Application of bacteria from *Agrobacterium* genus for gentians genome modification

Anna Wójcik, Diana Szczygieł, Jan J. Rybczyński

Botanical Garden - CBDC PAS, Warsaw, Poland
e-mail: ai.wojcik@wp.pl

Introduction

Agrobacterium-mediated genetic transformation is still the most popular method of producing genetically modified plants. It's simple, not expensive and not requiring to use equipment that's why it wins competition with direct gene transfer techniques into plant cells. Efficient transgene integration into the recipient genome, which is possible thanks to natural *Agrobacterium* ability to plants infection, is also of great importance.

Material and methods

Explants were obtained from seedlings, stems and young leaves of *G. cruciata* L., *G. kurroo* Royle and *G. tibetica* King. Explants were infected by immersion and incubation into bacterial suspension containing MS salts and vitamins, 200 µM acetosyringon and different L-glutamine concentration (0.25 - 2 g l^{-1}) from 15 minutes to 120 hours or by puncturing with a needle submerged in bacterial suspension. Three *Agrobacterium rhizogenes* and four *Agrobacterium tumefaciens* strains were applied in presented work (Table 1). Eexplants were cultured on MS medium containing 30 g l^{-1} sucrose, acetosyringon and enriched (*A. tumefaciens* transformation) or not (*A. rhizogenes* transformation) with plant growth regulators PGR (0.5 mg l^{-1} 2.4-D + 1.0 mg l^{-1} kinetin for seedlings explants, 1.0 mg l^{-1} NAA + 0.5 mg l^{-1} BAP or 1.0 mg l^{-1} NAA + 0.25 mg l^{-1} kinetin for *G. cruciata* leaf explants, 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BAP - *G. kurroo*, 2.0 mg l^{-1} NAA + 1.0 mg l^{-1} CPPU - *G. tibetica*). 48 hours after transformation plant material was transferred onto medium with the same PGR composition and sucrose concentration with timentin. Two weeks later explants transformed by means of modified bacteria strains were transferred on medium containing kanamycin or BASTA (depending on reporter gene used).

Three different media for *Gentiana tibetica* plant regeneration were tested (Figure 1) on which explants were transferred 14 days after transformation.

Cell suspension aggregates were infected by its incubation in medium containing *Agrobacterium* cells, acetosyringon and/or not glutamine. After transformation tissue was rinsed in MS solution and transferred into fresh culture medium with antibiotics. Cell suspension cultures were passaged every week onto fresh medium enriched with antibiotics and selection factors.

Table 1. Usefulness of various explants type and *Agrobacterium* strains in gentians transformation

		G. cruciata	*G. kurroo*	*G. tibetica*
Leaves	AT LBA 4404a	-	-	-
	AT C58C1a	+	-	+
	AT C58C1b	-	-	-
	AR ATCC 15834	+	-	+
Stems	AR ATCC 15834	-	-	-
Seedlings	AT LBA 4404a	-	nt	-
	AT LBA 4404b	-	nt	-
	AT C58C1a	-	nt	+
	AT C58C1b	-	nt	-
	AR LBA 9402	-	nt	-
	AR A4	-	nt	-
	AR ATCC 15834	-	nt	-
Cell suspension aggregates	AT C58C1a	+	+	-
	AT C58C1b	-	-	-
	AR LBA 9402	nt	nt	-

Gentiana cruciata

A. rhizogenes ATCC 15834 in L-glutamine presence allowed to obtain callus tissue and hairy roots on leaf explants (Figure 2). Transformation was made by puncturing with a infected needle and incubation into bacterial suspension. When only one infection method was used no reaction was observed .

Plants were regenerated after transformation by C58C1a.

Modified cell suspension culture had ability to growth in kanamycin presence. Aggregates and regenerated somatic embryos revealed positive GUS reaction (Figure 3).

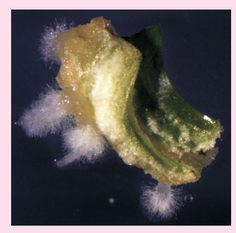

Figure 2. Hairy roots on *G. tibetica* leaf explant

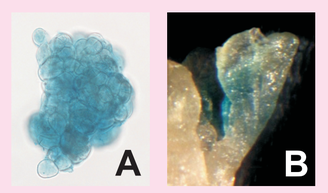

Figure 3. GUS-stained cell suspension aggregate (A) and somatic embryo (B)

Gentiana kurroo

Genetic modified cell suspension transformed by C58C1a was obtained. Cell aggregates showed β-glucuronidase activity and growth on medium supplemented with kanamycin. The transformation efficiency increased with L-glutamine content in medium (Figure 1) but high aminoacid concentration was making *Agrobacterium* elimination very difficult. 0.5 g l^{-1} L-glutamine and 7 days incubation with bacterial cells turned out to be the most useful.

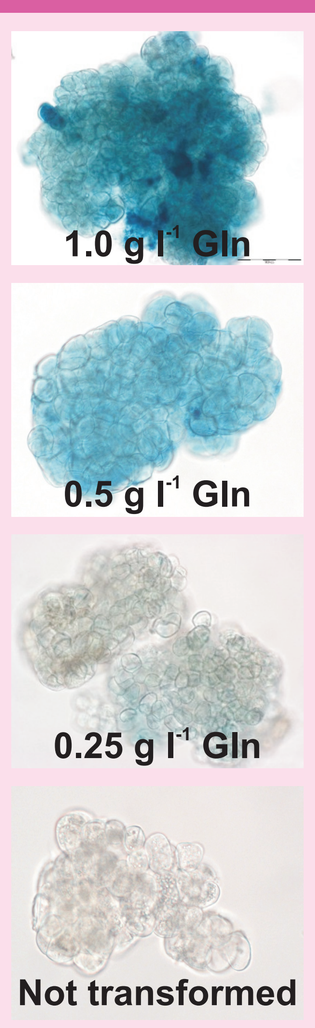

Figure 1. β-glucuronidase activity in transformed cell suspension aggregates

Gentiana tibetica

From tested *A.rhizogenes* strains only ATCC 15834 allowed to obtain transformed tissue from leaf explants. L-glutamine in medium and infection by mechanical injuries preceding incubation with bacterial cells were essential for modified tissue regeneration. Hairy roots were formed on transformed explants.

C58C1a was useful in seedling explants transformation. Callus tissue on transformed cotyledon explants was developed (Figure 4). The transformation efficiency amounted 20 %.

The same strain was helpful in leaf explants transformation. Callus tissue and regenerated plants were formed. Transformation efficiency increased with L-glutamine concentration. The best results were observed in 1.0 or 2.0 g l^{-1} L-glutamine presence (Figure 5) but their use was not possible because of difficulty with bacterial cells elimination. Incubation time over 4 hours caused rapid *Agrobacterium* cells proliferation and death of explants. 30 minutes incubation in medium with 2.0 g l^{-1} glutamine was the most effective for leaf explants transformation (Figure 6) but genetic modified plants were regenerated in the presence of 0.25 or 0.5 g l^{-1} glutamine and one hour incubation time (Figure 7).

The most effective plant regeneration was observed on WPM (Figure 8).

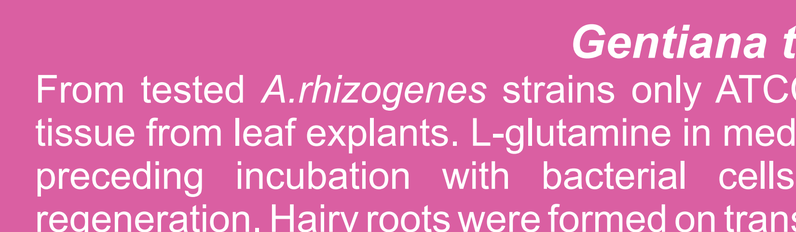

Figure 5. Transformation efficiency investigated in regenerated callus tissue

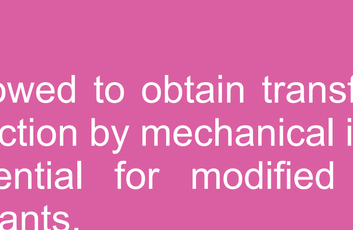

Figure 4. Histochemical analysis of GUS activity in formed on cotyledon explants callus tissue

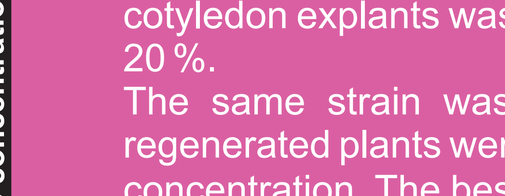

Figure 6. Transformation efficiency 7 days after explants infection for 15 minutes incubation time

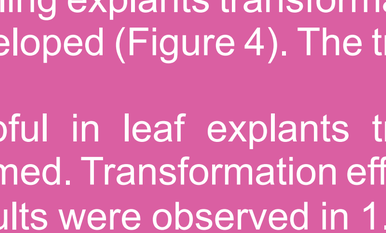

Figure 7.GUS expression in leaf from regenerated plant

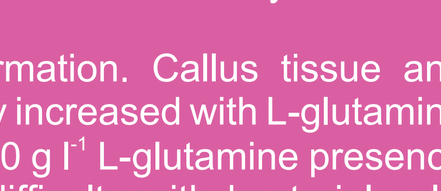

Figure 8. Plant regeneration on various media

Conclusions

1. Only C58C1 *A. tumefaciens* and ATCC 15834 *A. rhizogenes* strains were useful in gentians genetic transformation
2. Effective T-DNA integration into plant genome required L-glutamine presence in inoculation and coculture medium
3. Hairy roots, modified callus tissue and regenerated plants were obtained as a result of done experiments