Korean J Biol Sci 2: 239-242, 1998

Protoplast Isolation and Regeneration of Fertile Plants from *Arabidopsis Trp* Mutant, *trp1-100*

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Key Words:
Arabidopsis thaliana
PAT
Trp1-100
Protoplast
Plant regeneration

***Arabidopsis trp1* mutant plants, deficient in phosphoribosyl anthranilate transferase (PAT) activity, accumulate anthranilate compounds, which render them blue fluorescence. The visible phenotype of *trp1* makes the *PAT* gene an excellent reporter gene in the mutant. In order to develop a system for the homologous recombination using the phenotypic characteristic of *trp1-100*, we established optimum conditions for the isolation and regeneration of protoplast from auxin-conditioned, *trp1-100* root cultures. Tryptophan had to be supplemented in the germination medium for the efficient cell division and subsequent plant regeneration. When 10 μM tryptophan was added to the germination medium, we obtained the highest yield of protoplasts (3×10^6 cells/g) and the best viability (92%). Thirty percent of root protoplast derived from meristematic cells underwent cell division within 5 days in callus-induction medium. Regenerated rosette leaves (2-3 mm) were transferred to rooting medium and finally acclimated to the soil for flowering.**

An *Arabidopsis* blue fluorescent tryptophan mutant (*trp1-100*) has a defective phosphoribosyl anthranilate transferase (*PAT1*) gene (Rose et al., 1992; Niyogi, 1993). The *trp1-1* plant containing the most severe *trp*1 allele is a tryptophan auxotroph and displays small and bush-type growth morphology. Unlike *trp1-1*, the *trp1-100* mutant does not require exogenous tryptophan for growth and has normal growth morphology and fertility. The mutants are resistant to 5-methylanthranilate, which is converted to a toxic 5-methyltryptophan in wild type plant (Last and Fink, 1988). These characteristics can be used to develop the *trp1-100* mutant as a host line for the homologous recombination system using *PAT1* gene as a selectable marker.

Development of 'knock-out' plants by homologous recombination will greatly facilitate the analysis of gene function in plants. Due to the low frequency of the homologous recombination event, it requires an efficient way of generating and screening a large number of transformants (Shortle et al., 1982; Masson and Paszkowski, 1997). Plant protoplasts can be successfully transformed and screened for this kind of rare occurrence (Offringa et al., 1990; Peterhans et al., 1990).

To develop *trp1-100* as a host line for the homologous recombination system, we optimized the main parameters that are important for the efficient isolation and regeneration of *trp1-100* protoplasts. Protoplasts were isolated from auxin-conditioned root culture. It was important that protoplasts should be derived from healthy plants. Addition of tryptophan to the germinating seeds enhanced the viability of protoplasts and increased divisions of isolated protoplasts for the subsequnent handling.

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Materials and Methods

Plant material

A mutant (*trp1-100*) derived from *A. thaliana* ecotype Columbia was used in this study (Rose et al., 1992).

Root culture

Sterilized seeds were germinated in 500 ml Erlenmeyer flasks containing 100 ml liquid 0.5 x MS medium with continuous shaking at 80 rpm for 7 days. Roots were excised from seedlings and transferred into solidified callus-inducing medium (CIM) supplemented with IAA (2.0 mg/l), 2,4-D (0.5 mg/l) and IPAR (0.5 mg/l) in 0.5 x MS medium.

Determination of optimal concentrations of tryptophan

To optimize the tryptophan concentration for protoplast cultures, we examined the yield and viability of isolated protoplasts according to the concentration of tryptophan. Initially, the liquid medium contained 0, 1, 5, 10,

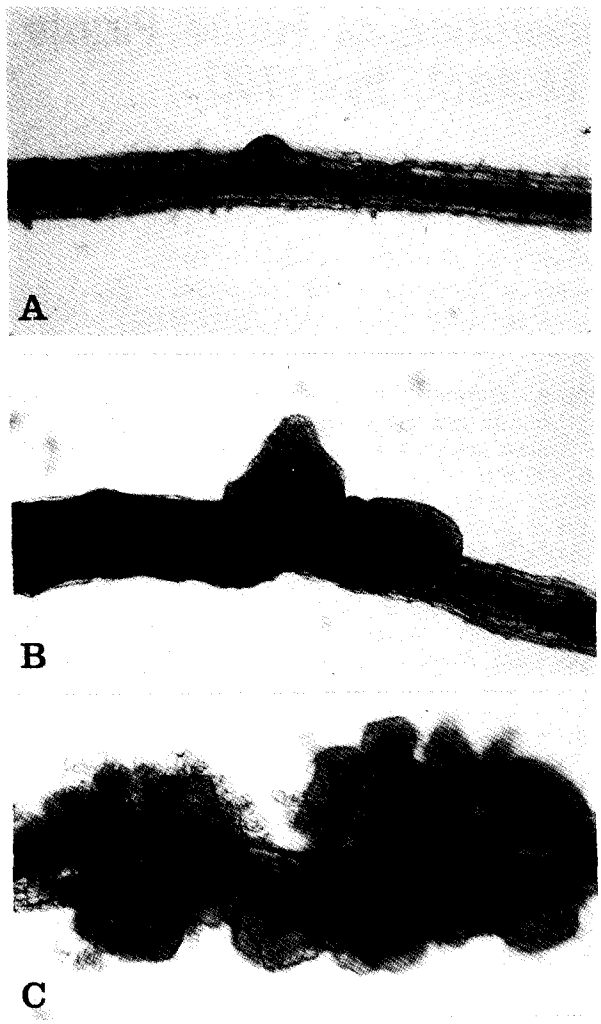

Fig. 1. Protoplast regeneration from *trp* mutant. The *trp1-100* root explants cultured in callus induction medium. The root was derived from 7 day-old seedlings grown in liquid 0.5X MS medium containing 10 μM tryptophan. A, 3 day-old root explant. B, 5 day-old root explant. C, 7 day-old root explant.

50, and 100 μM tryptophan for seed germination, respectively. The root explants were transferred to solid CIM medium and then digested to isolate protoplasts. The viability was scored by counting green fluorescent cells stained with fluorescein diacetate. Also, cell division was observed 5 days after protoplast isolation.

Protoplast isolation and culture

Proliferating root cultures were resuspended in a enzyme solution containing 2.5% cellulase Onozuka R-10 and incubated for 4 h at 24℃ with shaking (40 rpm). Protoplasts were filtered through 50 μm steel meshes, pelleted by centrifugation (80 rpm for 5 min), and washed twice with 0.5 x MS medium containing 0.4 M glucose. The sedimented protoplasts were resuspended in liquid CIM containing 0.4 M glucose (PCIM) at a density of 1×10^6 cells/ml. The protoplast suspension was distributed in 1 ml aliquots into a petridish and incubated at 24℃ under dim light (700 lux). Gradual dilution of protoplasts was performed by adding 1 ml of the same medium at 5, 10, and 15 days after protoplast isolation. Then, 2 ml of liquid CIM was added at 21, 27, and 33 days of culture.

Table 1. Comparison of protoplast yield and division rate in the *Arabidopsis* tryptophan mutant

Tryptophan concentration (μM)[1]	Protoplast yield (10^6 cells/g ± SD)	Division rate (%)[2]
0	1.85 ± 0.19	5-10
1	2.25 ± 0.28	15-25
5	3.20 ± 0.32	25-35
10	5.37 ± 0.41	30-40
50	1.70 ± 0.25	13-20
100	1.40 ± 0.30	5-10

[1]Tryptophan concentration in the medium for seed germination.
[2]Scored after 5 days of culture in PCIM.

Plant regeneration

When their diameters were approximately 1 mm, proliferating colonies were subcultured onto MS medium containing 7 mg IPAR and 0.5 mg IAA to induce shoot regeneration (SRM) at 24℃ with 16 h photoperiod at 3000 lux. The regenerating shoots were individually transferred to MS medium containing 1 mg/l IBA (RIM) to promote root formation. The plants were grown to flower and set seeds.

Results

Arabidopsis seedlings during the first few days following germination exhibited a characteristic pattern of red pigmentation on the distal edges of the cotyledons and in the epidermal layers of the hypocotyl. Particularly, seedlings of *trp1-100* appear to be excessively pigmented. Addition of tryptophan to the germination medium led to greening of the cotyledons. The high concentration of tryptophan (100 μM), however, caused

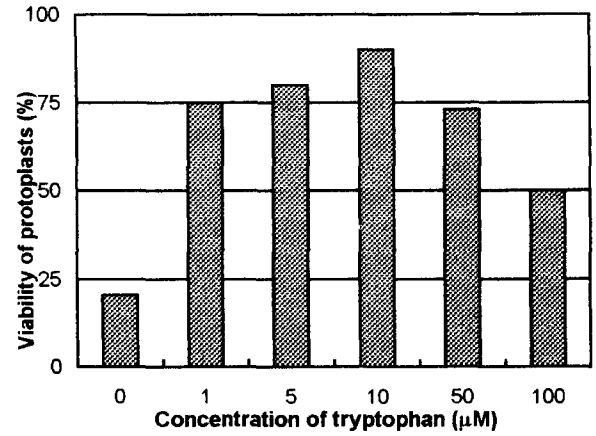

Fig. 2. The viability of protoplasts isolated from *trp1-100* root culture. The explants were derived from seedlings germinated in liquid medium containing various concentrations of tryptophan and incubated on callus induction medium.

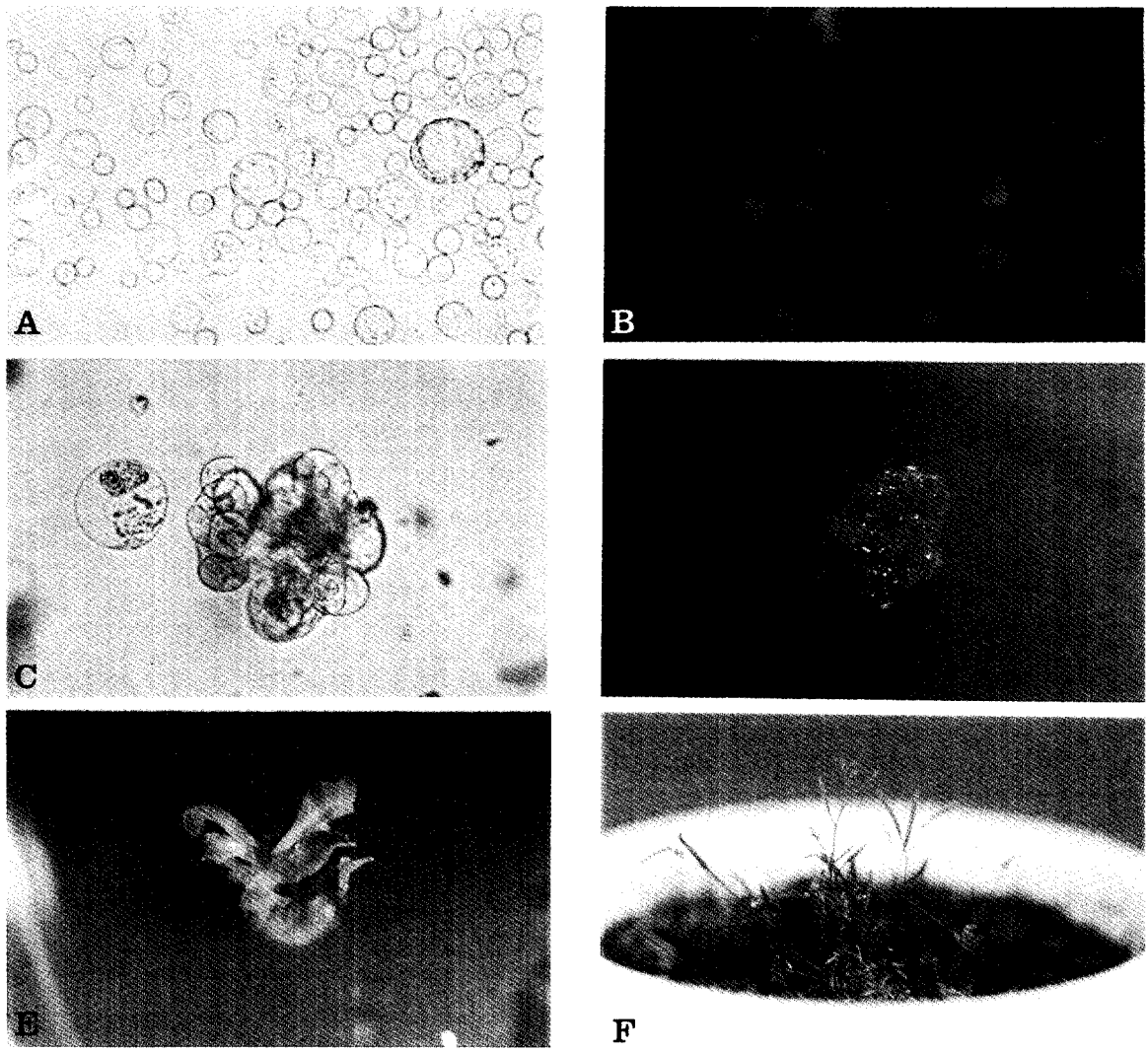

Fig. 3. Isolation, culture, and regeneration of *A. thaliana trp1-100* root protoplasts. A, Protoplast isolated from 7 day-old CIM root cultures. B, Root protoplasts in the presence of fluorescein diacetate illuminated with UV. Viable protoplasts emitted fluorescence. C, Microcolony formation in 3 week old liquid culture. D, Green callus developed from a protoplast. E, Shoot differentiation from green callus. F, Fertile *trp1-100* plant from root protoplasts.

some growth retardation of the seedlings (data not shown).

Protoplasts were isolated from 7 day-old root cultures. After the seeds were geminated in liquid medium containing tryptophan, the root explants were transferred to solid CIM. After 3 days, the explants showed intense cell divisions starting from the central vascular cortex of roots (Fig. 1A), where globular meristematic structures were developed (Fig. 1B, C). Root cultures showing cell proliferation were harvested for the protoplast isolation (Fig. 1C). The viable protoplasts were obtained by digesting the root cultures with 2.5% cellulase Onozuka R-10 for 4 h. A short period of digestion generated better quality of protoplasts (data not shown). Most of the protoplasts were small and did not possess vacuoles, which is indicative of meristematic origin (Fig. 3A).

In the absence of tryptophan pretreatment, the protocol yielded protoplasts at a density of 1.85×10^6 cells/ml from 1 g of root cultures. The protoplast yield was enhanced to about three times when the roots were germinated in the medium containing 10 μM tryptophan. Higher concentrations of tryptophan in the medium resulted in the reduction of the yield. The viability of isolated protoplasts were determined by green fluorescence after staining with 5% fluorescein diacetate (Figs. 2 and 3B).

Tryptophan pretreatment enhanced the protoplast viability regardless of tryptophan concentrations. When 10 μM of tryptophan was added to liquid medium for seed germination, we observed the highest yield of dividing protoplasts and the best viability (Table 1 and Fig. 2).

The high initial protoplast density (1×10^6 cells/ml),

required for the induction of cell division, had to be gradually reduced because undiluted protoplasts tended to aggregate and collapse. A proper dilution schedule resulted in colony formations composed of 20-60 cells within 4 weeks of culture (Fig. 3C). Plating of colonies on solid SRM induced the development of green calli (Fig. 3D). About 80% of them regenerated rosette leaves in light within 2-3 weeks of culture (Fig. 3E). Because the high cytokinin concentration of the medium inhibited the root development, rosettes were first transferred to root induction medium. Then, 4-6 days later, they were subcultured to 0.5 x MS medium with 0.5% sucrose and finally, transferred to soil for the flowering (Fig. 3F).

Discussion

A short life cycle and small genome size of *Arabidopsis* make it an excellent model plant for genetic studies (Meyerowitz, 1992). The application of many useful tissue culture techniques in *Arabidpopsis*, however, lagged considerably behind those (Wench and Marton, 1995). In the previous reports, several efficient methods were developed for the regeneration of protoplasts in *A. thaliana* (Damm and Willmitzer, 1988). These protocols were applicable to most ecotypes but sustained division of the protoplasts was achieved only in limited conditions like in alginate bead.

To take the advantage of the *trp1-100* mutant for the homologous recombination system, it is necessary to isolate viable protoplasts and to regenerate fertile plants. Isolation of a large number of viable protoplasts from *trp1-100*, however, were difficult due to its intrinsic metabolism; *Trp1-100* has defects not only in PAT activity but also in related secondary metabolites, including the phytohormone (IAA), antimicrobial phytoalexins, and other indolic molecules.

We described a protocol that allows an efficient regeneration of morphologically normal and fertile plants from protoplasts of *A. thaliana trp1-100*. Tryptophan was added to germination seeds. Protoplasts were isolated from auxin-conditioned root cultures according to the Mathur et al. (1995). Protoplasts from the meristematic centers of the root explants were released by a brief treatment with cellulase. The meristem-derived protoplasts underwent cell division with a high division rate up to 35%. For the regeneration, cells were gradually diluted through a period of 3-5 weeks.

In conclusion, the most important aspect of the regeneration of *trp1-100* protoplasts is the careful pre-culture of donor plants treated with both tryptophan and auxin. The factor contributing to the high division rate and regeneration was the highly meristematic nature of the starting material. Therefore, the root protoplast protocol appears to be suitable for regeneration of the *trp1-100* plant. The method described thus represents a first and nesessary step toward the genetic modification of *A. thaliana* through the transformation of protoplasts.

Acknowledgements

This work was supported by the research fund for the Genetic engineering Program from the Ministry of Education (1996).

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[Received March 7, 1998; accepted April 3, 1998]