dark with 30 rpm agitation. The released protoplasts were filtered through a plastic sieve (45 μm) and centrifuged for 10 min at 180 rpm on an MPW-360 centrifuge (MPW Med. Instruments, Warsaw, Poland). The protoplast pellet was resuspended and washed three times with a protoplast washing solution supplemented with 90.0 g/l mannitol. The protoplast yield and densities were determined with a Fuchs-Rosenthal hermocytometer. Protoplasts were embedded in Sea Plaque agarose-gelled PCM medium (MS without NH_4NO_3 + 30.0 g/l glucose + 3.0 g/l Glutamine + 0.5 mg/l 2,4-D + 1.0 mg/l Kin + 9.0% mannitol, pH = 5.8) and cultured in droplets of 100 μl. 10 droplets per 5.0 cm Petri plate were covered by 2.0 ml of a liquid PCM medium.

The effect of kanamycin concentration on protoplast survival

Freshly isolated protoplasts were embedded in agarose PCM and, in the form of 100 μl drops of medium containing protoplasts, these were dropped into a ⌀ 5.0 cm plastic Petri plate. Later, once the droplets (10 per plate) had jellified, Petri plates were filled with 2.0 ml of PCM. After seven days of culture, the medium was exchanged for the PCM supplemented with 25.0, 50.0, 75.0 or 100.0 mg/l kanamycin for transformant selection. The experiment was repeated five times for each antibiotic concentration. The culture period lasted four weeks. The effect of kanamycin concentration on the growth and development of cultured protoplasts was evaluated using a Vanox Olympus light microscope, and this served as the basis for the choice of the antibiotic concentration for further experiments.

Esherichia coli culture and plasmid DNA isolation

Escherihia coli strain HB 101 with plasmid pBI (Jefferson et al., 1987) carrying an *npt II* gene encoding neomycin phosphotransferase under a *nos* promoter and a *gus* gene encoding β-glucuronidase under a CaMV35S promoter was the source of DNA used for electroporation experiments.

E. coli HB 101 cells were maintained on LB medium (Schaad, 1988) containing 5.0 g/l yeast extract, 10.0 g/l tryptophan, and 10.0 g/l NaCl, pH 7.2. Plasmids were isolated by alkaline lysis with some modifications (Sambrook and Russell, 2001). The bacteria cells were treated with 15 μl RNase (10mg/ml) in solution I (50 mM glucose, 25 mM Tris-HCL, pH = 8.0, 10 mM EDTA pH = 8.0). Later, the sample was supplemented with 200 μl solution II (0.2 MNaOH, 1% SDS), shaken gently and incubated in ice for 5 min, and then 150 μl of frozen 7.5 M ammonium acetate was added. The sample was left for 10 min on ice, followed by centrifugation at 14,000 rpm. The DNA was precipitated with 900 μl of 96% EtOH for 2 min. at room temperature and centrifuged at 13,000 rpm for 5 min. The next step of preparation consisted of the washing of the DNA sample with 70% EtOH and centrifugation at 13K rpm for 5 min. Finally, the sample was dried at room temperature and diluted in 30 μl H_20. All centrifugations in this procedure were performed in 1.5 ml Eppendorf tubes and on a SIGMA 2-16PK centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany).

Protoplasts were electroporated using the Generator of Electric Pulses BTX ECM 2001 Electro Cell Manipulator together with Cuvette Safety Stand 630B in BTX Electroporation Cuvettes Plus with 2.0 mm Gap Cuvettes. Protoplasts freshly isolated from the embryogenic cell suspension were suspended in an electroporation buffer at a density of 4×10^5 per ml in the presence of 50 μg/ml plasmid DNA. Two electroporation buffers were tested: EB1 (70 mM KCl, 5 mM $MgCl_2$, 0.1% MES, 9% mannitol, pH = 5.8) and EB2 (4 mM $MgCl_2$, 0.5% MES, 9% mannitol, pH = 5.6). Before electric current treatment, the cuvettes with protoplasts and plasmids in the electroporation buffer were placed in ice for 10 min.

The electroporation parameters tested were: the length of the electric pulse (20, 30, 40 μs and 1.0, 2.0, and 5 ms), the number of pulses (1 or 2), the electric field strength (0.0-1.75 kv/cm), and the temperature (on ice; about 0°C and room temperature; about 20°C) treatment after electroporation. The experiments were repeated five times for each of the above experimental conditions.

After the electric current treatment, protoplasts were incubated at room temperature or on ice for 10 min, followed by protoplasts being washed with liquid

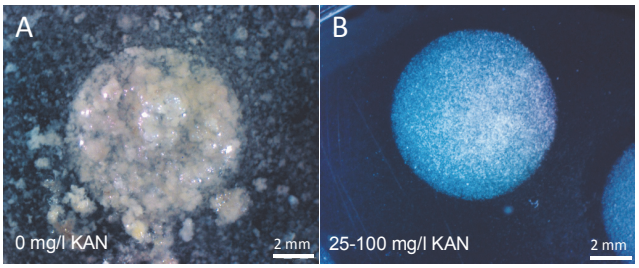

Fig. 2. Sea Plague Agarose embedded control protoplast and kanamycin treated protoplasts of *G. kurroo* after four weeks of the experiment. A) The overgrowth of callus formed by control protoplast culture. Notice spontaneous cell aggregates – shedding from agarose bead into liquid medium. B) Protoplast-derived cell division suppressed culture exposed to 25-100 mg/l kanamycin applied after 7 days of culture

PCM medium and left at room temperature for 24hrs. Then, the liquid medium was substituted by a PCM agarose gelling medium, and cultures were maintained as droplets with a protoplast density of 1×10^5/ml. A total of 2.0 ml of liquid PCM medium was then added to each Petri plate. After one week, the PCM medium was substituted with the same medium supplemented with 50 mg/l kanamycin. Complete osmotic pressure reduction was achieved as follows: mannitol was reduced from 9% to 6% after 3 weeks and then to 3% two weeks later with final reduction of osmotic pressure by week 8.

Agarose droplets with protoplasts already developed into multicellular aggregates were transferred to CSIM medium (MS + 0.5 mg/l 2,4-D + 1.0 mg/l Kinetin) supplemented with the same kanamycin concentration. Mock electroporation was performed as above, except that the electric current was not used. The protoplast morphogenic potential evaluation required a control culture which was carried out as described elsewhere (Fiuk and Rybczyński, 2007).

Protoplast viability and transient gene expression evaluation in the presence of kanamycin

Protoplast viability evaluation was performed twice using 0.01% FDA (Fluorescein diacetate) and UV light λ = 485 on a Vanox (Olympus) microscope: after 24 hrs and after 7 days of culture. After 7 days, the frequency of cell divisions was also scored. The transient gene expression was evaluated by the β-glucuronidase test. The histochemical activity of β-glucuronidase was evaluated again when the calli reached a prominent size and dissection of small pieces from the tissue did not harm subsequent proliferation.

Transformant regeneration from protoplast culture exposed to electroporation

After seven weeks of culture, agarose droplets with prominent calli were transferred to CSIM + Kan medium. The regenerated structures were transferred to a TPM medium (MS + 30.0 g/l sucrose + 8.0 g/l agar, pH = 5.8) supplemented with 50 mg/l kanamycin.

Evaluation of GUS activity

The expression of *gus* in cells, calli and leaf fragments of transformants was assayed using 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) as a substrate. The tissues were incubated in the reaction mixture including 1.0 mM X-Gluc in DMSO, 20.0 mM phosphate buffer pH = 7.0, 0.25 mM Triton X-100, 3.12 mM methanol. The plant material was covered with a reaction mixture and maintained at 37°C for 24hrs in the dark. After that, the tissues were treated with 70% ethanol (three times) to extract chlorophyll from them in order to visualize a blue pigment (dichloro-dibromoindigo). The blue stain is the effect of GUS activity. Finally, the tissues were fixed with Carnoy's fixative (acetic acid/ethanol: v/v 1:3).

PCR analysis of regenerated plants

In order to confirm the transgenesis, primers for the marker *nptII* gene (selection gene) and for the gene *uidA* (*gus* reporter gene) were used for testing. For the PCR amplification of a 700 bp fragment of the *nptII* gene, the following starters have been used: (5′-GAGGCTATTCGG CTATGACTG-3′) and (5′-ATCGGGAGCGGCGATACCG TA-3′) (Dong and McHughen, 1993). Next, the following thermal profile was adopted: 95°C 15 min., 30× (94°C 30 sec., 56°C 30 sec., 72°C 60 sec.), 72°C 5 min and 4°C. For the *uidA* gene, the following starters were tested: 1 – (5′-TTATCTCTATGAACTGTGCGTCA-3′) and 2 – (5′- TTGGACATACCATCCGTAATAA-3′) with amplification of 679 bp. Primers were designed according to the Primer3 program on the basis of *uidA* gene sequences found in the NCBI database (National Center for Biotechnology Information 2008). After that, the following thermal profile was adopted: 95°C 15 min., 30× (94°C 30 sec., 51°C 1 min., 72°C 2 min), 72°C 10 min and 4°C.

Statistical analysis

A statistical analysis was performed with the application of the Statgraphics Plus4.1 program. The number of samples and their size, and the number of repetitions of experiments are presented.

Results

Electroporation experiments were carried out on freshly isolated protoplasts of *Gentiana kurroo* embryogenic cell suspensions. Electroporated DNA plasmid derived from the HB101 *E. coli* strain carried two gene markers: *nptII* (selection) and *gus* (reporter). Successful electroporation experiments required the determination of the protoplast viability, the content of the electroporation buffer, the length of the electric pulse, the number of pulses and the strength of the electric fields applied. Long-term kanamycin treatment of calli resulted in a selection of regenerants.

The effect of kanamycin on the growth and development of protoplasts in control cultures

To assess the effect of kanamycin on the cell division and growth of protoplast cultures, kanamycin was used at 0.0, 25.0, 50.0, 75.0, and 100.0 mg/l. The use of kanamycin significantly blocked cell divisions of protoplasts and, at 25-75 mg/l, it decreased cell division in protoplast-derived cultures four-fold. The highest level of kanamycin (100.0 mg/l) completely blocked cell division and no further development of the culture was observed. In contrast, those control cultures which expressed a morphogenic potential by forming a callus and regenerating plants when treated with kanamycin did not form a callus and were not able to regenerate plants (Fig. 2).

The effect of the electroporation buffer on protoplast survival

The effect of the buffer composition on protoplast survival was studied after 24 hrs and at 7 days of culture of freshly isolated protoplasts. After one week of culture, protoplast electroporation in the richer EB1 medium resulted in a protoplast viability of 70.1%, while EB2 buffer supported a significantly lower level of survival (55.8%). Protoplast viability was higher at room temperature than on ice, where it dropped to as low as 60%. Conversely, when assessed after 7 days, which led to nutrient protoplast starvation, protoplast survival was higher on ice than at room temperature (Fig. 3).

Effect of electric field on protoplast survival and plating efficiency

The effect of the electric field voltage on protoplast survival was evaluated twice: at 24 hrs and 7 days. A high electric field voltage significantly decreased the protoplast survival, irrespective of the temperature at

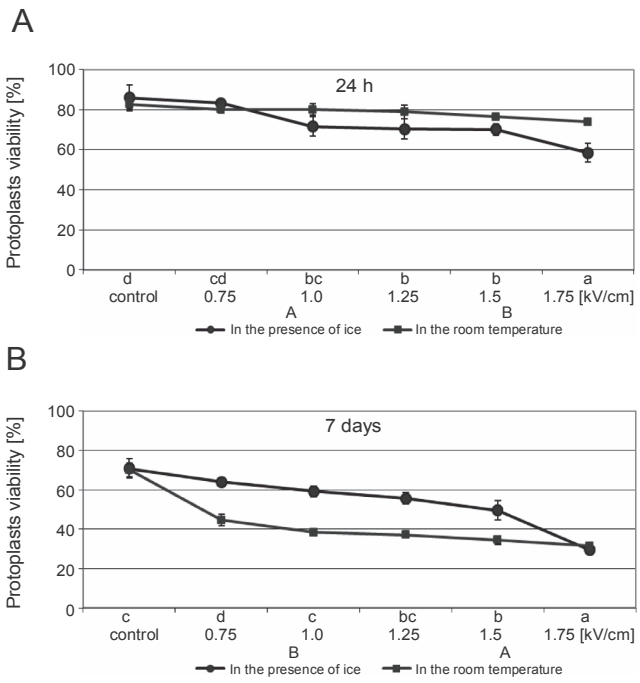

Fig. 3. The effect of temperature (at 0°C and 20°C) and electric field current (1 puls, 20 μs long) on viability of *G. kurroo* protoplasts evaluated A) 24 hrs and B) 7 days after electroporation (values described by the same letters and the size of characters are not significantly different, $p > 0.05$)

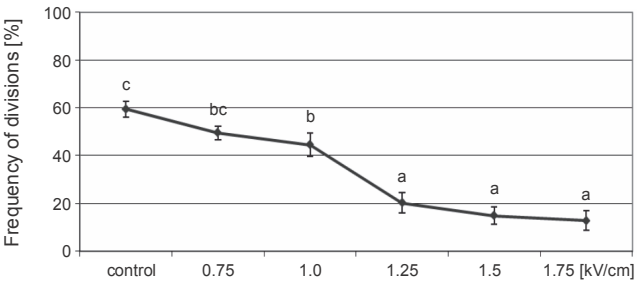

Fig. 4. The effect of electric field on frequency of *G. kurroo* protoplast cell division after one pulse of 20 μs duration. Protoplasts were incubated on ice (data marked by the same letters are not statistically different, $p > 0.05$)

which protoplasts were maintained following electroporation. At the lowest voltage – 0.75 kV/cm – 44.6% of protoplasts survived, while at the highest voltage (1.75 kV/cm) only 31.4% survived. Protoplast incubation on ice produced similar results, with a protoplast survival of 63.8% at 0.75 kV/cm and 29.3% at 1.75 kV/cm after one week of culture (Fig. 4).

Non-electroporated protoplasts showed a plating efficiency of 59.4%. The lowest electric pulse voltage (0.75 kV/cm) reduced the protoplast plating efficiency to

49.5%, while further increases in the electric field strength up to 1 kV/cm resulted in a protoplast division reduction to 44.53%. These data, however, were not significantly different. The elevation of electric field voltage above 1 kV/cm dramatically decreased protoplast division, but results were not significantly different from those for 1.25 or 1.75 kV/cm.

Number of pulses

The number of pulses had a significant effect on the viability and frequency of the cell division of protoplasts. Thus, the percentage of viable protoplasts was nine-fold lower with two successive electric pulses as compared to a single one. With one pulse, the frequency of protoplast division was 44.53%, and this dropped to 6.8% with two pulses.

The effect of short and long time electric pulse treatment on protoplast viability and division

To study the effect of the length of an electric pulse on protoplast viability, five durations of pulses were selected: from 20, 30, 40 μs; and 1.0, 2.0 and 5 ms. The two longest pulses significantly reduced protoplast viability counted after 24 hrs of culture (Fig. 4). After one week of culture, the longest pulse treatments killed all the protoplasts. Protoplasts electroporated for 20, 30 and 40 μs survived at 70, 64 and 60%, respectively, after 24 hrs of culture. After 7 days, the highest level of survival was observed for those samples treated with the shortest electric pulses (20 μs) (Fig. 5).

Long electric pulses decreased the number of dividing protoplasts, and a pulse longer than 40 μs totally blocked the division of electroporated protoplast-derived cells. The highest percentage (44.5%) of protoplast division occurred in the case of 20 μs pulses. Already, a 30 μs long pulse resulted in a drastic decrease to 5.6% in cell division, which was reduced further to 2.9% with 40 μs pulses, although no significant differences were observed between 30 and 40 μs.

Plant regeneration from electroporated protoplasts

Among the experimental conditions for the electroporation of embryogenic cell suspension protoplasts of *G. kurroo* (Fig. 6A and Fig. 6B), only one combination led to callus formation and plant regeneration; namely, one single 20 μs long pulse with a field strength of 1 kV/cm. It is noteworthy, that the control protoplast culture underwent numerous cell divisions and by six

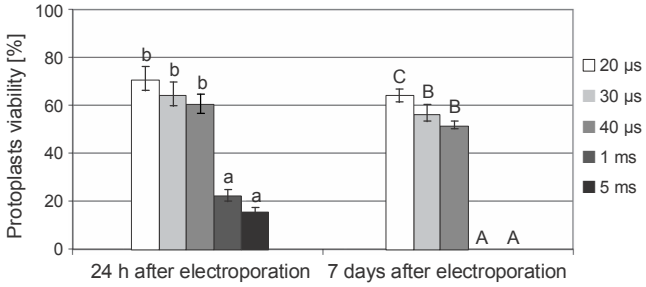

Fig. 5. The effect of the duration of electric pulse on the viability of *G. kurroo* protoplasts after application of one pulse of 1 kV/cm and incubated on ice (data marked with the same letters and size of characters are not significantly different, $p > 0.05$)

weeks the beginning of small callus formation was noticed. Further, the change of culture conditions from liquid to solidified medium resulted in plant regeneration.

Electroporated protoplasts required embedding in agarose droplets supplemented with plant growth medium to start cell division. The initial division of protoplast-derived cells was observed after 5 days of culture (Fig. 6C and Fig. 6D). As expected, supplementing kanamycin to the culture medium after 7 days suppressed cell divisions of non-transformed protoplasts. A microscopic analysis confirmed the mitotic activity of transformed protoplasts which were able to reach a few cell aggregates. However, only an extremely low percentage of protoplasts in kanamycin selection conditions were able to divide and form a callus (Fig. 6E). Two months of protoplast culture in agarose droplets was sufficient for prominent callus formation (Fig. 6F and Fig. 6G). Initially, the transgenic callus consisted of hydrated cells which were white or pale yellow (Fig. 6H). Only the embryogenic regions of the callus were of a deeper yellow color. On the proliferation medium, these regions underwent an intense growth and this resulted in plant regeneration of *G. kurroo*. Indirect somatic embryogenesis from the callus was the only morphogenic pathway observed in these cultures (Fig. 6I and Fig. 6J). Finally, 9 plants were regenerated and later their resistance to kanamycin was confirmed.

Transgenesis confirmation assessment of* gus *gene expression

No expression of *gus* genes on cells and calli or in collected leaf fragments was observed. In contrast to the results obtained for the transformation of cell suspensions (data not published) with *A. tumefaciens*, proto-

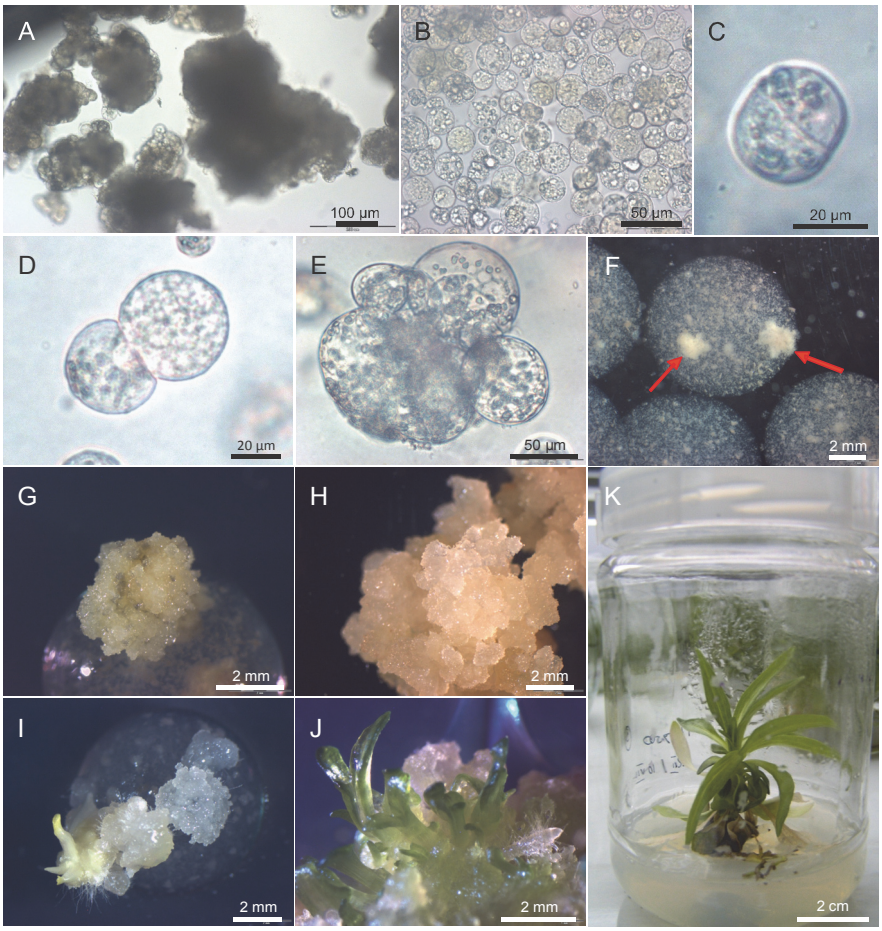

Fig. 6. *G. kurroo* transformant regeneration after embryogenic cell suspension protoplast electroporation: A) General view of plant cell suspension used for protoplast production, B) Freshly isolated protoplasts ready for electroporation experiments, C-D) First cell division of electroporated protoplasts after initial cell wall regeneration on 5th day of culture, E) Few cell aggregates, F) Agarose droplet with developing mini calli, G-H) Overgrowth of transformed callus on the agar proliferation medium, I-J) Somatic embryo formation from callus derived from electroporated protoplasts, K) Transgenic plantlet of *G. kurroo* cultured on 50 mg/l kanamycin selection medium. All cultures were carried out in the presence of 50 mg/l kanamycin

plast transformants did not show any expression of gene coding for β-glucuronidase (blue color).

Discussion

Protoplast transformation opens the possibility of obtaining genetically modified plants from single cells, thus excluding chimerism of tissue and regenerants derived from multicellular explants (Puonti-Kaerlas et al., 1992). The absence of a cell wall makes the protoplast an almost ideal explant for the exploration of electroporation (Tagu et al., 1988; Saunders et al., 1995). However, the most important prerequisite is the development of an efficient methodology of plant regeneration from protoplast cultures. In our experiments, electroporation was carried out on protoplasts derived from embryogenic cell suspension cultures, for which the conditions for protoplast isolation, culture and plant regeneration had previously been described (Fiuk and Rybczyński, 2007). In the discussed paper, protoplasts were placed under the extremely tough stress conditions of an electric field, various buffers and kanamycin treatment. However, this stress was partly compensated by the culture conditions. Our attempts resulted in the expression of genes of the selection agent and the establishment of a callus culture with embryogenic potential.

The chemical composition of the electroporation buffer plays an important role in protoplast survival, as does the electric field. Gupta and coworkers (1988) showed that protoplast viability of conifer plants after electroporation was reduced almost by half. In our experiments,

buffers differed in their composition. These differences resulted in a higher survival (70.1%) in EB1 buffer and lower (55.82%) in EB2 buffer. High protoplast survival is related to the application of the same pH during protoplast isolation and electroporation. In this study, it is necessary to remember that divalent cations ($Ca^{2+}$and Mg^{2+}) play an important role in membrane stabilization, which has been discovered in protoplast fusion with both PEG and electrofusion experiments (Fromm at al., 1985; Niedz et al., 2003). Working with friable structures such as protoplasts, their plasmolemma requires some physical protection in the case of any manipulations, so incubation on ice appeared to act as the agent for biological stabilization. However, the death of the protoplast may be the result of the overheating of a sample which is located between two electrodes, and irreversible membrane damage resulting from the electric field applied. Hence, pre-cooling of the electroporation cuvette lessened the effects of such drastic protoplast treatments. It has been proved that low temperature supports the duration of the pore opening and the efficiency of transformation is higher than with an uncooled sample (Tsong and Kinosita, 1977). This research contradicted the electroporation of citrus protoplasts, where heat shock treatment at 49°C before an electric pulse greatly enhances transient transformation expression (Hidaka and Omura, 1993).

The only condition of electroporation with *nptII* + *uidA* genes that permitted the development of a callus and regenerated plants in the presence of kanamycin selection medium was 1 pulse with a voltage of 1kV/cm and a duration of 20 μs. Other experimental conditions studied resulted in a drastic decrease in the number of dividing protoplasts. A significant decrease in the number of the dividing protoplasts was found when the voltage was higher than 1.0 kV/cm. We did not observe any significant differences between 0.75 and 1.0 kV/cm. It has previously been shown that the voltage used for electroporation will change with the electroporated plant material. For *Stysanthes guianensis* a voltage of 0.25 kV/cm (Quecini et al., 2002) is the best, while for *Citrus sinensis, Daucus carota* (Bower and Birch, 1990; Niedz et al., 2003) it is 0.45 kV/cm, 0.5 kV/cm for *Nicotiana tabacum, Glycine max* (Bates, 1989; Dhir et al. 1991), and 1.0 kV/cm for *Pisum sativum* (Hashimoto et al., 1992). The last result was similar to our results. Most frequently, only one pulse is used; however, sometimes 6 or 10 pulses are required (Bower and Birch, 1990). The pulse duration is experimentally specified and species dependent. For protoplasts of Chinese orange a duration as long as 70-80 ms is the best (Niedz et al., 2003).

In our experiments, only DNA at a concentration of 50 μg/ml was used. A number of papers indicate 100 μg/ml as the most successful concentration of DNA in transient expression. The most effective transformation of *Citrus* protoplasts occurred in the presence of that concentration, while doubling it to 200 μg/ml resulted in a drastic reduction of the phenomenon (Hidaka and Omura, 1993). Not only does the concentration of the plasmid play an important role, but the form (circular or linear) of DNA should also be considered (Quecini et al., 2002). In some plants, linear DNA allowed better results to be obtained than the circular vector (Negrutiu et al., 1987; Shillito et al., 1985).

Because of the inner structure of DNA and the lack of distortions resulting from the structure of a double helix, a circular DNA is significantly less mobile in the electric field (Courey and Wang, 1983; Drew et al., 1988). The size of the linear molecule is significantly smaller because of the lack of its tertiary and quaternary structure, which helps DNA molecules to penetrate the pores of cell membranes (Tanaka, 1988).

The number of plants, only nine, derived from electroporated embryogenic protoplasts is low in comparison to the morphogenic potential of untreated protoplasts (Fiuk and Rybczyński, 2007). However, the system of selection based on kanamycin resistance seems to be very stringent and only the expression of the *nptII* gene ensures the survival of the protoplast and callus in the presence of 50.0 mg/l kanamycin. The lack of a solid cell wall at the initial stages of protoplast cultures that could act as a physical barrier, combined with a relatively early application of kanamycin, could be considered as the factors that inhibit the formation of "escapes".

The experimental system presented here is very labor-intensive and requires knowledge of protoplast culture and plant regeneration techniques. It is nevertheless worthwhile to invest in research into technology for sourcing the whole culture from a single cell.

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Authors' contribution

The authors' contributions to the research are: laboratory experiments – A. Wójcik, writing of the manuscript – J. J. Rybczyński.

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