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Full Length Article

The Induction of Polyploid Hairy Roots in *Petunia hybrida* Using Root Transformation of *Agrobacterium rhizogenes* K599 and Colchicine

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Abstract

In general, polyploid plant tends to have larger size, increased amounts of secondary metabolites, and stronger resistance to stress. On the other hand, hairy root has the advantages of rapid propagation and convenience to genetic manipulation. Thus, considering these two beneficial features, we used *Agribacterium rhizogenes* K599 to infect the leaves of petunia tissue culture seedlings to induce hairy root, and the hairy roots were subsequently treated with 0.05%-0.20% colchicine for 12-36 h. Among them, 0.1% colchicine for 24 h treatment resulted in 55.0% survival rate and 26.70% tetraploid induction rate, which led to chromosome doubling events while maintaining a high survival rate. Microscopic observation of root tip cells confirmed that the polyploid hairy roots were mostly tetraploid 4n = 28. This study provides a theoretical basis for obtaining polyploid plants using root transformation and colchicine.

Keywords: *Petunia hybrida*; Hairy root; Chromosome doubling; Root transformation

Introduction

Polyploids are widely spread in the plant kingdom. Compared with haploid and diploid plants, polyploids have thicker rhizomes and larger leaves and flowers. Moreover, polyploid can produce more vegetative and reproductive organs (Chao *et al.* 2013; Hou *et al*. 2014; Yang *et al.* 2014), and more resistant to stresses. Also, polyploidy has often been considered to confer plants a better adaptation to environmental stress (Tan *et al.* 2015; Shin *et al.* 2017). In addition, polyploid contains more contents in cells, which can significantly enhance production of the secondary metabolites (Dhawan and Lavania 1996).

On the other hand, hairy root is an adventitious root induced by *Agribacterium rhizogenes*, which infect plant cells, and insert their T-DNA into the genomic DNA of plant cells. Hairy root can not only overcome the disadvantages of plant slow growth and limited accumulation of effective components, but also can grow vigorously on medium without exogenous plant hormones. Moreover, hairy root can be directly used to analyze the function of functional genes, and can regenerate plants under certain conditions (Xiang *et al*. 2016). Lastly, hairy root can propagate rapidly and are easy to handle. In this study, we used the model flower plant petunia as the material and used *Agrobacterium rhizogenes* K599 strain to infect the leaves of petunia to induce hairy root. The hairy roots were treated with colchicine to obtain polyploid hairy roots. This study provides a theoretical basis for obtaining polyploid plant using root transformation and colchicine.

Materials and Methods

Experimental material

Diploid petunia (2n=14) tissue culture seedlings were obtained from our previous studies (Wang *et al*. 2015; Wu *et al*. 2018). *Agrobacterium rhizogenes* K599 strain was stored at -80°C freezer in our laboratory.

Induction, culture and identification of hairy roots

A small amount of *Agrobacterium rhizogenes* K599 was picked with a sterile inoculation needle, streaked on a LB plate containing 50 mg/L streptomycin (Str) and incubated at 28°C for 2-3 days in dark. Then, a single colony was picked and inoculated in liquid LB medium with 50 mg/L Str for 24 h at 200 r/min and 28°C. When the *OD* value reached about 0.5, 1 mL volume of the bacterial culture was transferred into a 1.5 mL centrifuge tube and centrifuged at 4000 r/min for 5 min. After the supernatant was removed, the bacteria pellet was washed twice with MS liquid medium, and diluted to *OD* of about 0.1. This solution was used as infection solution. The leaves of sterile petunia seedling were cut off, and multiple wounds were made on each leaf with scalpel.

These leaves were then immersed in bacteria solution for 10 min at room temperature and 100 r/min. After the bacterial solution was wiped off, leaves were incubated in MS medium plus 10 mg/L acetosyringone (As) for 2 days. Then, the leaves were washed with sterile water, and transferred to MS+10 mg/L As+500 mg/L cefotaxime (Cef) to induce hairy roots. After the hairy roots that were induced at the leaf wounds reached about 2 cm in length, they were cut and cultured onto MS+500 mg/L Cef for sterilization and propagation.

PCR identification of hairy roots

The hairy root DNA was extracted using the plant genomic DNA extraction kit (Shanghai Sangon Biotech, Co. Ltd., China). Based on *rolB* gene sequences carried by *Agrobacterium rhizogenes* K599 T-DNA, we designed and synthesized primers of rolB-P1: 5'-gccagcatttttggtgaact-3' and rolB-P2: 5'-ctggcccatcgttctaaaaaa-3' (Zhang *et al.* 2011). The 35 μL PCR reaction contained 3.5 μL 10 × buffer (containing 15 mmol/L Mg^{2+}), 2 μL dNTP (2 mmol/L), 2 μL each of rolB-P1 and rolB-P2 (10 pmol/μL), and 2 μL DNA template (50-100 ng/μL). The PCR cycles were: denaturing at 94°C for 5 min, followed by 30 cycles of 94°C 45 s, 55°C 45 s, and 72°C 90 s, then extension at 72°C for 10 min. The PCR products were electrophoresed on a 1.2% agarose gel for 1.5 h (5 V/cm), stained with EtBr, and imaged with BioRad gel imaging system.

Colchicine treatment on hairy roots and the identification of its ploidy level

The hairy roots grown on culture medium were taken at 7:00 a.m., 8:00 a.m., 9:00 a.m., 10:00 a.m., and 11:00 a.m. in the morning, and immersed in colchicine solutions containing 0%, 0.05%, 0.1%, 0.15%, and 0.2% (W/V) colchicine for 12, 24 and 36 h respectively. Then, the roots were rinsed with MS liquid medium for three times to remove the residual colchicine on the surface and dried with sterile absorbent paper. The roots were then transferred back to solid MS medium to continue cultivation. After the hairy roots treated with colchicine resumed growth, they were put into a finger bottle containing 0.05% colchicine, incubated at 4°C for 4 h. Then the roots were rinsed with distilled water for 3 times, and fixed in Carnot-type fixation solution for 24 h. After the fixation, the roots were rinsed with 75% alcohol for 5 min, and then rinsed with distilled water for three times and soaked for 10 min, followed by 1 mol/L HCl treatment at 60°C for 15 min. After rinsed with distilled water for three times, the roots were stained with a modified pectic carbonic acid-fuchsine solution for 10 min, and then mounted and examined by microscopy.

Results

Induction, propagation, and PCR identification of petunia hairy roots

After 7-10 days of K599 infection, white protrusions started to appear at the wounds of petunia leaves. About 15 days after infection, white hairy roots were obviously visible by naked eye (Fig. 1A). Then, about 30 days after infection, a large number of hairy roots had been grown out (Fig. 1B). The fast-grown hairy roots were cut and transferred to MS medium containing 500 mg/L Cef and these roots were transferred to fresh medium every 30 days. After 3-4 times subculture, all agrobacterium in hairy roots can be killed. Then hairy roots could grow well on the MS medium without Cef (Fig. 1C).

We randomly selected five sterilized hairy roots and extracted their genomic DNA. Then, PCR of these hairy roots were amplified with primers of *rolB* gene, and showed the 450 bp characteristic band. As the control, the genomic DNA from petunia tissue culture seedlings did not show any bands (Fig. 2).

The cultivation and identification of hairy roots ploidy after colchicine treatment

The hairy roots were treated with 0.05%-0.2% colchicine solution. As the colchicine concentration and treatment time increased, the survival rates of hairy roots gradually decreased (Table 1). For example, the survival rate of hairy root was 20.0% with 0.2% colchicine treatment for 24 h, and no hairy root survived after 36 h of the treatment.

The color of hairy roots turned dark after colchicine treatment. During the cultivation process, no obvious growth was seen in the first 15 days or so; but after that, there were new white roots gradually grew out from the hairy root tips (Fig. 3A and B). The new roots could continue to grow and quickly proliferate (Fig. 3C and D). There was no significant difference in the morphology and growth rate of hairy root recovered from colchicine-treated and untreated hairy roots on MS medium.

The chromosomes of root-tip cells were stained, and examined under microscope. For samples that were collected at 7-8 am in the morning, their chromosomes could be clearly stained and observed. Chromosome doubling events appeared in the hairy roots treated with 0.1% colchicine or higher. The chromosome doubling was mostly tetraploidy (4n=28) (Fig. 4), but aneuploidy was also observed. Among all the conditions, 0.2% colchicine treatment for 24 h led to the highest tetraploid induction rate, which was 40.0% (Table 1).

Discussion

In this study, we used colchicine treatment to induce chromosome doubling and obtained tetraploid hairy roots in petunia. Evaluating together both of the survival rate and chromosome doubling events of hairy roots, we concluded that 0.1% colchicine treatment for 24 h is optimum to achieve chromosomes doubling effect while maintaining a high survival rate. Moreover, the polyploids we obtained were mostly tetraploids, which is consistent with the report from Hou *et al*. (2014). Previous studies have shown

Table 1: The survival rates and polyploid induction rate of hairy roots under different colchicine treatment

Colchicine Concentration (%)	Treatment Time (h)	Time Required to Visualize New Roots (D)	Survival rate (%)	Tetraploidy induction rate（%）
0.05	12	18-25	66.7	0
	24	25-32	60.0	0
	36	32-39	46.7	0
0.10	12	15-20	60.0	0
	24	20-25	55.0	26.7
	36	25-30	10.0	33.3
0.15	12	15-20	30.0	0
	24	20-27	26.7	30.0
	36	27-30	6.7	35.0
0.20	12	17-20	36.6	13.3
	24	22-27	20.0	40.0
	36	27-33	0	—

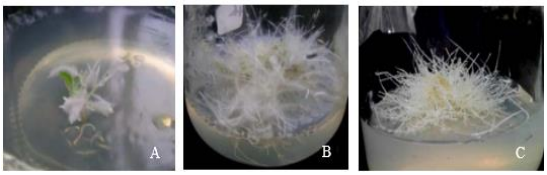

Fig. 1: The hairy roots induced from petunia leaves. **A**: Hairy roots were induced at the leaf wound; **B**: The fast-grown hairy roots. **C**: The hairy roots grew well after sterilization

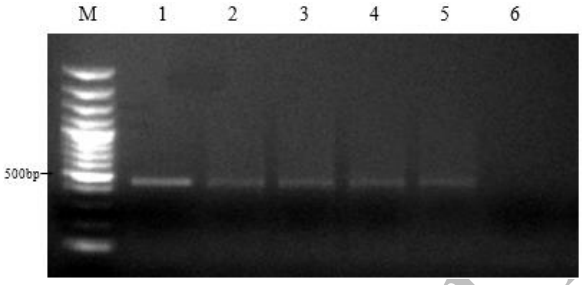

Fig. 2: PCR identification of hairy roots. M: 100 bp DNA molecular ladder; 1-5: different lines of hairy roots; 6: root from petunia tissue culture seedlings

significant differences between diploid and tetraploid plants, and polyploidy often confers emergent properties. Paterson *et al*. (2012) reported that tetraploid cottons had higher fiber productivity and quality than diploid cotton in the same environment. Moreover, extracts from tetraploids showed higher amounts of amino acids, while the extracts from diploids contained more organic acids and sugars (Shin *et al*. 2017). Zhao *et al*. (2018) reported that the fruits of tetraploid grapes were significantly bigger than diploid grapes. In addition, hairy root can regenerate plants under certain conditions (Xiang *et al*. 2016). Thus, the polyploid hairy root obtained in this study could be used to further regenerate and obtain polyploid petunia with bigger floral organs and more ornamental values. Since hairy root can be used as bioreactors for exogenous genes to produce medicinal ingredients (Du *et al.* 2015), this study can help to improve the production of medicinal ingredients using chromosomal doubled hairy roots in medicinal plants (Jesus-Gonzalez and Weathers 2003).

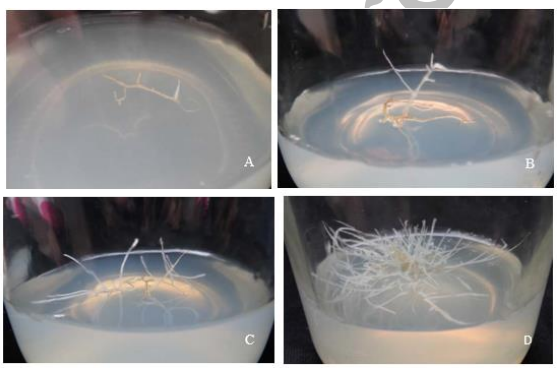

Fig. 3: The recovery process of hairy roots after colchicine treatment. **A** and **B**: The newly grown white roots; **C** and **D**: The new hairy roots grew quickly

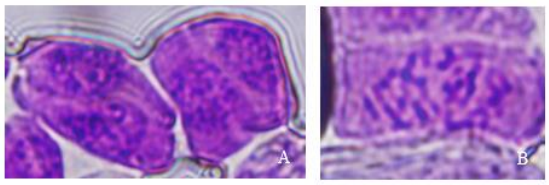

Fig. 4: Microscopic examination of hairy root ploidy. **A**: Tetraploid hairy roots obtained from colchicine treatment (4n = 28); **B**: Diploid hairy roots from untreated plants (2n = 14)

Conclusion

Evaluating together both of the survival rate and chromosome doubling events of hairy roots in petunia, 0.1% colchicine treatment for 24 h is optimum to achieve chromosomes doubling effect while maintaining a high survival rate. Moreover, the polyploids obtained were mostly tetraploids 4n=28. This study provides a theoretical basis for obtaining polyploid plants using root transformation and colchicine.

Acknowledgments

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