Regeneration of intergeneric somatic hybrids by protoplast fusion between *Onobrychis viciaefolia* and *Medicago sativa* *

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Abstract Protoplast fusion was induced between sainfoin and alfalfa by an improved polyethyleneglycol (PEG) method. The intergeneric somatic calluses were selected based on complementation of hydroxyproline-resistance of sainfoin and hormone autonomy growth of alfalfa transformation cell line. 17 somatic hybrid plantlets were regenerated. PEG could induce the tight agglutination of protoplasts. During diluting and washing process, cyclization of the linked membrane and formation of vesicle-like structures were observed, resulting in protoplast fusion. 5%—10% glycerol supplemented in the fusion inducing solution markedly increased the frequency of heterogeneous fusion. Better fusion results were obtained when mixed protoplast suspension was dripped in petri dishes in which PEG solution was previously placed. Chromosome number of regenerated hybrid buds varied from 30 to 60. The genome of hybrids included the small chromosome from sainfoin and two chromosomes with two clear constrictions from alfalfa. The hybridity of obtained hybrid calluses was confirmed by their isozyme banding patterns and their nopaline synthetase activity.

Keywords: hydroxyproline-resistant cell line of sainfoin (Hyp^r), *Agrobacterium tumefaciens* 702 transformed cell line of alfalfa (M_7), PEG, glycerol, vesicle, protoplast fusion, somatic hybrid, plantlet.

Sainfoin is a perennial "bloat-safe" pasture plant because of its high tannin content. However, the ability of nitrogen fixation of sainfoin is rather low, and insufficiency of nitrogen element that it obtained in symbiotic nitrogen fixation process limits elaboration of its high-production potentialities. In contrast to this, alfalfa exhibits higher ability of nitrogen fixation. Plant somatic hybridization could be adopted to combine characteristics such as "bloat-safe" tannin synthesis of sainfoin and high nitrogen fixation ability of alfalfa.

Limited success has been achieved in intergeneric somatic hybridization of legumes, besides somatic hybrids of *Lotus corniculatus* and *Glycine max*[1]. The present paper describes protoplast fusion between Hyp^r and M_7 by PEG[2] method, with hydroxyproline resistance and hormone autonomy being used to recover somatic tissues from which plantlets were subsequently regenerated.

1 Materials and methods

1.1 Plant materials

A hydroxyproline-resistant cell line of sainfoin (*Onobrychis viciaefolia* Scop, Hyp^r, $2n=4x$

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$=28$) selected in our laboratory was used as one parent. This variant was resistant to 10 mmol/L *trans*-4-hydroxy-L-proline (Hyp), or 20% PEG-4000, or 1% NaCl, and was regenerable[3]. *Agrobacterium tumefaciens* 702 transformed alfalfa (*Medicago sativa*) cell line (M_7, $2n=4x=32$) obtained in our laboratory was used as another parent, which was unable to regenerate and grew vigorously on medium without phytohormone.

1.2 Protoplast isolation

2 g granular yellow-greenish calluses of M_7 taken from 12 day-old subcultures were placed in 10 mL enzyme solution 1 (table 1), while 1.5 g Hyp^r calluses taken from 9—10-day-old subcultures in 10 mL enzyme solution 2 (table 1). After being incubated for 6 h at 25℃ on a shaker at 50 r/min, the enzyme-protoplast mixture was filtered through a 60-μm nylon sieve. Protoplasts were pelleted by centrifugation (80 g, 5 min) and resuspended in 2 mL CPW9[4] solution. The protoplasts were purified by sedimentation over 6—8 mL 18% sucrose at 500 r/min for 10 min.

Table 1 Enzyme composition used for protoplast isolation

Enzyme composition	Solution 1	Solution 2
Onozuka R-10	2%	1%
Macerozyme R-10	0.5%	0
Pectinase (Serva)	0	0.5%
Hemicellulase (Serva)	0.5%	0.2%
2(N-morpholino) ethanesulfolic acid (MES)	0.1%	0
$CaCl_2 \cdot 2H_2O$/mol·L^{-1}	0.015	0.015
Mannitol/mol·L^{-1}	0.4	0.45
pH	5.6	5.8

1.3 Selection of hybrid cells

The selection system was established upon complementation of hydroxyproline-resistance of sainfoin and hormone autonomy growth of alfalfa transformation cell line. Somatic cells were selected in hormone-free SH medium[5] (supplemented with Hyp, 0.45 mol/L glucose, 2% sucrose and pH was 5.8—6.2); protoplasts of each parent and the products of homogeneous fusion could not survive in this medium.

1.4 Protoplast fusion

Purified Hyp^r and M_7 protoplasts were mixed in equal numbers to give a final density of 2.0×10^6 protoplast/mL. After centrifugation at 50 r/min, the pellets were rinsed with one of the following fusion pretreatment solutions twice and placed statically for 5 min. The precipitated protoplasts were resuspended in a little amount of the same solution. The pretreatment solutions were: (ⅰ) CPW9; (ⅱ) W_5[6]; (ⅲ) 0.16 mol/L $CaCl_2 \cdot 2H_2O$, pH 5.6; (ⅳ) 0.4 mol/L mannitol, 0.05 mol/L $CaCl_2 \cdot 2H_2O$.

The pretreated protoplasts were fused in the following different ways:

(a) Centrifuging method: 0.5 mL fusion-inducing solution was dripped into 1 mL mixed protoplast suspension; 4-fold volume of diluting and washing solution was added twice after 5-min staying. The protoplasts after fusion treatment were collected by centrifugation at 500 r/min.

(b) Pre-dripping protoplast method: The mixed protoplasts were dripped regularly onto petri dishes (6 cm in diameter), and placed motionlessly for 5 min to precipitate the protoplasts on the dish bottom. Fusion-inducing solution was added carefully to side of each protoplast drop, and made the drops connect each other. These dishes were placed at 37℃ for 5—30 min in the dark, and 2 mL diluting and washing solution was added twice at 5 min interval. After being placed for 2.5—3 h at room temperature, the precipitated protoplasts on dish bottom were washed with SH medium for 3 times, and then cultured in 2 mL SH medium.

(c) Post-dripping protoplast method: Fusion inducing solution was dripped beforehand on the petri dish bottom. The mixed protoplast suspension was then dripped carefully to the fusion inducing solution drops. Incubating, diluting and washing operation was the same as described in (b).

The fusion-inducing solution was made up by the following solutions: (ⅰ) PEG solution: 25%—45% (*W*/*V*) PEG (MW = 6 000) supplemented with 0.1 mol/L $CaCl_2 \cdot 2H_2O$ and 5 mmol/L KH_2PO_4, pH 5.7; (ⅱ) high Ca^{2+}-high pH solution: 0.05 mol/L $CaCl_2 \cdot 2H_2O$, 0.4 mol/L mannitol dissolved in 0.05 mol/L glycine-NaOH buffer (pH = 10.5); (ⅲ) glycerol; (ⅳ) DMSO (dimethylsulfoxide). The constitution of fusion inducing solution was ⅰ : ⅱ : ⅲ (or ⅳ) = (8.5—9): 0.5: (1—0.5) (*V*/*V*/*V*). Solution (ⅰ) could be used solely.

After fusion treatment, the mixtures were diluted and washed with W_5 solution supplemented with 50 mmol/L MES.

1.5 Identification of hybrid cells

Hyp^r protoplasts were labelled with fluorescein isothiocyanate (dissolved in liquid medium), and M_7 protoplasts with Rhodamine B isothiocyanate (dissolved in liquid medium). The nuclei of Hyp^r protoplasts gave out yellow-greenish fluorescence, and the nuclei of M_7 protoplasts gave out red fluorescence. The hybrid cells, i.e. heterokaryons, would show both of yellow-green and red fluorescences under a fluorescent inverted microscope.

$$\text{Fusion frequency between heterogeneous protoplasts (\%)} = \frac{\text{Protoplasts giving out two kinds of fluorescences}}{\text{Total protoplasts observed}} \times 100\%$$

1.6 Culture of fusion products and selection of hybrid cells

Protoplasts after fusion treatment were cultured in SH liquid medium at (25 ± 2)℃ in the dark or dim light. After two weeks of culture, 0.5 mL dilution medium with half concentration of glucose was added. After 20 d, the liquid cultures were picked out and centrifuged. The pellets were washed with dilution medium of one-fourth glucose concentration twice, and then resuspended in a little amount of the same medium. The suspension was spread onto 0.6% agar solidified hormone-free MS medium supplemented with 10 mmol/L Hyp. The growing calluses were individually proliferated on the same medium. Differentiation was carried out on MS medium without phytohormone or MS medium supplemented with 0.5—2 mg/L 6-benzylaminopurine (6-BA) and 0.2—2 mg/L indole-3-acetic acid (IAA).

Suitable controls were also set up, consisting of protoplasts of each parent, the individual

protoplast suspensions subjected to the fusion treatment and untreated mixtures of two parental protoplasts.

1.7 Identification of somatic hybrids

Regenerated hybrid buds were cut and pretreated for 4 h in saturated P-dichlorobenzene solution, and stained with haematoxylin. After being squashed, chromosomes were observed and counted under a light microscope. Isozyme patterns of peroxidase and esterase were compared between calluses of two parents and somatic hybrids[7]. The improved procedure of Xu *et al*.[8] was used for determining the nopaline synthetase activity.

2 Results and discussion

2.1 Factors influencing protoplast fusion

The pretreatment of mixed protoplasts was efficient. Among the four pretreatment solutions tested, W_5 was the most effective. Repeated experiments revealed that centrifuging method was not suitable to protoplast fusion of sainfoin and alfalfa. After fusion treatment and centrifugation, most protoplasts were injured and could not recover. When using pre-dripping protoplast method, protoplasts usually precipitated and adhered to the dish bottom after being statically placed for a few minutes, and it was difficult for them to recover in diluting and washing process even if they could fuse. A great deal of experiments revealed that post-dripping protoplast method (identical to pre-dripping PEG method) was much more effective. When passing thick PEG solution, large amounts of protoplasts would agglutinate together, but they would not adhere to the dish bottom.

The frequency of protoplast fusion was markedly affected by PEG concentration. If PEG level preceded over 35%, most protoplasts would break into pieces and floated on the surface of the solution. Although the protoplasts agglutinated tightly were able to recover in diluting and washing process, they scarcely survived in later culture process because of heavy injury of plasma membrane. When PEG concentration was below 25%, protoplasts agglutinated slowly, and the fusion frequency was very low. As shown in table 2, 30% PEG concentration was suitable for protoplast fusion between sainfoin and alfalfa. The frequency of heterogeneous fusion could reach 2.6%.

Table 2 Influence of PEG (MW = 6 000) concentration on fusion frequency

PEG (%)	Total number of protoplasts observed	Number of heterokaryons	Fusion frequency of heterogeneous protoplasts	Surviving situation
45	451	10	2.2	browned and died after 3 d
40	405	14	3.5	browned and died after 3 d
35	390	11	2.8	a few protoplasts survived after one week
30	421	11	2.6	survived
25	473	5	1.1	survived
20	425	0	-	-

Simultaneously with the induction of protoplast agglutination, PEG could cause cyclization of plasma membrane, resulting in vesicle formation and cell fusion. Two situations were observed in the process: (ⅰ) the cyclization happened in many sites of the linked membrane; (ⅱ) the cyclization happened at the centre of the linked membrane.

Table 3 shows the effects of several combined fusion inducing solutions. It was indicated that 5%—10% glycerol and DMSO apparently increased the frequency of heterogeneous fusion, and glycerol was much more effective. According to the previous reports, a certain amount of DMSO and glycerol could increase surviving rate of frozen protoplasts, and protect their plasma membrane against damage of freeze[9]. DMSO was used in research of protoplast fusion and could increase fusion frequency effectively[10]. However, glycerol was not used in fusion research up to now.

Table 3 Influence of stabilizing agent on protoplast fusion between sainfoin and alfalfa

Composition of fusion inducing solution (1 mL)				Total number of protoplasts observed	Heterokaryon number	Frequency of heterogeneous fusion (%)	Surviving situation
3% PEG	high Ca^{2+} – high pH solution	glycerol	DMSO				
1	5%	0	0	421	11	2.6	survived
0.9	0.05	0.05	0	432	28	6.4	survived
0.85	0.05	0.1	0	417	34	8.2	survived
0.9	0.05	0	0.05	379	22	5.7	survived
0.85	0.05	0	0.1	405	25	6.1	survived

2.2 Culture of fused cells

Protoplast mixtures after fusion treatment consisted of heterokaryons of 1:1 or over 1:2 of heterogeneous protoplasts, homokaryons and unfused protoplasts. The shape of fused cells was irregular originally and changed into globularity in subsequent washing process. After one week of culture, some large cells began to divide. In contrast to this, the mixed protoplasts of two parents (with a small volume) and homogeneous protoplasts subjected to the fusion treatment browned and died after one week in the selection medium.

Cell clusters formed after 10 d of culture. About 20 d later, cell colonies consisting of 10—20 cells formed , and grew slowly. In order to further promote growth of these cell colonies, all cultures were picked out and centrifuged at 50 r/min. The pellets were washed twice with hormone-free SH medium of one-fourth glucose concentration and resuspended in a little amount of the same medium. The suspension was spread onto solid MS medium containing identical supplementary substances. 7 pieces of calluses were selected after 40 d of culture. One of them (named R_1, fig. 1(a)) differentiated buds spontaneously on hormone-free MS medium plus 10 mmol/L Hyp, and 17 plantlets were regenerated (fig. 1(b)). Besides, two pieces of the calluses (named R_2, R_3) formed buds on MS medium containing 6-BA and IAA. These buds could develop into shoots, but could not form main stalk. Other four pieces of calluses had no regeneration ability.

The above-mentioned three regenerable cell lines were regarded as putative somatic hybrids; their hybridity was identified by biochemical and cytological method.

2.3 Identification of somatic hybrids

2.3.1 Chromosome observation and count. Cytological examination revealed that 86% cells of Hyp^r calluses had chromosome number of $2n = 4x = 28$; other cells were aneuploid and their chromosome numbers were less than 40. The regenerated plants of Hyp^r also had normal chromo-

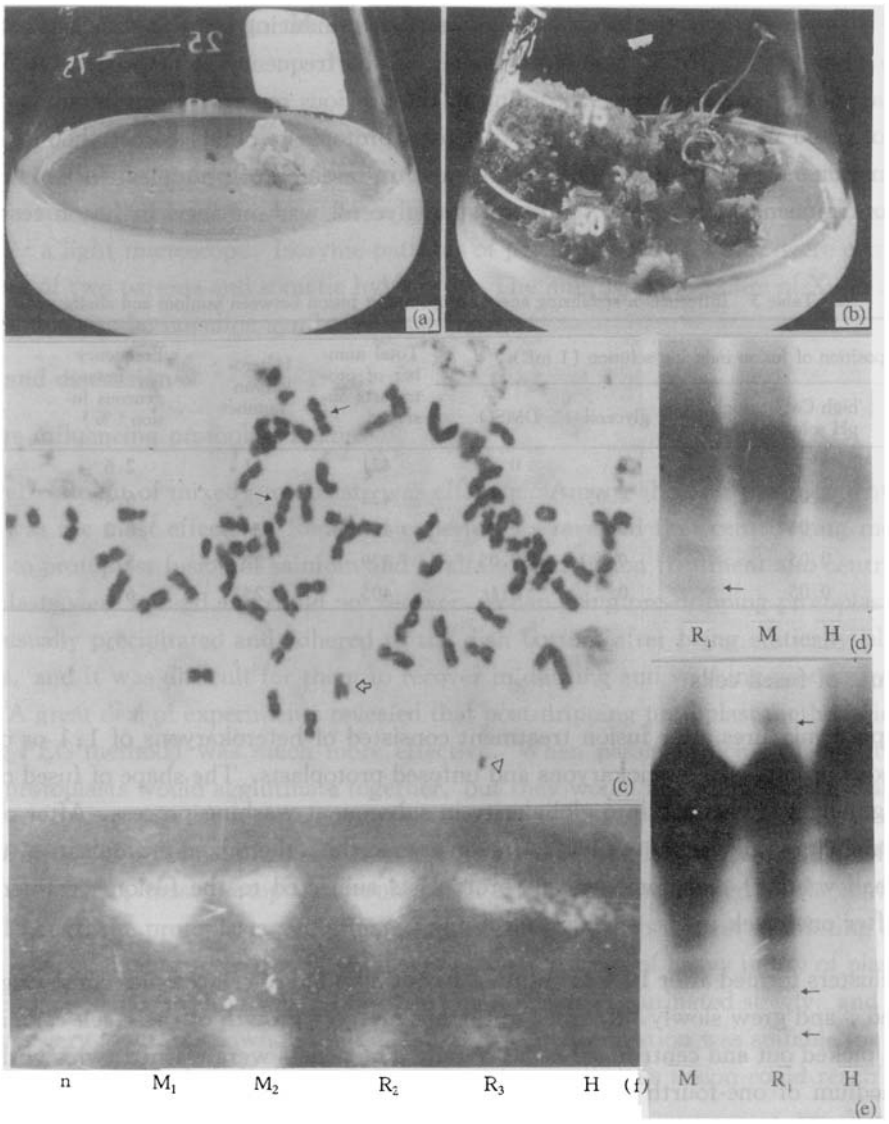

Fig. 1 (a) R_1 hybrid callus; (b) regenerated plantlet from R_1 calluses; (c) chromosomes in hybrid bud ($2n = 60$, arrows indicate the two chromosomes with two constrictions of alfalfa); (d) isozyme banding pattern of peroxidase (M, M_7 calluses; R_1, R_1 hybrid calluses; H, Hyp^r calluses); (e) isozyme banding pattern of esterase (M, M_7 calluses; R_1, R_1 hybrid calluses; H, Hyp^r calluses); (f) nopaline analysis (n, nopaline; M, M_7 calluses; R_2, R_3, R_2 and R_3 hybrid calluses; H, Hyp^r calluses).

some number. Chromosome numbers of M_7 cells were below 32, and most of them were $2n = 4x = 32$. Two chromosomes of alfalfa showed two clear constrictions. Regenerated buds of the three putative hybrids revealed 30—60 chromosomes. Certain hybrid buds had 60 chromosomes corresponding to the sum of chromosome numbers of two fusion partners (fig. 1(c)), simultaneously with the small chromosome (using triangular arrow as a sign) of sainfoin and two alfalfa chromosomes with two clear constrictions. One monosome of a chromosome (signed by empty arrow) did not duplicate completely (fig. 1(c)). This irregular chromosome would be lost in later division process. In R_2 and R_3, regenerated buds merely developed into shoots, but could not produce

main stalk, which might be related with variation of chromosomes[11,12].

2.3.2 Isozyme analysis. Peroxidase and esterase were compared between somatic hybrid calluses and two parental calluses. Somatic hybrids showed characteristic bands of the parents, together with additional bands (fig. 1(d), (e), using arrow as signs). R_1 and M_7 grew on MS medium without phytohormone; Hyp^r grew on MS medium supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2, 4-D) and 0.5 mg/L 6-BA.

2.3.3 Nopaline analysis. Results of paper electrophoresis (figs. 1(f) and 2) revealed that nopaline synthetase gene of M_7 had been integrated into R_1, R_2 and R_3.

3 Conclusion

Fig. 2. Nopaline analysis of R_1 calluses. a, arginine; n, nopaline; o, octopine; H, Hyp^r calluses; M, M_7 calluses; R_1, hybrid calluses.

Combination of sainfoin and alfalfa was intergeneric and distant, along with the elimination of chromosomes; aneuploidy was the inexorable trend. Nevertheless, certain hybrid buds had 60 chromosomes corresponding to the sum of chromosome numbers of two fusion partners, and the characteristic chromosomes, such as small chromosome of sainfoin and two alfalfa chromosomes with two clear constrictions coexisted. Isozyme analysis of peroxidase and esterase revealed that the hybrid not only possessed the characteristic bands of two parents, but also formed new bands. Analysis of nopaline synthetase showed that the transformation characteristic of alfalfa was transferred into hybrids. In addition, the hybrids had regeneration ability identical to sainfoin. All these results suggested that protoplast fusion could be carried out between resistant cell line and transformation cell line, and selection medium could be designed using mutation characteristics of two distantly related species. Somatic hybrid of sainfoin and alfalfa was a new reconstruction. If the time of culture and selection was shortened further, it was possible to increase the regeneration ability and acquired progeny had practical potentiality in forage legume breeding.

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