Zygote **13** (February), pp. 39–44. © 2005 Cambridge University Press
doi:10.1017/S0967199405003102 Printed in the United Kingdom

Production of rabbit chimeric embryos by aggregation of zona-free nuclear transfer blastomeres

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Date submitted: 25.10.04. Date accepted: 25.01.05

Summary

The objective of this study was to compare *in vitro* developmental capacity of zona-free aggregated rabbit chimeric embryos and the allocation of EGFP (enhanced green fluorescence protein) gene expression to the inner cell mass (ICM). We produced chimeric embryos by synchronous aggregation of zona-free blastomeres from embryonic cell nuclear transfer (EMB-NT) or somatic cell nuclear transfer (SC-NT) and blastomeres from normal zona-free embryos (N) at the 16-cell stage. In the control group, transgenic (TR) and normal zona-free embryos were used to produce chimeric embryos (TR<>N). EMB-NT embryos were produced by fusion of enucleated oocytes with embryonic cells, which were derived from 32-cell stage transgenic embryos bearing the EGFP gene. The SC-NT embryos were produced by fusing enucleated oocytes with cumulus cells, which were derived from homozygotes transgenic for the EGFP gene female oocytes at 16 h *post-coitum*. Nuclei of transgenic blastomeres emitted a green signal under fluorescence microscopy. Zona-free EMB-NT or zona-free SC-NT rabbit embryos, both with EGFP fluorescence, as well as TR and zona-free rabbit embryos with no fluorescence (EMB-NT<>N, SC-NT<>N, TR<>N) were aggregated on day 2.5 and evaluated on day 5. The proportion of EMB-NT<>N embryos that developed to the blastocyst stage was significantly higher compared with SC-NT derived cells ($p < 0.05$), but significantly lower than in TR<>N chimeric blastocysts ($p < 0.001$). Similarly, a higher proportion ($p < 0.001$) of EGFP-positive cells allocated to ICM of chimeric blastocysts was revealed in TR<>N chimeras (55%), compared with EMB-NT<>N (35%) and SC-NT<>N (21%). Our results indicate that synchronous chimeric embryos reconstructed from TR embryos were better able to develop and colonize the ICM area than EMB-NT and SC-NT embryos. In this study we have demonstrated for the first time that rabbit NT-derived embryos are able to develop into chimeric blastocysts and participate in the ICM area.

Keywords: Chimera, EGFP, Embryo, Embryonic cells, Nuclear transfer, Rabbit, Somatic cells

Introduction

Reprogramming the nuclei of differentiated cells using the technique of nuclear transfer (NT) has been proposed as an alternative method for the production of transgenic animals, particularly at gene targeting.

Transgenic nuclei from embryonic cells have been used for the production of rabbit transgenic cloned embryos by transfer of asynchronous blastomeres into host embryos (Chrenek *et al*., 1998, 2000; Kang *et al*., 2000), demonstrating the potential of this approach. Although remodelling with subsequent development to term using rabbit embryonic and somatic cell nuclei has been reported (Stice & Robl, 1988, Chesne *et al*., 2002), there are have been no reports of successful reprogramming of transgenic somatic cell nuclei. Recently a novel method of producing transgenic cloned rabbit embryos was demonstrated, in which about three somatic cell nuclear transfer (SC-NT) transgenic blastomeres, dissociated from a morula-stage embryo, were transferred into host embryos at the 4- to 8-cell stage (Matsuda *et al*., 2002). This technique, using asynchronously staged cells, demonstrated that SC-NT embryos have the ability to develop and differentiate *in vivo* to form a fetus. To increase the probability of a higher cell number in NT-derived embryos, Boiani *et al*. (2003) presented a method based

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on the aggregation of genetically identical clone–clone embryos at the 4-cell stage. Clone–clone aggregates did not form more blastocysts, but most of them expressed the transgene and had a higher rate of fetal and postnatal development.

In our study we used another technique of chimera production: aggregation of zona-free synchronous rabbit embryos. The aim of our experiments was to compare *in vitro* preimplantation development of rabbit transgenic chimeric embryos reconstructed from: (1) transgenic embryonic cell nuclear transfer (EMB-NT) embryos and normal (N) embryos (EMB-NT<>N), (2) transgenic somatic cell nuclear transfer (SC-NT) embryos and normal embryos (SC-NT<>N), and (3) transgenic (TR) and normal embryos (TR<>N) at about the 16-cell stage. Allocation of NT-derived blastomeres to the inner cell mass (ICM) was monitored according to the presence of EGFP (enhanced green fluorescence protein) expression.

Materials and methods

Oocyte and embryo collection

Mature New Zealand White rabbit females were superovulated with six subcutaneous injections of FSH: two of 0.625 mg per animal and four of 0.250 mg per animal injection given 12 h apart. HCG at a dose of 100 IU was injected intravenously 12 h after the last dose of FSH. At 16 h after the injection of HCG, females were laparotomized and mature MII oocytes were flushed from the oviduct with Dulbecco's phosphate-buffered saline (PBS) supplemented with 3 mg/ml of bovine serum albumin (BSA; Sigma). Cumulus cells were dispersed using 0.5% hyaluronidase (Sigma) treatment and gentle pipetting.

For embryo collection, superovulated does were mated with a nontransgenic or transgenic (EGFP gene) male. Then embryos at the 2-cell or 16-cell stage were flushed, using the same procedure as for oocytes, at 24 h or 48 h after HCG treatment, respectively.

Subsequently, oocytes and embryos were cultured in k-DMEM medium (Gibco BRL) supplemented with 10% FBS-ES (Gibco BRL) in a humidified atmosphere of 5% CO_2, in air at 37 °C.

Preparation of nuclear donor cells

Transgenic (the EGFP gene) embryonic cells (EMB), as a source of nuclei, were deep-frozen using 1.5 M dimethylsulfoxide (DMSO) (Chrenek *et al*., 2000) and stored in liquid nitrogen until the day of nuclear transfer. After rapid thawing, embryos were released from their mucin coat and zona pellucida by 0.5% pronase treatment (Sigma), rinsed two times in CIM medium (Gibco BRL) supplemented with 10% FBS-ES and incubated in a Ca^{2+}- and Mg^{2+}-free PBS medium (Gibco BRL) for 5 min at 38 °C. All blastomeres from the embryos were then dissociated using a gauged pipette and kept in CIM medium supplemented with 10% FBS-ES at room temperature until nuclear transfer.

Transgenic (EGFP gene) cumulus cells (SC), as a source of somatic nuclei, were detached from the oocytes of a transgenic homozygous female. Immediately after flushing of the oocytes, cumulus cells were dispersed by 4 min of treatment with 0.5% hyaluronidase in CIM medium and incubated in a Ca^{2+}- and Mg^{2+}-free PBS medium containing 2% polyvinylpyrrolidone (PVP; Sigma) at room temperature until nuclear transfer.

Nuclear transfer

Recipient oocytes, free of cumulus cells, were incubated in CIM medium containing 0.5 μg/ml Hoechst 33342 (Sigma) for 30 min. Metaphase chromosomes and the polar body for enucleation were visualized under ultraviolet light of a fluorescence microscope. The first polar body and metaphase plate with approximately 5% of its surrounding cytoplasm were removed by treatment of the oocytes with cytochalasin B (Sigma) and by aspiration through a specially beveled pipette (20 μm inner diameter) without rupturing the plasma membrane.

Single donor transgenic cells (EMB or SC) were individually introduced into the perivitelline space of enucleated oocytes. The eggs were submitted to electrostimulation in a solution containing 0.3 M mannitol (Sigma), 0.1 mM calcium chloride and 0.1 mM magnesium chloride to achieve fusion of membranes (3 DC pulses of 3.2 kV/cm for 20 μs). Fused NT embryos were activated by electric pulse (3.2 kV/cm, 3 × 20 μs, 0.3 M mannitol solution) followed by 1 h of culture in k-DMEM medium containing 10% FBS-ES and 2 mM 6-DMAP (Sigma). The embryos were subsequently cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C until the aggregation procedure (up to the 16-cell stage).

Chimeric embryo production

For the production of chimeric embryos all the embryos were released from the zona pellucida as described above. We produced chimeric embryos by synchronous aggregation of zona-free embryos derived from the EMB-NT or SC-NT embryos or transgenic (TR) embryos with zona-free normal (N) embryos at the 16-cell stage (EMB-NT<>N or SC-NT<>N or TR<>N) in microdrops of k-DMEM medium containing 10% FBS-ES under mineral oil. All the embryos were placed in microdrops, and were manipulated with a tip-drawn glass pipette to contact in a linear fashion. The embryos

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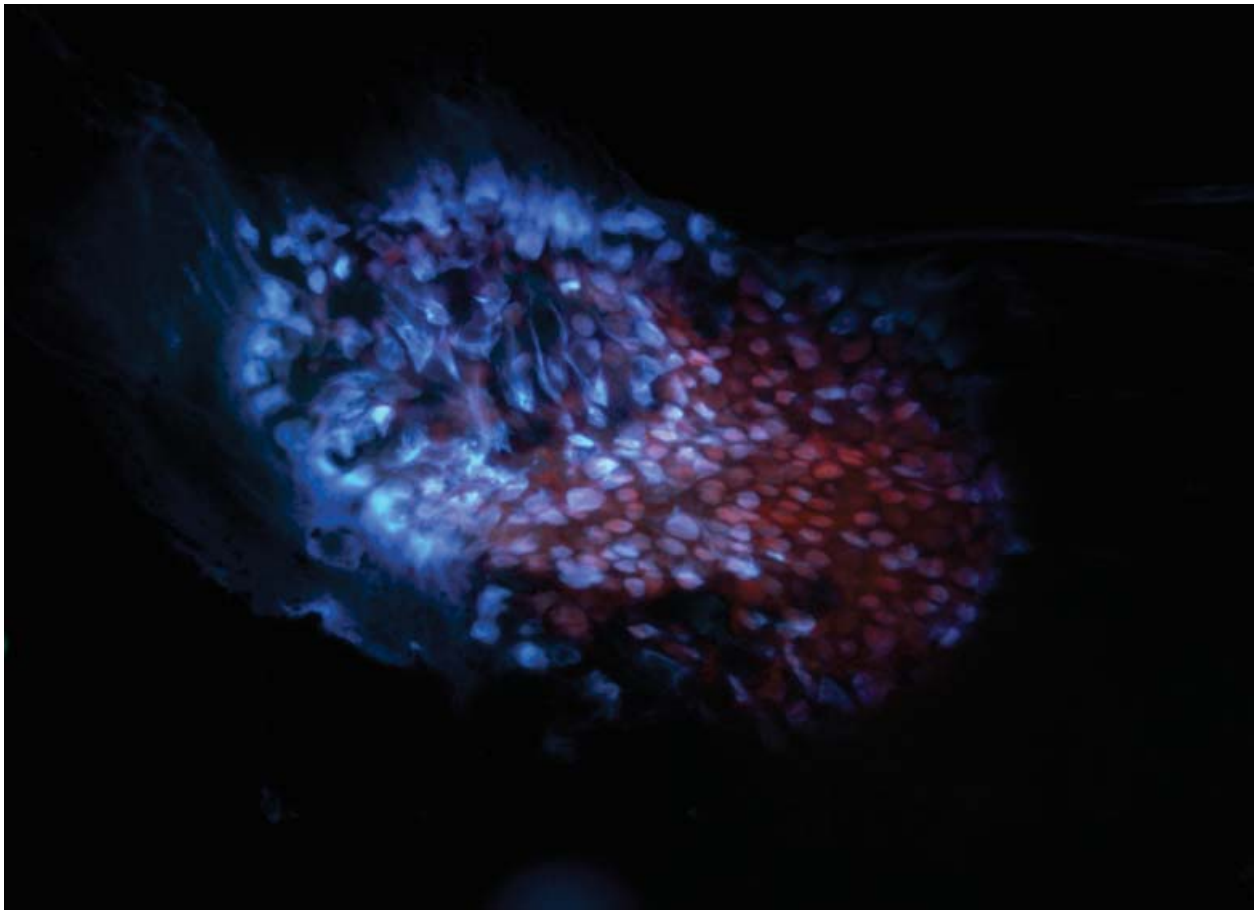

Figure 1 Differential staining of chimeric rabbit embryos. Trophectroderm cells are pink-stained; inner cell mass cells are blue-stained.

were kept in this position, in a humidified atmosphere of 5% CO_2 in air at 37 °C up to the blastocyst stage.

EGFP expression

EGFP expression in transgenic and transgenic NT-derived embryo blastomeres was monitored under a Leica fluorescence microscope (excitation filter 460–495 nm).

Cell number and differential staining analysis

For the determination of cell number, blastocysts were stained for 20 min with 1 μg/ml of Hoechst 33342 (Sigma), mounted on a microslide in Vectashield (Vector Laboratories, Burlingame, CA) and analysed under a Leica fluorescence microscope (excitation filter 340–380 nm).

For the ICM allocation, the blastocysts were differentially stained according to Thouas (2001) with slight modification. Briefly, embryos were incubated in 0.5% Triton X-100 and 25 μg/ml propidium iodide (PI) diluted in BSA-free Hepes-buffered TCM 199 medium for 10–15 s. They were then washed briefly in 2 drops of PBS-PVP solution and transferred to an ice-cold solution of 25 μg/ml Hoechst 33342 diluted in 100% ethanol. In this solution the embryos were fixed and stained for 2 h. Afterwards they were examined under a Leica fluorescence microscope (Fig. 1) using excitation filters of 340–380 nm (for Hoechst 33342) and 515–560 nm (for PI).

Statistics

Development of aggregated embryos up to the blastocyst stage and the expression of EGFP were analysed using the chi-square test. Cell numbers were processed using analysis of variance (ANOVA).

Results

Table 1 summarizes the developmental rates of NT embryos produced using different sources of donor cell nuclei. Although the proportion of fused eggs did not differ between embryonic (EMB; 85%) and somatic cell nuclei (SC; 81%), the cleavage rate of

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Table 1 *In vitro* development of NT-derived and parthenogenetic rabbit embryos

Donor cell	No. of oocytes	No. fused *n*/*N* (%)	No. of 2- to 4-cell *n*/*N* (%)	No. of 8-cell *n*/*N* (%)	No. of 16-cell *n*/*N* (%)
EMB	130	110/130 (85)	75/110 (68)	68/110 (62)	68/110 (62)[a]
SC	78	63/78 (81)	58/63 (92)	55/63 (87)	51/63 (81)[b]
Parthenogenetic activation	30	–	28/30 (93)	26/30 (87)	26/30 (87)[c]

[a] versus[b] difference is significant at $p < 0.05$; [a] versus[c] difference is significant at $p < 0.01$.

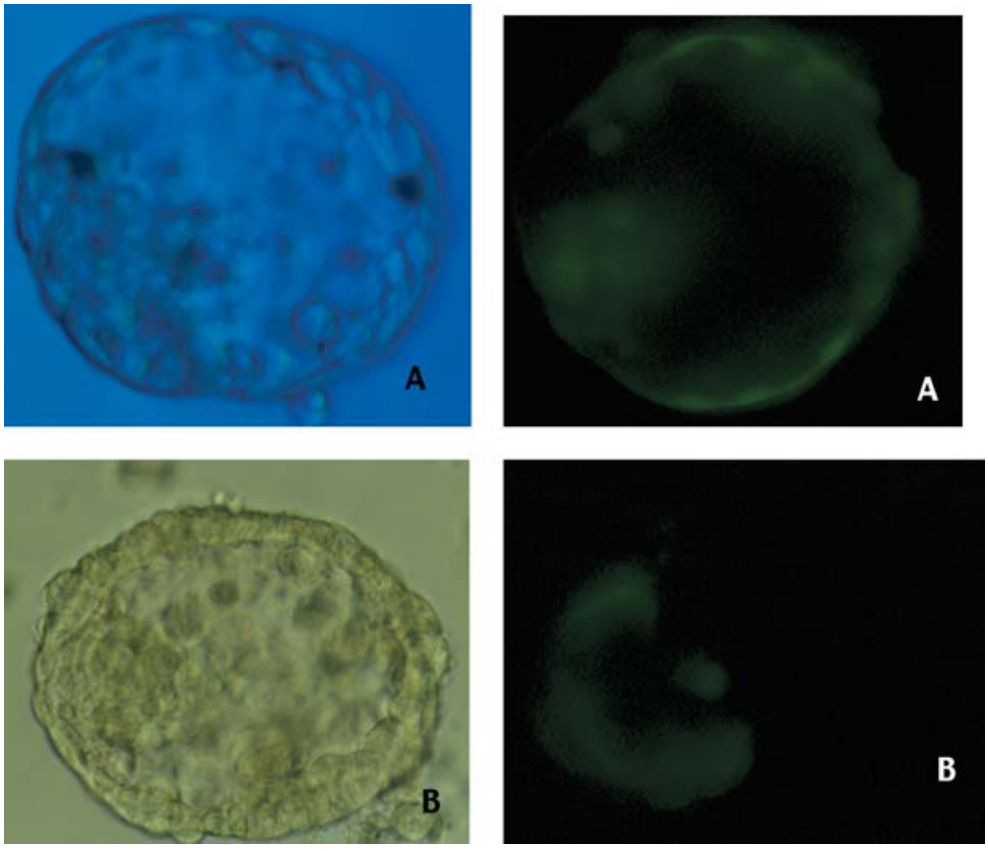

Figure 2 Representative results of rabbit chimera blastocyst production. (*A*) Rabbit chimeric transgenic blastocyst after aggregation of zona-free EMB-NT<>N embryos. (*B*) Rabbit chimeric transgenic blastocyst after aggregation of zona-free SC-NT<>N embryos.

cloned embryos to the 16-cell stage was significantly higher in SC-NT embryos (81% vs 62%, $p < 0.05$). A significantly higher cleavage rate (87%) was obtained in parthenogenetic rabbit embryos than in EMB-NT-derived embryos ($p < 0.01$). All NT-derived embryos exhibited green signal (EGFP gene expression) under fluorescence microscopy.

Production of chimeric embryos

The proportion of EMB-NT<>N embryos that developed to blastocyst stage (Fig. 2) was higher than in SC-NT<>N embryos ($p < 0.05$) but lower than in the TR<>N group ($p < 0.001$), where all embryos reached blastocyst stage (Table 2).

The total cell number and allocation of EGFP-positive (green) blastomeres in aggregated blastocysts are shown in Table 3. No differences were observed in the total cell number and ICM area cell number between the groups. The proportion of green NT blastomeres in the ICM area was different for each group. A significantly higher ($p < 0.001$) number of EGFP cells in the ICM area was revealed in TR<>N blastocysts compared with the two other aggregates. A significant

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Table 2 *In vitro* development of rabbit chimeric embryos up to blastocyst stage

Groups of chimeras	No. of reconstructed embryos *N*	No. aggregated *n*/*N* (%)	No. of blastocysts *n*/*N* (%)
EMB-NT<>N	30	20/30 (67)	20/30 (67)[a]
SC-NT<>N	20	14/20 (70)	10/20 (50)[b]
TR<>N	10	10/10 (100)	10/10 (100)[c]

[a] versus[b] difference is significant at $p < 0.05$; [a,b] versus[c] difference is significant at $p < 0.001$.

difference ($p < 0.001$) in the number of green NT cells in the ICM area was also observed between EMB-NT and SC-NT embryos.

Discussion

There are two approaches for the aggregation of chimeric embryos: introduction of halves of embryos into a common zona pellucida or aggregation of zona-free embryos. In our study we used synchronous aggregation of two zona-free rabbit embryos derived from differently manipulated embryos. Some previous experimentation had suggested that the culture of zona-free embryos could be problematic from two viewpoints. First, embryo aggregates would need to be cultured individually to prevent further aggregation during culture and to allow assessment of blastocyst building. The second concern is that the zona-free embryos would attach to the dish or co-culture cells and would then begin to spread in a monolayer.

Experiments carried out in recent decades have shown that it is possible to use zona-free aggregation in different embryos species (Nagy *et al*., 1993, Wells & Powell, 2000). More than 50% blastocyst yield from aggregated zona-free rabbit embryos supports this possibility. In rabbits, zona-free embryo manipulation is reasonable only in *in vitro* conditions. *In vivo*, rabbit embryos developing in the oviduct produce a mucin coat which is important during implantation of embryos in the uterus (Murakami & Imai, 1996). Therefore, it is unlikely, that chimeras formed by the aggregation of zona-free cleavage-stage embryos will be successful in the rabbit, as it has been shown that zona-free blastocysts fail to implant. Our aggregation approach was tested for developmental potential and allocation of transgenic NT-derived cells in the ICM area, but was not examined for embryo transfer of aggregated chimeric embryos.

In our previous work we have reported the production of an asynchronous and a synchronous rabbit chimera, where two EMB-NT-derived blastomeres were transferred into a host embryo (Chrenek *et al*., 1998, 2000). Using both types of aggregation, chimeric embryos from NT blastomeres showed a lower developmental rate than those constructed from transgenic or non-transgenic blastomeres. The developmental rate to blastocyst stage of chimeric embryos in those reports was about 98%, but participation of transgenic EMB-NT-derived blastomeres in production of the ICM area was lower than in the present study. Wells & Powell (2000) state that murine and bovine embryos 24 h asynchronous in development could be used to make aggregated chimeras with preferential allocation to the trophectoderm. However, asynchronous aggregation using bovine NT-derived embryos was not successful relative to synchronous aggregation. For this reason we also used synchronous aggregation in rabbit embryos.

In this study we firstly show that blastomeres of rabbit NT-derived embryos are able to develop and participate in the ICM area of synchronous zona-free aggregated chimeric embryos. We found that the EMB-NT embryos developed better up to the blastocyst stage than did SC-NT embryos, with a higher contribution to the ICM area but with a lower efficiency compared with TR non-NT embryos. More likely the reason for the failure of rabbit NT-derived blastomeres to develop and participate in the ICM of chimeric embryos results from chromosomal abnormalities of

Table 3 Distribution of blastomeres in an aggregated chimeric blastocyst

Group of chimeras	No. of chimeric blastocyst, *N*	All cells		EGFP-positive NT cells	
		Total *N*	ICM area *n* (%)	Total *N*	ICM area *n* (%)
EMB-NT<>N	20	240 ± 14.02	45 ± 2.63 (19)	85 ± 8.50	30 ± 2.80[a] (35)
SC-NT<>N	10	260 ± 15.95	49 ± 3.01 (19)	68 ± 7.02	14 ± 3.02[b] (21)
TR<>N	10	275 ± 13.10	58 ± 2.76 (21)	95 ± 8.93	52 ± 5.02[c] (55)

Values are the mean ± SEM.
Values with different superscripts within columns are significantly different ($p < 0.001$).

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NT-derived embryos. Although we did not carry out a chromosomal analysis of our NT-derived rabbit embryos, Shi *et al*. (2004) demonstrated higher chromosomal aneuploidy rates in cumulus cell NT-derived rabbit embryos compared with *in vivo* fertilized embryos. This incidence of chromosomal abnormalities was correlated with subsequent developmental failure. Another reason could be the lower developmental capacity of rabbit NT-derived blastomeres as a result of insufficient nuclear reprogramming. Chromatin remodelling and processes of reprogramming of somatic nuclei in embryonic development can also be assessed through the evaluation of genomic methylation, which has a major role in epigenetic regulation in cells. Insufficient reprogramming of nuclei and activation factors for ontogeny can also influence developmental rate of cloned rabbit embryos (Matsuda *et al*., 2002). These results agree with several reports which show that the developmental ability of rabbit SC-NT embryos *in vitro* and *in vivo* seems to be complicated (Pennisi & Vogel, 2000, Dinnyes *et al*., 2001). Boiani *et al*. (2003) found that mouse cloned blastocysts have less than half the normal cell number, and that higher cell number correlates with correct transgene expression. They proposed the use of clone<>clone aggregates as an alternative way to improve developmental rate and gene expression. On the other hand, Wells & Powell (2000) showed poor developmental ability of bovine NT<>NT chimeric embryos in *in vitro* conditions. Therefore, techniques of chimera production *in vitro* clearly still require further optimization.

In conclusion, rabbit TR blastomeres have a higher ability to develop and colonize the ICM area in synchronous chimeric embryos than do EMB-NT or SC-NT embryos, probably because TR blastomeres do not need reprogramming.

Acknowledgement

This work was supported by the grant from the Science and Technology Assistance Agency (APVT), Slovak Republic No. APVT-27-027902 and from the Ministry of Agriculture, Slovak Republic (grant CU 01- EU01-01).

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