Biotechnological methods utilized in *Cucumis* research – A review

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Abstract

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Our biotechnological research on selected *Cucumis* species has encompassed interspecific hybridization via embryo-rescue, *in vitro* pollination, somatic hybridization and cytogenetics of protoplasts. Embryo-rescue and *in vitro* pollination are suitable *in vitro* techniques for production of hybrid embryos. These methods were tested and optimized for cucurbits. Protoplast culture is another valuable tool for biotechnological applications, such as somatic hybridization and genetic transformation. We study protoplast dedifferentiation not only as a biotechnological application in breeding systems, but mainly to describe mechanisms of obtaining totipotency. Protoplasts of cucurbits were studied cytogenetically to observe changes in nuclear architecture during protoplastization and regeneration and for comparison with the expression profile obtained using cDNA-AFLP techniques and reverse transcription for the specific genes involved.

Keywords: *Cucumis* spp.; expression profile; interspecific hybridization; *in vitro* biotechnological methods

A number of biotechnological techniques make possible to obtain interspecific hybrids within the genus *Cucumis*. The most interesting hybridization partner for cucumber (*Cucumis sativus*) is muskmelon (*C. melo*, MR-1) because of its genes for resistance to downy mildew (*Pseudoperonospora cubensis*) (Lebeda et al. 2007). For successful hybridization, it is possible to use embryo-rescue techniques, *in vitro* pollination, and somatic hybridization by isolation and fusion of protoplasts. By optimizing these methods, it should be possible to obtain successful results for application to cucumber breeding programmes.

The method of embryo-rescue makes possible to obtain plants from immature embryos on culture media that plays a key role in this process. The presence of special components, such as an undefined mixture of organic components as caseinhydrolysate, coconut water, and growth regulators, is necessary for supporting successful embryogenesis (Ondřej, Navrátilová 2000; Skálová et al. 2008a). In other than *in vivo* pollination followed by embryo-rescue, there exists the possibility of *in vitro* pollination and fertilization. This area needs more positive results in Cucurbitaceae, because only callus formation was obtained (Ondřej et al. 2002a; Skálová et al. 2008b).

The method of protoplast culture is a unique and simple system enabling the study of the structure and function of cell organelles, cytoplasmic membrane transport, cell wall formation, influences of abiotic and biotic stress on plant cells, and characterization of their responses to genetic manipulation (Davey et al. 2005). Protoplast cultures also represent an efficient tool for overcoming hybridization barriers by somatic hybridization, where the

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Table 1. Frequency (%) of regeneration of *Cucumis* genotypes on various media

Cucumis spp.	Age of embryos															
	3 days				7 days				14 days				21 days			
	OK	ON	CW	GA	OK	ON	CW	GA	OK	ON	CW	GA	OK	ON	CW	GA
C. sativus	22	61	72	72	56	50	50	60	20	78	78	40	10	86	86	–
C. melo	6	11	6	17	60	25	60	80	89	89	100	100	50	45	55	40
C. anguria	35	7	27	–	35	7	27	–	50	20	50	40	90	80	80	–
C. zeyheri	6	8	12	4	10	10	20	60	55	40	70	40	80	90	75	60
C. metuliferus	20	30	30	23	10	10	25	280	0	5	20	15	10	10	50	75

OK – MS, 20 mg/l ascorbic acid, 0.01 mg/l IBA, 0.01 mg/l BA, 20 g/l sucrose, 8 g/l agar; ON – MS, 1 g/l casein hydrolysate, 0.01 mg/l IBA, 0.01 mg/l BA, 20 g/l sucrose, 6 g/l agar; CW – MS, 5% coconut water, 200 mg/l glutamine, 0.01 mg/l IBA, 0.01 mg/l BA, 60 g/l sucrose, 6 g/l agar; GA – MS, 0.3 mg/l GA3, 0.01 mg/l IBA, 0.01 mg/l BA, 20 g/l sucrose, 8 g/l agar; media used for embryogenesis (Skálová et al. 2008a)

physiological and genetic differences of the fusing partners determine the ability of the hybrid cells to survive. Protoplastization must also be followed by somatic plant cell dedifferentiation and obtaining the ability to proliferate and develop into shoots or somatic embryos. Plant regeneration from protoplasts of *Cucumis sativus* was reported for the first time by Orczyk and Malepszy (1985) but a number of experiments showed that plant regeneration after isolation or fusion is very difficult (Gajdová et al. 2004, 2007a). Herewith is an overview of our results concerning embryo-rescue, *in vitro* pollination, protoplasts isolation and fusion, and the study of protoplast dedifferentiation.

EMBRYO-RESCUE AND *IN VITRO* POLLINATION

Embryo-rescue

Zygotic embryogenesis *in vitro* in *Cucumis* species was studied over the last two decades (Lebeda et al. 1996, 1999; Ondřej, Navrátilová 2000; Ondřej et al. 2000, 2002b; Skálová et al. 2004, 2008a) and used for *in vivo* intraspecific and interspecific hybridization. Cucumber (*C. sativus*), muskmelon (*C. melo*) and selected wild *Cucumis* species (*C. metuliferus*, *C. zeyheri* and *C. anguria*) were used for experimentation.

Successful embryo-rescue is influenced mainly by accession type and age, and the composition of culture media. Ten embryos of chosen *Cucumis* genotypes were cultivated repeatedly in Petri dishes with various media (the minimum of repetition was three times per one genotype and per one type of medium). The results of embryo-rescue experiments are summarized in the Table 1 and Fig. 1a. The youngest (3-day-old) embryos, that are able to cultivate only *in ovulo*, showed the worst results during culture, as expressed by lower frequency and level of regeneration (Ondřej, Navrátilová 2000; Ondřej et al. 2002b; Skálová et al. 2008a). Nevertheless, immature embryos are the most important for interspecific hybridization, because of the early abortion of hybrid embryos at the globular stage (Ondřej et al. 2001). Considering the youngest embryos, from the set of studied wild *Cucumis* spp. accessions, the highest frequency of regeneration showed *C. metuliferus*. It seems that this accession was also a good partner for interspecific hybridization with cucumber.

During experiments of cross pollination, positive results were observed using melon (*C. melo*, MR-1). The outcomes concerning interspecific hybridization between *C. sativus* and other *Cucumis* spp. are summarized in the Table 2. Plant formation was detected after intraspecific hybridization within the genus *Cucumis*. However, only the callus formation was observed after interspecific hybridization between cucumber and several other *Cucumis* species (Skálová et al. 2008a). Nevertheless one case of interspecific hybridization was successful; hybrid plants were obtained after cross pollination of *C. anguria* with *C. zeyheri.* The hybrids were confirmed by isozyme and morphological analyses (Skálová et al. 2008c).

As regards the composition of cultivation media, the undefined mixture of organic components, mainly coconut water, and growth regulators, especially gibberellic acid, had the most positive effect on embryogenesis of isolated embryos, non-hybrids and hybrids (Ondřej, Navrátilová 2000; Skálová et al. 2008a).

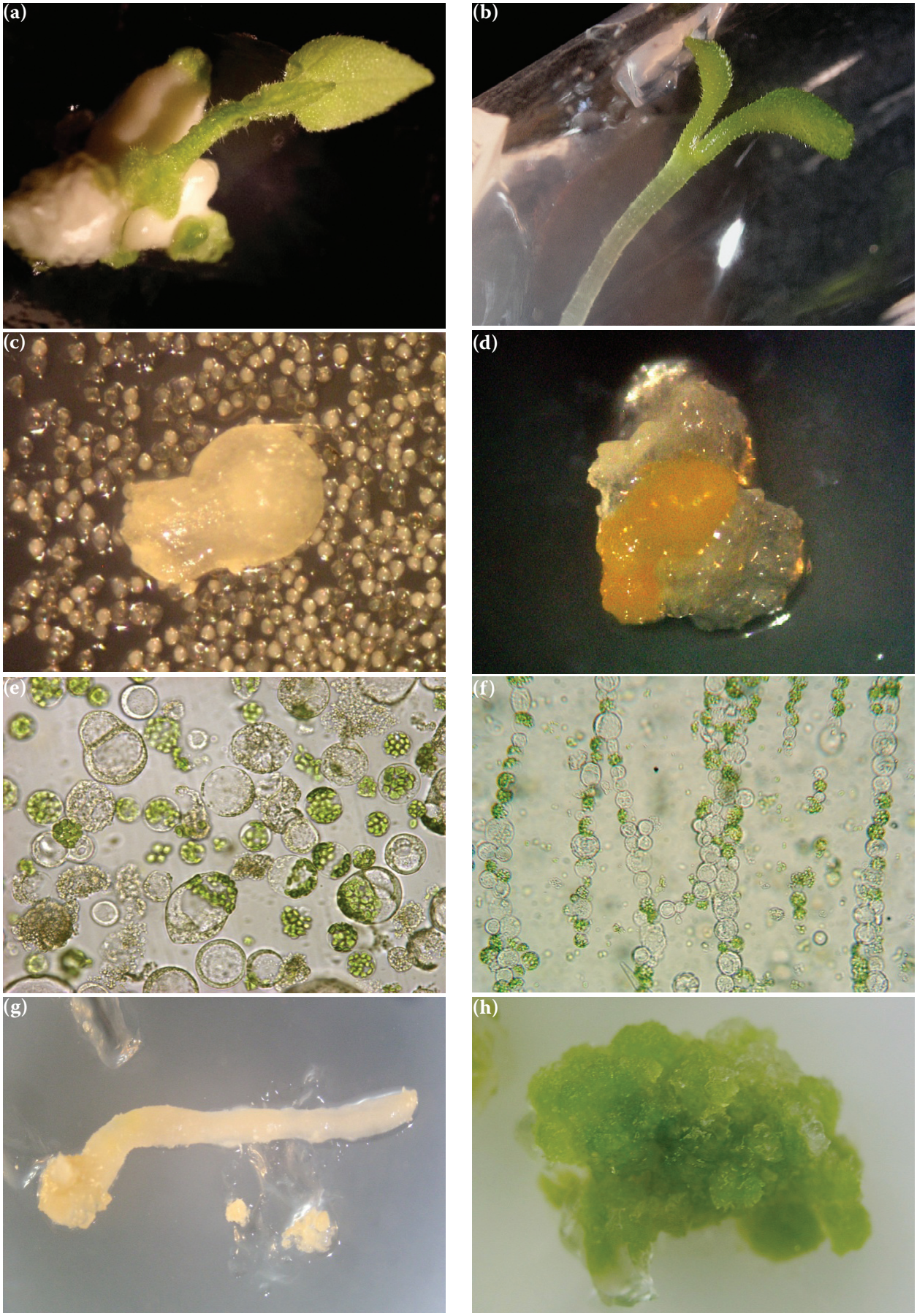
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

Fig. 1. (a) plant formation from *C. anguria* embryos on CW medium; (b) plant formation of embryo (*C. anguria* × *C. zeyheri*) on ON medium; (c) isolated *C. sativus* ovule with *C. melo* pollen grains on YS medium; (d) callus formation of *C. sativus* ovule after *in vitro* pollination with *C. melo* pollen grains on YS medium; (e) chemical fusion of *C. sativus* and *C. melo*; (f) protoplast alignment by electrofusion of *C. sativus* and *C. melo*; (g) regenerated callus after chemical fusion *C. sativus* + *C. melo*; (h) root regeneration after electrofusion *C. sativus* + *C. melo*

Embryo-rescue method was successful for cultivation of immature and mature embryos obtained by intraspecific hybridization, vital plants were recovered. The embryo-rescue after interspecific hybridization was not so successful; nevertheless, the calluses were obtained from immature embryos (Skálová et al. 2008a).

In vitro pollination

In vivo pollination followed by *in vitro* embryo-rescue can be replaced by *in vitro* pollination and fertilization with subsequent *in vitro* embryogenesis. Mature cucumber ovules were isolated and cultivated together with cucumber and melon pollen grains on special media.

With respect to this type of pollination, no plant formation was observed during our experiments. The highest level of regeneration of isolated ovules (after culture with pollen grains) was callus formation, however, proembryos and globular embryos were found in fertilized ovules (Ondřej et al. 2002a; Skálová et al. 2008b). The organic mixture, which included caseinhydrolysate in the culture medium, had a positive effect on *in vitro* fertiliza-

Table 2. Results of interspecific hybridization of *C. sativus* with *Cucumis* species

Interspecific partner	No. of pollinations	No. of obtained fruits	No. of isolated seeds	No. of isolated embryos	No. of regeneration
C. melo (MR-1)	27	13	260	260	8
C. melo (var. Charentais)	18	8	120	160	0
C. anguria	11	2	80	0	0
C. zeyheri	16	7	280	0	0
C. metuliferus	8	6	240	0	4

Table 3. Number of isolated ovules (♀) with pollen grains (♂), number of successful *in vitro* pollinations and progressed ovules in CP and YS-media

Medium		No. of isolated ovules (♀) – cultivated with pollen grains (♂)		No. of successful fertilization		No. of progressed ovules (ovules becoming green; calluses formation)	
		CP	YS	CP	YS	CP	YS
♀	♂	isolated by centrifugation					
CS	CS	660	660	150	190	95	57
CS	CM	160	160	100	100	41	17
Total No.		960	960	310	400	184	108
isolated directly							
CS	CS	90	90	50	50	22	43
CS	CM	90	90	60	60	8	18
Total No.		180	180	110	110	30	61

CP – MS, 9.5 mg/l glycine, 500 mg/l casein hydrolysáte, 4 mg/l IAA, 0.5 mg/l KIN, 4 mg/l GA3; 40 g/l sucrose; 10 g/l agar; YS – 600 mg/l Ca $(NO_3)_2$, 100 mg H_3BO_3, 80 g/l sucrose, 10 g/l agar; CM – *Cucumis melo*; CS – *Cucumis sativus*; media used for *in vitro* pollination (Skálová et al. 2008b)

tion and embryogenesis (Skálová et al. 2008b). The final numbers of used ovules and the results obtained in this area are summarized in Tables 1–3 and in Fig. 1a–d.

PROTOPLAST ISOLATION, CULTURE AND FUSION

Protoplast isolation and cultivation

The efficiency of protoplast isolation and culture depends on many factors: genotype, type of explants, density of protoplasts, viability, method of cultivation and composition of the medium (Navrátilová et al. 2000; Rokytová et al. 2001; Gajdová et al. 2004, 2007a, b).

In our experiments, the protoplasts were isolated from leaves and leaf-derived calluses of four *Cucumis* species, *C. melo*, *C. metuliferus*, *C. sativus* and *C. zeyheri*. The evaluating criteria for successful experiments were the density (yield of protoplasts per 1 g of fresh mass) and the viability of protoplasts immediately after isolation. Differences among genotypes and explant types (mesophyll, callus) are presented in Table 4. Viability was determined to be approximately 80% and the density of mesophyll protoplasts was 10 times higher than callus protoplasts, the callus protoplasts of *C. sativus* having an unusually low density.

The most optimal results for isolation of cucumber mesophyll protoplasts were obtained from 4-week-old *in vitro* plants. Older ones started to flower, and they were not suitable for experimentation. Thus, cucumber plants were germinated for every experiment. Genotypes of *C. metuliferus*, *C. melo* and *C. zeyheri* had optimal results 2 to 3 weeks after subculture, their plantlets not flowering (Gajdová et al. 2007b; Navrátilová et al. 2008).

Calluses that appeared to be soft, fine, and white, optimally 6–10 weeks old and 10 days after subculture, were suitable for protoplast isolation and culture. Leaf-derived calluses of *C. metuliferus* and *C. sativus* were compact, soon necrotized and were not as suitable for long culture and protoplast isolation as calluses of *C. melo* or *C. zeyheri* (Gajdová et al. 2007b).

Cucumber mesophyll protoplasts cultures were used to study the effect of UV-C irradiation on protoplast physiology. This method can be utilized in asymmetric hybridization (Greplová et al. 2006; Navrátilová et al. 2008).

Protoplast fusion

In *Cucumis* species, somatic hybridization by protoplast fusion was described between *C. sativus* and *C. melo* (electrofusion: Jarl et al. 1995), *C. metuliferus* and *C. melo* (electrofusion: Debeaujon, Branchard 1990; chemical fusion: Roig et al. 1986), *C. melo* and *C. anguria* (electrofusion: Dabauza et al. 1998), and *C. melo* and *C. myriocarpus* (electrofusion: Bordas et al. 1998).

Mesophyll and callus protoplasts in two types of fusion were tested and compared: chemical fusion

Table 4. Comparison of viability and density of isolated *Cucumis* spp. protoplasts

Genotype	Code+	Mesophyll		Callus	
		viability (%)	density* (10^6 prot./ml)	viability (%)	density* (10^6 prot./ml)
C. sativus	CZ09H390768	87.63	7.63	undetected	0.01
	CZ09H390056	79.89	3.66		
	CZ09H390121	86.09	4.36		
C. melo	CZ09H401114	81.85	3.29	84.17	0.32
	CZ09H401116	84.42	3.82	83.92	0.44
C. metuliferus	CZ09H410586	87.06	15.03	84.17	0.32
	CZ09H410587	85.45	15.33	87.11	0.43
C. zeyheri	CZ09H410196	86.89	11.85	84.54	0.37

Experiments were repeated minimally three times; + www.vurv.cz, part Databases, Evigez; *Density (the yield of protoplast) was compared onto 1 g of fresh weight of leaves or calluses

Table 5. The level of regeneration after chemical fusions of protoplasts among *Cucumis* spp.

Mesophyll protoplast	Callus protoplast			
	C. melo	*C. metuliferus*	*C. sativus*	*C. zeyherii*
C. melo	not tested	cell division	cell division	cell division
C. metuliferus	callus	not tested	no regeneration	microcallus
C. sativus	callus	no regeneration	not tested	callus
C. zeyheri	callus	microcallus	microcallus	not tested

Each fusion combination was repeated minimally three times

by polyethylene glycol (PEG 6000, Fluka, Sigma-Aldrich Co. LLC., St. Louis, USA) and electrofusion (using apparatus ECM 2001, BTX, Inc., San Diego, USA). Protoplasts were viable and undamaged after isolation and fusion experiments. The hybrid products after fusion contained rich vacuolar systems and many chloroplasts, that is, characteristics of both fusion partners (Navrátilová et al. 2006a, b).

Polyethylene glycol (PEG) is a fusogen introduced in 1974 by Kao and Michayluk to increase the frequency of the fused protoplasts of lucerne (*Medicago sativa*). Fusing partners are mixed (mesophyll

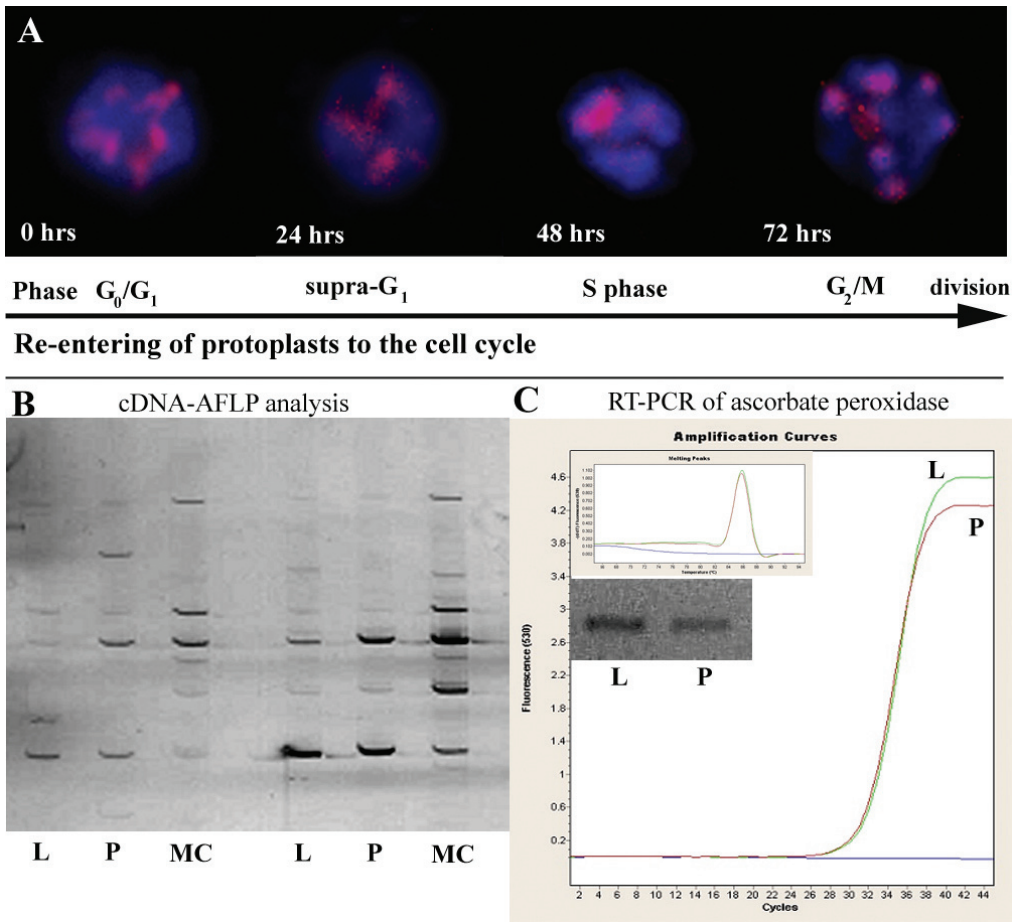

Fig. 2. Reassembly of the decondensed satellite type I repeats within cell nuclei to the chromocenters during protoplast cultivation (up to 72 h after protoplasts isolation)

(A) The recondensation process of chromatin is connected with re-entry of protoplasts to the cell cycle and cell division; (B) cDNA-AFLP patterns of the transcription in leaves (L), freshly isolated protoplasts (P), protoplasts derived microcallusses (MC); (C) amplification curves of the real-time PCR analysis of a selected gene (ascorbate peroxidase) in leaves (L), freshly isolated protoplasts (P). The amplification was checked by melting analysis and electrophoresis (attached to the subset C). Results indicate no significant changes in the expression of the selected gene

and callus, mesophyll and mesophyll) and treated with PEG of different molecular mass (1,500–6,000) at a concentration range of 15–45% for 15–30 min, to the final concentration of 10^6 protoplasts per ml (Navrátilová 2004).

In our experiments, chemical fusion was performed using 33% PEG for 15 min (Christey et al. 1991; Navrátilová et al. 2006a; Fig. 1e). Electrofusion was realized in electroporation chamber for 400–500 µl of volume with the 3, 2 mm electrode distance and following parameters: protoplast alignment 30 V AC and 1 pulse 90 V DC of length 80 µs (Navrátilová et al. 2006a). The level of regeneration was categorised: regeneration of cell wall, cell division, microcallus and callus formation, and plant regeneration (Table 5). We detected the regeneration of cell walls after 24 h by Calcofluor White. In a different version of protoplast cultivation and fusion, we recorded also the first divisions (3–5 days in culture), microcalluses (0.2–2 mm after 2–3 weeks in culture) and calluses (more than 2 mm). In the protoplast fusion experiments across the *Cucumis* species, the regeneration stopped at the stage of calluses. Only on one occasion the root was regenerated after electrofusion of *C. sativus* + *C. melo* (Fig. 1f, g).

The obtained calluses grew for several months until two years of *in vitro* culture. They grew well, were compact to crumbly, and white to green (Fig. 1h). Many experiments showed that plant regeneration after isolation or fusion is very difficult (Gajdová et al. 2004, 2007a, b), however, recently we reported somatic proembryo formation derived from cucumber (*C. sativus*) protoplasts (Ondřej et al. 2009a).

CYTOGENETIC ASPECTS OF PROTOPLAST CULTURES

Protoplast cultures are acceptable as a model for developmental and cytogenetic studies (Ondřej et al. 2009a, b) and also studies of abiotic and biotic stresses, including heavy metals on plant cells (Luhová et al. 2006, 2008a, b, c). It was found that during protoplastization, the heterochromatin blocks, known as chromocenters, decondensed (Fig. 2a) (Tessadori et al. 2007; Ondřej et al. 2009a, b). The chromatin decondensation affected repetitive sequences like satellite DNA type I (Ondřej et al. 2009b) and 5S rDNA repeats. The reassembly of chromocenters and repetitive sequences is required to re-enter protoplast into the cell cycle and start proliferation, as was described using FISH and flow cytometry analyses (Ondřej et al. 2009b). Although the level of heterochromatin reassembly correlates with the level of cell differentiation (Ondřej et al. 2009a), the large-scale chromatin decondensation did not dramatically influence gene transcription (Ondřej et al. 2009b), (Fig. 2b). However, the expression of genes involved in oxidative stress is in some freshly isolated protoplast cultures similar to donor tissues (Fig. 2c) or undergoes to dramatic down-regulation (Ondřej et al. 2010).

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