Procedure of asymmetric somatic hybridization (in *Solanum* genus)

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1 Introduction

Symmetric and asymmetric somatic hybridization can circumvent naturally occurring sexual incompatibility barriers and generate genetically novel germplasm for breeders (Davey et al. 2005). Symmetric protoplast fusion results in cells containing a mixture of the organelles of both fusion partners. In interspecific symmetric somatic hybrids however, there is a problem of the abundance of undesirable wild species characteristics. This can be overcome by a partial transfer of genes using asymmetric protoplast fusion techniques (Xia et al. 2003, Liu et al. 2005). In some asymmetric fusions both donors and recipients are subjected to treatment to limit the input of nuclear genome into the hybrid (the term donor refers to the partner which suffers chromosomal fragmentation or loss due to irradiation treatment, Xia et al. 2003). However in most cases, treatment is only given to the donor (Liu et al. 2005). A new nucleo-cytoplasmic combination - cytoplasmic hybrid (cybrid) is developed by the elimination of the nucleus of one partner (Yarrow 1999, Guo et al. 2004). Irreversible breakage or fragmentation of chromosomes to minimize introducing genomes from the fusion parents into the hybrids is mainly achieved using irradiation with X or γ rays (Zubko et al. 2002). UV is also often used for its easy access and for creating chromosomal breakages (Jain et al. 1988, Vlahova et al. 1997).

Another way of asymmetric somatic hybridization is double treatment of protoplasts. The protoplasts of cytoplasmic donor (wild species) are treated with UV irradiation. The protoplasts of nuclear donor (cultivated species) are treated with metabolic inhibitors (i.e. iodoacetic acid, iodoacetamide and rhodamine, Matibiri and Mantell 1994, Yarrow 1999, Polzerová and Greplová 2008). The ionising irradiation destroys chromatin, but in contrast, this treatment has apparently no effect on cytoplasm (Oberwalder et al. 1997, 2000, Orczyk et al. 2003) while treatment with metabolic inhibitors destroys only cytoplasmic enzymes (Orczyk et al. 2003). Such treatment is an effective selection system (Matibiri and Mantell 1994) because only heterofused protoplasts can grow, due to metabolic complementation (Liu et al. 2005). It is noteworthy that the elimination of a part of donor genome results in reduction of backcrosses (Fosberg et al. 1998).

2 Methodical procedure

The asymmetric protoplast fusion by electric field requires sufficient amount of suitable plant material for isolation and treatment of protoplast. The procedure involves five discreet stages.

- Cultivation of donor plants
- Protoplast isolation
- Verification of protoplast viability and protoplast treatment
- Protoplast electrofusion
- Cultivation of fusion products

2.1 Culture of donor plants

In vitro plantlets are grown in a culture chamber with a 16 h/8 h light/dark photoperiod (light conditions: fluorescent tubes, daylight, 60 μmol m^{-2} s^{-1}), at 22 °C. Plantlets are cultivated on hormone-free MS medium (Murashige and Skoog 1962).

Plants for protoplast isolations (from 10 to 20 plantlets from each parental genotype) are propagated on SH hormone free medium (Schenk and Hildebrand 1972) supplemented with $AgNO_3$ and Alar 85 (succinic acid dimethylhydrazide) for 4 - 6 weeks. Plants are cultured at 10 °C in darkness for 24 hours one day before the protoplast isolation; these stress conditions enable integration of cell cycle and this is positively reflected in subsequent protoplast regeneration.

2.2 Protoplast isolation

The whole process of protoplast isolation is performed under sterile conditions.
Detach fully expanded leaves (from 10 plantlets, 0.5 – 1 g). Cut them into small pieces with a sharp scalpel in one Petri dish (Ø = 5 cm) containing 5 – 6 ml of enzymatic solution (Bříza and Machová 1991), pack the Petri dish with Parafilm.
Incubate Petri dishes overnight in dark (14 – 16 h) in a thermostat at 25 °C. After this period, investigate the degree of plant tissue digestion under microscope, if it is necessary (depending on genotype) place Petri dishes onto a shaker for about 15 minutes.
Transfer enzymatic solution with the plant tissue by a disposable serological pipette with open end onto a small sieve (75 µm pore size) placed on Petri dish (Ø = 5 cm). Rinse the sieve with a sterile solution of 0.5 M sucrose while gentle stirring. By this step released protoplasts are separated from plant tissue debris. Divide protoplast suspension derived in this way into centrifugation tubes (8 ml) and overlay with 1 ml of W5 solution (Menczel et al. 1981).
Centrifuge (700 rpm, 12 min).
Carefully take away floating protoplasts forming a ring on the interface of applied solutions by a Pasteur pipette and put into a new centrifugation tube, add required amount of W5 (5 ml) and resuspend.
Centrifuge (500 rpm, 6 min)
Remove the supernatant with a Pasteur pipette, add W5 (2 – 5 ml) and resuspend the pellet.
Centrifuge (500 rpm, 6 min) and remove the supernatant with a Pasteur pipette.
Take a sample (5 µl) for viability verification (see 2.3.1). Give ¼ of protoplasts in tube (control sample). Give the rest of protoplasts in second tube. These protoplasts will be treated either with UV irradiation (wild parent) or with metabolic inhibitor (cultural parent).
Add medium SW_{11} (Bříza and Machová 1991) to obtain required density 6×10^5/ml (see 2.3.2) to control sample.

2.3 Verification of protoplast viability and protoplast treatment

2.3.1 Evaluation of protoplast viability prior to fusion

Mix equal volumes (20 µl) of diluted FDA solution and protoplast suspension on a slide and form microscope preparation. After 4-5 minutes, examine under a fluorescence microscope. Determine protoplast viability (count protoplasts in selected visual field without UV light and then green fluorescing protoplasts in UV light (green fluorescing protoplasts are metabolically active): % protoplast viability = no. of green fluorescing protoplasts/total no. of protoplasts × 100
When viability is higher than 50 %, investigated suspension is usable for fusion.

2.3.2 Determination of protoplast density using a Bürker chamber

Adjustment of protoplast concentration prior to fusion (prior to culture) is done under sterile conditions.
Add adequate amount (2 – 4 ml) of pre-fusion solution (Schilde-Rentschler and Ninnemann 1988) to isolated protoplasts with a Pasteur pipette, resuspend (protoplasts can stay in this

solution up to 2 h in a refrigerator at 6 – 7 °C) and count protoplast using a Bürker chamber (haemocytometer).

Count protoplasts in five triple-lined squares (i.e. a square of 1 mm side; in the left and above all cells touching the middle line are counted, in the right and below these ones are not counted). Determine an average number of protoplasts from five squares - **"X".** Cell concentration in 1 ml is: **"X" × 10^4** (1 square represents volume of 0.1 mm^3, i.e. 10^{-4} cm^3 [1 cm^3 = 1 ml]).
Total yield = $\mathbf{V_{suspension}}$ **× "X" × 10^4**
Dilution factor $\mathbf{F_d}$ **= required density / "X" × 10^4**
Suspension volume at required density = $\mathbf{V_{suspension}}$ **/$\mathbf{F_d}$**
If needed, concentration is adjusted for fusions (see 2.4.1) or culture of protoplasts.

2.3.3 UV irradiation of protoplasts
An application of UV irradiation is performed by a trained person dressed in protective clothing (a long sleeve coat, resistant nitrile gloves, laboratory trousers and laboratory shoes).
During handling it is necessary to keep all safety measures according to valid rules.
The whole process of UV irradiation is carried out under sterile conditions.
Add adequate amount (2 – 4 ml) of pre-fusion solution (Schilde-Rentschler and Ninnemann 1988) to isolated protoplasts with a Pasteur pipette, resuspend (protoplasts can stay in this solution up to 2 h in a refrigerator at 6 – 7 °C) and irradiate with UV-C (wave length 240 nm) with dosage 370 µW cm^{-2} for 10 minute. Irradiation conditions are given according to:

$E = I \times \cos\alpha / r^2$ [W / m^2]

I is irradiance: $I = P / 4\pi$ [W / st]
P is power of source [W]; r is vertical distance from the source to the irradiated surface [m] and α is angle from vertical line
Fig. Experiment arrangement

Count protoplast density (see 2.3.2). Give ¼ of protoplasts in tube (control sample). Add medium SW_{11} (Bříza and Machová 1991) to obtain required density 6 × 10^5/ml. Give the rest of protoplasts in second tube and continue according 2.4.1.

2.3.4 Application of metabolic inhibitors
An application of metabolic inhibitors is performed by a trained person dressed in protective clothing (a whole face filter mask, a long sleeve coat, resistant nitrile gloves, laboratory trousers and laboratory shoes).
During handling it is necessary to keep all safety measures according to valid rules.
The whole process of mitotic polyploidization is carried out under sterile conditions.
Add 5 ml of metabolic inhibitor solution to1 ml of protoplast suspension. Gently resuspend with Pasteur pipette.
After 30 minute, centrifuge (500 rpm, 6 min)
Remove the supernatant with a Pasteur pipette, add W5 (8 – 9 ml) and resuspend the pellet.
Centrifuge (500 rpm, 6 min) and remove the supernatant with a Pasteur pipette. Repeat the washing in W5.
Centrifuge (500 rpm, 6 min) and remove the supernatant. Give ¼ of protoplasts in tube (control sample). Add medium SW_{11} (Bříza and Machová 1991) to obtain required density 6 × 10^5/ml. Give the rest of protoplasts in second tube and continue according 2.3.2.

2.4 Protoplast electrofusion

2.4.1 Adjustment of protoplast concentration prior to fusion

The whole procedure of protoplast fusion is performed under sterile conditions.
Centrifuge protoplast suspension in pre-fusion solution (500 rpm, 3 min) and remove the supernatant with a Pasteur pipette.
Add a fusion solution (Schilde-Rentschler and Ninneman 1988) with pipette to obtain desired protoplast concentration for fusion 6×10^5/ml and resuspend protoplasts.
Mix protoplast suspensions of two fusion partners (UV irradiated and metabolic inhibited) in the ratio 1:1.

2.4.2 Protoplast fusion

Protoplast fusion is done using of electroporator (ECM 2001, BTX, Inc., San Diego, CA) and facilities (microscope, holder of fusion chambers, fusion chambers).
Put microscope into a flow-box, sterilize chambers with 70% alcohol prior to fusion and left to dry out. After drying, transfer 20 – 30 µl protoplast suspension with a Pasteur pipette into an electroporation chamber placing into the holder. Observe the whole process of protoplast fusion under invert microscope.
Process of protoplast fusion: protoplasts are aligned by acting of alternate current (AC field), then a pulse of direct current (DC pulse) is applied, which results in fusion.

Basic parameters of electric field

AC	5 V/chamber with 0.5 mm electrode distance	2 – 18s
DC pulse	10 V/chamber with 0.5 mm electrode distance	80 µs
AC	30 V/chamber with 3.2 mm electrode distance	2 – 18s
DC pulse	60 V/chamber with 3.2 mm electrode distance	80 µs

After fusion, transfer protoplasts from five chambers with a Pasteur pipette into one Petri dish (Ø = 3.5 cm) and add 1 ml of culture medium (liquid SW_{11}, Bříza and Machová 1991) to protoplast suspension. Pack Petri dishes with parafilm, transfer into the thermostat and culture in dark at 25 °C. Further culture is done according to the culture protocol for protoplast fusion products.

2. 5 Cultivation of protoplast fusion products

The whole process of fusion product culture is performed under sterile conditions.
The fist cell division usually occurs 2 – 3 days after fusion (you can observe it under microscope). After cell wall formation, add liquid SW_{11} medium with decreasing level of osmoticum in the interval of 7 – 10 days till the stage of microcalli (i.e. gradually SW_{11} 0.5 M mannitol, SW_{11} 0.4 M mannitol, SW_{11} 0.3M mannitol, Bříza and Machová 1991). After cell wall development, regenerating cells could be cultured in the light (16 h/8 h photoperiod; light conditions: fluorescent tubes, daylight, 150 µmol m^{-2} s^{-1}; 22 °C).
Microcalli are formed 3 – 4 days after fusion. When the calli are visible, remove liquid SW_{11} medium by pipette and add the liquid Shepard C medium (Shepard and Totten 1977). Repeat replacement of this medium twice in 7 – 10 days.
Subsequently, sterilely transfer calli to clean Petri dishes. Cultivate a part of calli in liquid Shepard D medium (Shepard and Totten 1977). Change the medium in 7 – 10 days till shoot regeneration. Cultivate the second part of calli in agar-solidified Shepard D medium. Transfer the calli onto fresh medium in 14 days till shoot regeneration.
First shoots are formed 4 – 12 weeks after culture initiation in Shepard D medium. Cut off regenerated shoots and left them rooting in MS medium (Murashige and Skoog 1962).

Cultivate the shoots under the same conditions as donor plants for protoplast isolation. Multiply the plantlets into pairs for subsequent testing of hybridity.

2.6 Used solutions and media – their preparation

2.6.1

● Murashige & Skoog medium

MS (Duchefa Biochemie)	4.4 g
sucrose	30 g
agar	8 g

Dissolve MS and sucrose in 450 ml of distilled water. Adjust pH to 5.6 – 5.8 and add distilled water to obtain volume of 500 ml. Dissolve agar in 400 ml of distilled water and microwave or boil in a water bath, make up to final volume (500 ml). Mix both solutions and dispense in culture vessels (to 1 – 2 cm height) and cover with lids. Autoclave them at 121 °C for 20 min.

● Schenk & Hildebrand medium

SH medium (Duchefa Biochemie)	3.2 g
Vitamins SH (Duchefa Biochemie)	1.01 g
sucrose	15 g
$AgNO_3$	0.003 g
Alar	0.0015 g
agar	8 g

Dissolve all substances except agar in 450 ml of distilled water. Adjust pH to 5.6 – 5.8 and add distilled water to obtain volume of 500 ml. Dissolve agar in 400 ml of distilled water and microwave or boil in a water bath, make up to final volume (500 ml). Mix both solutions and dispense in culture vessels (to 1 – 2 cm height) and cover with lids. Autoklave them at 114 °C for 20 min.

2.6.2

● Enzymatic solution

Stock solution:

$CaCl_2.2\ H_2O$	0.735 g
MES	1.952 g
sucrose	171.15 g
NAA	0.005 g (stock solution: 100 mg/100 ml, sterilization by filtration)
ZT	0.002 g (stock solution: 50 mg/100 ml, sterilization by filtration)

Dissolve ingredients without growth regulators in 480 ml of distilled water. Adjust pH to 5.6 – 5.8. Autoclave stock solution at 121 °C for 20 min.

After sterilization, add growth regulators and make up to volume 500 ml by sterile distilled water. Dispense in 50 ml aliquots for usage. Solution could be frozen to -20 °C.

(ZT – zeatin; NAA – alpha-naphthaleneacetic acid; MES – 2-(N-morpholino)ethanesulfonic acid)

Preparation of enzymes:

Celulase Onozuka R-10 (Duchefa Biochemie)	1 g (and/or 1 U)
Macerozyme R-10 (Duchefa Biochemie)	0.2 g (and/or 0.2 U)

Dissolve ingredients in distilled water. Adjust pH to 5.6 – 5.8 and add distilled water to obtain volume of 50 ml and filter sterilize. Adjust weight amount according to Units written on the label of individual package of enzyme.

Working enzymatic solution:

Mix 50 ml of enzymes with 50 ml of stock solution (the unexpended rest could be frozen to 20 °C up to 1 month).

● 0.5 M sucrose

sucrose	171.15 g

dissolve in distilled water to final volume of 1000 ml, autoclave at 121 °C for 20 min and cold-keep at 6 – 7 °C.

● W5 solution

NaCl	9.0 g
KCl	0.8 g
$CaCl_2.2\ H_2O$	18.4 g
glucose	1.0 g
glycine	1.0 g

dissolve in 990 ml of distilled water, adjust pH to 5.8, add distilled water to obtain volume of 1000 ml and autoclave at 121 °C for 20 min, cold-keep at 6 – 7 °C.

The concentrated W5 solution: dissolve ingredients in 400 ml of distilled water, adjust pH to 5.8, add distilled water to obtain volume of 500 ml and autoclave at 121 °C for 20 min, cold-keep at 6 – 7 °C.

2.6.3

● FDA solution (fluoresceindiacetate)

FDA	0.005 g

Prepare stock solution by dissolving of weighted amount in 1 ml of acetone. Working solution: add 20 µl of stock solution to 1 ml of culture solution, sucrose or W5.

● Solutions of metabolic inhibitors

An application of metabolic inhibitors is performed by a trained person dressed in protective clothing (a whole face filter mask, a long sleeve coat, resistant nitrile gloves, laboratory trousers and laboratory shoes).
During handling it is necessary to keep all safety measures according to valid rules.

Iodoacetic acid	0.0186 g

Stock solution: dissolve iodoacetic acid in 10 ml of distilled water and sterilize by filtration (0.22 µm).
Working solution: mix 200 µl of stock solution with 5 ml concentrated W5 solution and make up to 10 ml with sterile distilled water.

Iodoacetamide	0.0185 g

Stock solution: dissolve Iodoacetamide in 10 ml of distilled water and sterilize by filtration (0.22 µm).
Working solution: mix 400 µl of stock solution with 5 ml concentrated W5 solution and make up to 10 ml with sterile distilled water.

2.6.4

● Pre-fusion solution

Mannitol	7.4 g
$CaCl_2.2H2O$	0.011 g

dissolve in 90 ml of distilled water, adjust pH to 5.6 and add distilled water to obtain volume of 100 ml. Autoclave at 121 °C for 20 min.

● Fusion solution

Mannitol	7.4 g

dissolve in 90 ml of distilled water, adjust pH to 5.6 and add distilled water to obtain volume of 100 ml. Autoclave at 121 °C for 20 min.

2.6.5

● SW_{11} medium

SW_{11}	**SW_{11}**	**SW_{11} 0.4M**	**SW_{11} 0.3M**	**SW_{11} 0.2M**
KNO_3	950 mg			
$CaCl_2.2\ H_2O$	367.5 mg			
$MgSO_4.\ 2\ H_2O$	185 mg			
KH_2PO_4	85 mg			
NH_4Cl	133 mg			
myo-Inositol	100 mg			
Casein hydrolysate	500 mg			
L-glutamin	100 mg			
Yeast extract	100 mg			
Sucrose (0,01M)	3423 mg			
Mannitol	89.2682 **g**	72.87 **g**	54.65 **g**	36.44 **g**
Thiamine. HCl	10 mg	prepare stock solutions (100 ml) with concentration of 1 mg/1 ml		
Pyridoxine. HCl	1 mg			
Nicotinic acid	1 mg			
H_3BO_3	3 mg	(and adequate amount, e.g. Thiamine. HCl		
$MnSO_4.\ 4H_2O$	14 mg	pipette 10 ml into the medium)		
$ZnSO_4.\ 7H_2O$	2 mg			
$CuSO_4.5H_2O$	0.025 mg			
KI	0.75 mg			
$Na_2MoO_4.\ 2H_2O$	0.25 mg			
$CoCl_2.\ 6H_2O$	0.025 mg			
$FeSO_4.\ 7H_2O$	13.9 mg			
$Na_2EDTA.2H_2O$ Chelaton III	37.3 mg			
NAA	2 mg*			
2,4 D	0.2 mg			
Zeatin	0.5 mg*			

dissolve in 950 ml of distilled water, adjust pH to 5.6 – 5.8, autoclave at 121 °C for 20 min. Sterilely add Zeatin and NAA and sterilized distilled water to obtain volume of 1000 ml, cold-keep at 6 – 7 °C.

*Filter sterilise Zeatin – stock solution 50 mg/100 ml, NAA – stock solution 100 mg/100 ml.

● Shepard C and Shepard D medium

	$C_{0.3M}$	$C_{0.2M}$	$D_{0.2M}$	SG2
$NH_4 NO_3$			1650 mg	
KNO_3	1900 mg		1900 mg	
$CaCl_2.2 H_2O$	440 mg		440 mg	
$MgSO_4. 7 H_2O$	370 mg		370 mg	
KH_2PO_4	170 mg		170 mg	
Na_2EDTA	37.3 mg		37.3 mg	
$FeSO_4. 7H_2O$	27.8 mg		27.8 mg	
H_3BO_3	6.2 mg		6.2 mg	prepare stock solutions (100 ml)
$MnCl_2. 4H_2O$	19.8 mg		19.8 mg	with concentration of 1 mg/1 ml
$ZnSO_4. 7H_2O$	9.2 mg		9.2 mg	
KI	0.83 mg		0.83 mg	(and adequate amount, e.g. H_3BO_3
$Na_2MoO_4. 2H_2O$	0.25 mg		0.25 mg	pippete 6.2 ml into the medium)
$CuSO_4. 5H_2O$	0.025 mg		0.025 mg	
$CoSO_4.7H_2O$	0.03 mg		0.03 mg	
myo-Inositol	100 mg		100 mg	
Thiamine. HCl	0.5 mg		0.5 mg	
Glycine	2 mg		2 mg	
Nicotinic acid	5 mg		5 mg	
Pyridoxine. HCl	0.5 mg		0.5 mg	
Folic acid	0.5 mg		0.5 mg	
Biotin	0.05 mg		0.05 mg	
Casein hydrolysate	1000 mg		1000 mg	
Adenin sulfate	40 mg		40 mg	
NAA	0.05 mg*			
IAA			0.1 mg*	0 mg
BAP (6-benzylaminopurin)	0.5 mg*			2.25 mg*
Zeatin			0.5 mg*	4 mg*
Mannitol	54.654 **g**	36.44 **g**	36.44 **g**	
Sucrose 15 mM	5.1345 **g**		5.1345 **g**	
MES	0.976 **g**		0.976 **g**	
Agar (solidified medium)			7 **g**	

dissolve in 950 ml of distilled water, adjust pH to 5.8, autoclave at 121 °C for 20 min. Sterilely add NAA, IAA, zeatin, BAP and/or gibberellic acid (see above) and distilled water to obtain volume of 1000 ml. Cold-kept medium. Prepare Shepard D medium as liquid one or with agar. Dispense the agar-solidified medium in Petri dishes in a flow-box.
*Filter sterilise Zeatin – stock solution 50 mg/100 ml, NAA – stock solution 100 mg/100 ml, BAP – stock solution 50 mg/100 ml.

2.7 Sterility verification of used solutions

Verify each solution prior to use by a bacterial test: 1 ml of tested solution + 1 ml of provocation medium. Culture it in dark at 25 °C. Examine tested solutions in 3 – 4 days (presence of infection is indicated by milk discoloration of the solution).
Provocation medium: Standard I nutrient broth for microbiology (Merck) – 25 g/l

2.8 Regenerant evaluation

Hybridity of regenerants should be verified. For hybridity evaluation it is suitable to use flow-cytometry as pre-selection method for ploidy estimation (http://olomouc.ueb.cas.cz/book/dna-flow-cytometry). Asymmetric somatic hybrids should be verified by any of DNA analysis

methods e.g. RAPD (Greplová et al. 2007) and by analysis of mitochondrial and plastid DNA (Bastia et al. 2001, Harding and Millam 2000, Lössl et al. 2000).

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