

## BRIEF COMMUNICATION

## Plant regeneration from mesophyll protoplasts of *Agrobacterium rhizogenes*-transformed *Astragalus melilotoides*

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### Abstract

Plant regeneration from mesophyll protoplasts of *Agrobacterium rhizogenes*-transformed *Astragalus melilotoides* Pall. was here developed. The protoplasts were isolated directly from the leaves of the hairy root-induced plants. The highest yield of protoplasts was obtained from fully expanded leaves of young plants. Their viability was up to  $72 \pm 2.3$  %. The highest division frequency ( $32.4 \pm 0.13$  %) and sustained divisions were obtained in Durand, Potrykus and Donn (DPD) medium supplemented with  $2.0 \text{ mg dm}^{-3}$  2,4-dichlorophenoxyacetic acid,  $0.2 \text{ mg dm}^{-3}$  6-benzylaminopurine, 0.3 M mannitol, 2 % sucrose and  $500 \text{ mg dm}^{-3}$  casein hydrolysate at the plating density of  $3.0 \times 10^5 \text{ cm}^{-3}$ . The frequency of shoot differentiation from protocalli reached to  $91.75 \pm 3.1$  %. Opine synthesis and polymerase chain reaction analysis confirmed that T-DNA still existed in the protoplast regenerated plants.

*Additional key words:* growth regulators, opine, protoplast culture, T-DNA, transformation.

*Astragalus melilotoides* Pall. is a herbaceous perennial legume forage widely grown in the arid and semiarid area in northwest of China. The long growth period and rather late maturity often cause low yield and poor quality of leaves and seeds (Hou and Jia 2004). However, this species exhibits drought and cold resistances, water-holding and soil or sand stabilizing capacities (Guo *et al.* 1987). These useful traits of wild germplasm might be incorporated into cultivated forage species by means of genetic manipulation, such as gene transformation (Vinterhalter *et al.* 2006, Sretenović-Rajičić *et al.* 2006) and somatic hybridization (Brewer *et al.* 1999). A study on tissue culture of internode-segments of this plant has been reported (Chen and Jia 2001). Recently, a progress has been made in protoplast culture of this species (Hou and Jia 2004). Here we have produced transgenic plants of *A. melilotoides* Pall. through *Agrobacterium rhizogenes* A<sub>4</sub> mediated method. Because these transgenic plants possess selectable genetic marks for somatic hybrid selection, they could be used as a proper partner for protoplast fusion. Since the regeneration from protoplasts is a prerequisite

for somatic hybridization, an efficient system for plant regeneration from protoplasts of transgenic plants have been developed. To our knowledge, only few successful protoplast-to-plant regeneration systems have been established in the genus *Astragalus* until now (Luo and Jia 1998, Hou and Jia 2004).

*A. rhizogenes* A<sub>4</sub> harboring the wild type pRiA4 was cultured in  $10 \text{ cm}^{-3}$  YEB medium (Hooykaas *et al.* 1977) in  $50 \text{ cm}^{-3}$  flasks on a shaker with 200 rpm at 28 °C. Sterilized seeds of *A. melilotoides* were germinated on MS medium (Murashige and Skoog 1962) without any growth regulator (MS<sub>0</sub> medium). Co-cultivation of hypocotyl segments of these plantlets was performed with *A. rhizogenes* A<sub>4</sub> for 2 d. Then these segments were rinsed 5 times in sterile distilled water and cultured on MS medium added with  $500 \text{ mg dm}^{-3}$  cefotaxime. Cefotaxime concentration was gradually reduced to  $250 \text{ mg dm}^{-3}$ ,  $100 \text{ mg dm}^{-3}$ ,  $0 \text{ mg dm}^{-3}$  with each subculturing. Plant regeneration from transgenic hairy roots was induced on MS<sub>0</sub> medium at temperature of  $25 \pm 2$  °C and irradiance of  $100 \mu\text{mol m}^{-2}\text{s}^{-1}$  under 16-h photoperiod.

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*Abbreviations:* BAP - 6-benzylaminopurine; CH - casein hydrolysate; 2,4-D - 2,4-dichlorophenoxyacetic acid; DPD - Durand, Potrykus and Donn (1973) medium; Kn - kinetin; MES - 2[N-morpholino]ethane-sulfonic acid; NAA -  $\alpha$ -naphthaleneacetic acid; PCR - polymerase chain reaction; MS - Murashige and Skoog (1962) medium.

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The regenerated transgenic plants were further grown under irradiance of  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ , 12-h photoperiod, and temperature of  $25 \pm 2 \text{ }^\circ\text{C}$ . Then the fully expanded young leaves were sliced to approximately 2 mm size and pre-treated for 60 min in  $10 \text{ cm}^3$  hypertonic solution consisting of 0.3 M mannitol and 0.05 M  $\text{CaCl}_2$ . Further, the incubation was carried out for 4, 10, 16 h in the filter-sterilized enzyme solution containing 2 % (m/v) cellulase (*Onozuka R-10*), 0.5 % pectolyase (both from *Yakult*, Nishinomiya, Japan), 0.4 M mannitol, 0.05 M  $\text{CaCl}_2$  and 0.1 % MES (pH 5.8 - 6.0) on a shaker with 60 rpm at  $25 \text{ }^\circ\text{C}$ . After digestion, the enzyme-protoplast mixture was filtered through a  $74 \mu\text{m}$  stainless steel mesh. The released protoplasts were collected by centrifugation at 100 g for 10 min, and washed immediately with washing solution, *i.e.*, 0.16 M  $\text{CaCl}_2$  and 0.1 % MES (pH 5.8). Purification of the protoplasts was performed by centrifugation on an 18 % sucrose gradient. Viable and purified protoplasts, which formed a band at the interface between sucrose and  $\text{CaCl}_2$  solution following centrifugation at 100 g for 10 min, were removed to a new centrifugal tube and washed once with washing solution and once with DPD medium (Durand *et al.* 1973). Protoplast viability was assessed by phenosafranine

staining method (Widholm 1972).

The purified protoplasts were resuspended in DPD medium supplemented with different growth regulators (Table 1) at various densities ( $0.25, 0.5, 1.0$  or  $3 \times 10^5 \text{ cm}^{-3}$ ).  $2 \text{ cm}^3$  of protoplast suspension was put into each glass Petri dish, sealed with parafilm, and then incubated at  $25 \pm 2 \text{ }^\circ\text{C}$  in the dark. The culture dishes were gently shaken twice everyday. After 10 d, the cultures were diluted by adding  $0.5 \text{ cm}^3$  fresh DPD medium with 0.15 M mannitol. When the protoplast-derived calli formed, they were transformed onto agar-solidified DPD medium omitting mannitol, but with 2 % sucrose,  $2 \text{ mg dm}^{-3}$  2,4-D and  $0.2 \text{ mg dm}^{-3}$  BAP and placed at  $25 \pm 2 \text{ }^\circ\text{C}$ , 10-h photoperiod at irradiance of  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by daylight fluorescent tubes. The division percentage of protoplasts in liquid thin layer cultures was calculated on the 15<sup>th</sup> day in culture.

For induction of hairy roots, the protocalli were inoculated on a half-strength MS medium without growth regulator. For inducing shoots, they were transferred on the MS medium supplemented with different growth regulators (Table 2). When shoots grew up to height 2 - 3 cm, they were transplanted on the half-strength MS medium without or with  $0.2 \text{ mg dm}^{-3}$  NAA for rooting.

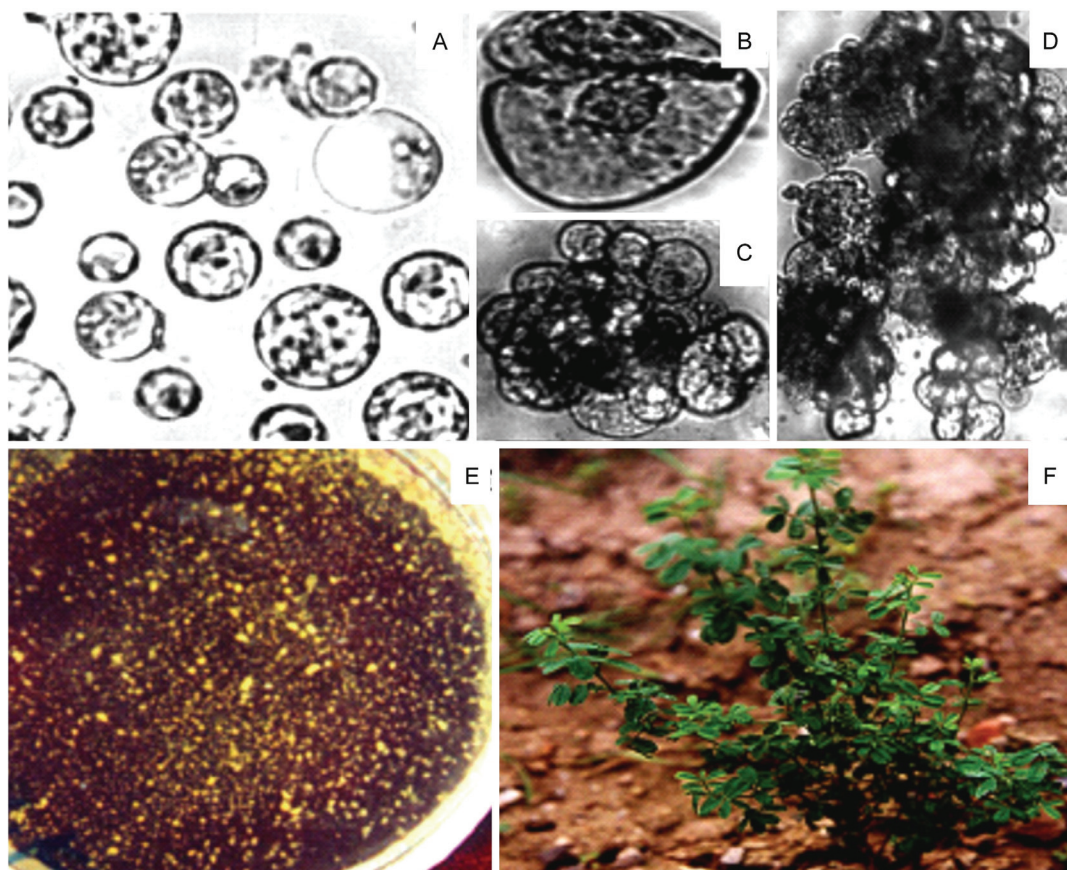


Fig. 1. *A* - Freshly isolated mesophyll protoplasts ( $\times 600$ ); *B* - first division of a protoplast ( $\times 790$ ); *C* - a cluster derived from a protoplast ( $\times 45$ ); *D* - a colony from a protoplast-derived cell ( $\times 40$ ); *E* - microcalli derived from protoplasts; *F* - a regenerated transgenic plant from protocalli.

For opine detection, 0.1 - 0.2 g fresh mass of the hairy roots or the leaves from protoplast-regenerated plants were taken and placed in an Eppendorf tube, and ground. After centrifugation at 5 300 g for 15 min, 0.01 cm<sup>3</sup> of supernatant together with standard mannopine (*Sigma*, St. Louis, USA) were spotted on *Waterman* chromatography paper and dried. Electrophoresis was performed at 45 V cm<sup>-1</sup> for 30 min in formic acid : acetic acid : water (30:60:910, v/v/v) at pH 2.1. The dried paper was stained with silver nitrate as described by Petit *et al.* (1983), dipped in Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> and washed for hours with running water.

Genomic DNA of regenerated plant was extracted from 50 mg of fresh tissue according to the method of Edwards *et al.* (1991). Integration of the T-DNA in plant genome was confirmed by PCR with two oligonucleotide primers: 5'-ATGGATCCCAAATTGCTATTCCTTCCA CGA-3' and 5'-TTAGGCTTCTTCTTCAGGTTT ACTGCAGC-3' according to the procedure of Hamill *et al.* (1991). Amplification products were analyzed by electrophoresis on 0.8 % agarose gel and detected by staining with ethidium bromide.

After 14 d in culture on MS medium containing cefotaxime, numerous hairy roots appeared on the cut sites in most of hypocotyl segments infected by *A. rhizogenes*. However, all the uninfected control explants became brown and subsequently died. The elongating hairy roots were well-proliferated on the MS<sub>0</sub> medium as cefotaxime concentration was gradually reduced until no colony of bacteria appeared. The cultures showed a typical hairy root phenotype, *e.g.*, rapid growth, reduced apical dominance and root plagiotropism. They spontaneously regenerated shoots on MS<sub>0</sub> medium in 15 d under irradiance of 100 μmol m<sup>-2</sup> s<sup>-1</sup>, 16-h photoperiod, and 25 ± 2 °C. When these shoots were transferred on root induction medium, the root formation occurred in about 5 d. The transformed plants also revealed some phenotypic alterations, such as dwarf, wrinkled leaves and numerous adventitious roots.

The protoplasts (Fig. 1A) started to divide after 3 d in culture (Fig. 1B) followed by sustained divisions (Fig. 1C). Proto-colonies, visible to the naked eyes (Fig. 1D,E), were formed in 30-d protoplast culture. The division frequency of protoplasts was 32.4 ± 0.13 % after 15 d in culture. The protoplast viability and yield were influenced by many factors. The most important factor was the age of leaves used for protoplast preparation. The best viability (72.3 ± 2.3 %) and highest yield of protoplasts were obtained from the fully expanded young leaves which were pretreated in hypertonic solution for 60 min. The protoplast division frequency was also dependent on protoplast density and optimum density was 3 × 10<sup>5</sup> cm<sup>-3</sup>. About 30 % of the isolated protoplasts underwent sustained divisions at this density. Moreover, both protoplast viability and division frequency were affected by the enzyme digestion duration. For our transformed plants, the appropriate duration of enzyme digestion was 4 h. As digestion lasted longer, the viability and the division frequency of protoplasts declined.

Table 1. Effect of different growth regulators [mg dm<sup>-3</sup>] on the division frequency of protoplasts incubated in DPD medium with 2 % sucrose and 0.3 M mannitol (pH 5.8 - 6.2). Means ± SE, *n* = 3.

2,4-D	BAP	Kn	NAA	Division frequency [%]
2.0	0.2	0.0	0.0	30.8 ± 0.09
2.0	0.5	0.0	0.0	32.4 ± 0.13
2.0	0.2	0.0	0.2	14.7 ± 0.06
2.0	0.0	0.2	0.0	26.9 ± 0.33
1.5	0.2	0.0	0.0	25.4 ± 0.09
1.5	0.0	0.2	0.0	18.4 ± 0.22
1.5	0.0	0.2	0.2	23.6 ± 0.12

Table 2. Effect of different growth regulators combination [mg dm<sup>-3</sup>] on differentiation of protoplast-derived calli on MS basal medium with 3 % sucrose + 0.7 % agar. Means ± SE, *n* = 8.

BAP	Kn	NAA	Number of inoculated calli	Number of calli producing shoots	Shoot regeneration [%]
1.0	0.0	0.2	92	78	84.78 ± 2.7
1.5	0.0	0.2	97	89	91.75 ± 3.1
3.0	0.0	0.2	94	37	39.36 ± 3.3
0.0	1.0	0.2	94	55	58.51 ± 1.5
0.0	1.5	0.2	102	67	65.69 ± 2.9
0.0	3.0	0.2	81	43	53.09 ± 1.9
1.0	0.0	0.5	83	60	72.28 ± 2.2
1.5	0.0	0.5	81	69	85.18 ± 1.8

Protoplast division and subsequent colony formation were also affected by growth regulators in liquid medium. The combinations of 2,4-D (2.0 mg dm<sup>-3</sup>) and BAP (0.2 - 0.5 mg dm<sup>-3</sup>) were the most suitable, and the highest division frequency was achieved (Table 1).

Gradual reduction of mannitol concentration in the medium promoted proliferation of protoplast-derived colonies. The protocalli were produced in 2 months after protoplast inoculation. The combination of BAP and NAA in the medium was more effective than that of Kn and NAA for shoot differentiation (Table 2). When 1.5 mg dm<sup>-3</sup> BAP in combination with 0.2 mg dm<sup>-3</sup> NAA was added in the medium, the rate of shoot formation reached up to 91.75 ± 3.1 %. All of regenerated shoots produced roots on the half-strength MS<sub>0</sub> medium (Fig. 1F).

The protoplast regenerated plants had transgenic phenotype similar to original plants used for protoplast isolation. The difference between protoplast regenerated plants and non-transformed plants may reflect the modification of plant morphological characters by integration of *rol* genes of T-DNA.

Agropine and mannopine are well known to be detectable opiines in hairy roots transformed with Ri plasmid (Petit *et al.* 1983). In this work, the presence of

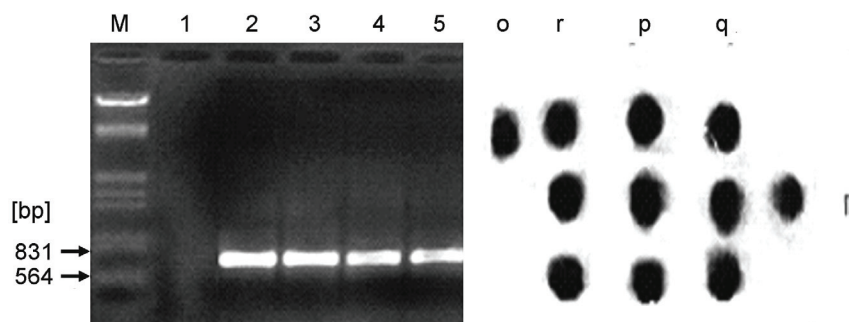


Fig. 2. PCR detection of the *roIB* gene (left) and paper electrophoretic pattern of extracts from hairy root cultures and regenerated plants (right). M -  $\lambda$ -DNA (*Hind*III + *Eco*RI digest), 1 - DNA from untransformed plants, 2 - plasmid DNA of pRiA4, 3 - DNA from primary hairy roots, 4 - DNA from roots of protoplast regenerated plants, 5 - DNA from leaves of protoplast regenerated plants, o - untransformed plants, r - hairy roots from protoplasts; p and q - leaves of protoplast regenerated plants; last column - standard mannopine (m).

agropine and mannopine in the roots and leaves of the regenerated plants was confirmed by paper electrophoresis followed by alkaline silver nitrate staining. Agropine band appeared below the band of mannopine on the electrophoresis pattern (Fig. 2). No bands of agropine and mannopine appeared in the untransformed plants. It indicated that the regenerated plants from mesophyll protoplasts still had the opine synthesis enzyme activity.

When PCR amplification was completed, the expected 780 bp fragment of *roIB* gene was observed on agarose TBE gel in the genomic DNA of the primary hairy roots, and the roots and leaves of protoplast regenerated plants (Fig. 2). Meanwhile no amplified products were obtained using the genomic DNA of control plant as

template DNA, *i.e.*, these sequences of *roIB* did not exist in non-transformed *A. melilotoides* plants.

In conclusion, this study presented a reliable procedure of plant regeneration from transformed plant protoplasts of *A. melilotoides*. The factors influencing the processes from protoplast preparation to shoot differentiation included plasmolysis of leaves prior to protoplast isolation, duration of protoplast isolation, protoplast density, combinations of growth regulators in the medium, *etc.* The results obtained also demonstrated that these transformants relatively easy regenerate to plants and the transgenic characters, especially the integration of T-DNA, were remained. These transgenic plants could be used for somatic hybridization with other species.

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