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Gentiobiose Feeding in Gentian *in vitro* Overwintering Buds or Plantlets

Hideyuki Takahashi* and Masahiro Nishihara

Iwate Biotechnology Research Center, Kitakami, Japan

*For correspondence: h-takahashi@ibrc.or.jp

[Abstract] To study the functions of sugars in plants, feeding experiment is one of the most common and easy methods. However, the traditional method, *e.g.*, a floating of leaf discs on sugar-containing solution seems to have an insufficient efficiency of sugar uptake, despite of high osmotic and injury effects. This is a protocol to feed oligosaccharide gentiobiose into *in vitro* cultured tissues of gentian. This protocol enables to incorporate gentiobiose into intact tissues without exposure to osmotic stress and may be useful to other plant species that are able to propagate by shoot tip culture.

Materials and Reagents

1. Gentian (*Gentiana triflora*) tissue culture plantlets
2. Sucrose
3. Gentiobiose (Sigma-Aldrich, catalog number: G3000-5G)
4. Gellan gum (Wako USA, catalog number: 075-03075)
5. Liquid nitrogen
6. Milli Q grade water
7. MS vitamins (see Recipes)
8. Propagation medium (see Recipes)
9. IOWB induction medium (see Recipes)
10. Gentiobiose medium (see Recipes)

Equipment

1. Sterile magenta boxes (sterilized by autoclaving)
2. Surgical tape (3M, catalog number: 1530-0)
3. Glass culture tubes (sterilized by autoclaving)
4. Silicon plugs (sterilized by autoclaving)
5. Sterile 15 ml plastic tubes (such as Greiner Bio-One GmbH, catalog number: 188271)
6. Sterile syringe filter (Millipore, catalog number: SLGP033RS)
7. Sterile petri dishes (IWAKI PUMPS, catalog number: SH90-20)
8. Cutoff filter (Millipore, catalog number: UFC5003BK)
9. Growth chambers
10. Clean bench

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11. Scalpel
12. Forceps
13. Ball miller
14. Centrifuge
15. Freeze dryer

Procedure

A. Preparation and gentiobiose feeding of gentian IOWB and plantlets

1. *In vitro* shoot cultures of gentian were prepared according to the method of Hosokawa *et al.* (1996).
2. Transfer shoot tips (approximately 1 cm) to magenta boxes containing 70 ml of propagation medium and culture at 20 °C for 1 to 2 months under a 16/8 h light/dark photoperiod (Figure 1A).
3. Transfer 3 to 5 shoot tips (approximately 1 cm) to a magenta box containing 70 ml of IOWB induction medium (Figure 1B) and culture at 20 °C for over 6 months under a 16/8 h light/dark photoperiod (Figure 1C).
4. Harvest the produced IOWBs and cut into approximately 2 cm length pieces using a scalpel and forceps on a plastic petri dish (Figure 2A).

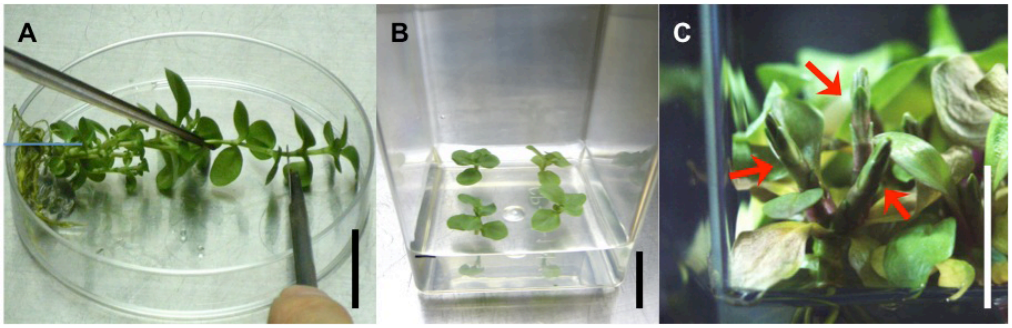

Figure 1. IOWBs induction from gentian plantlets. A. Cutting shoot tips from plantlet. B. Shoot tips placed on IOWB induction medium. C. IOWBs produced from gentian plantlets cultured for 6 months on IOWB induction medium. Arrows indicate IOWBs. Bar: 2 cm

5. Transfer IOWBs or plantlets to glass culture tubes containing 2 ml of a gentiobiose medium and seal the top of the tubes with silicon plugs (Figure 2B).

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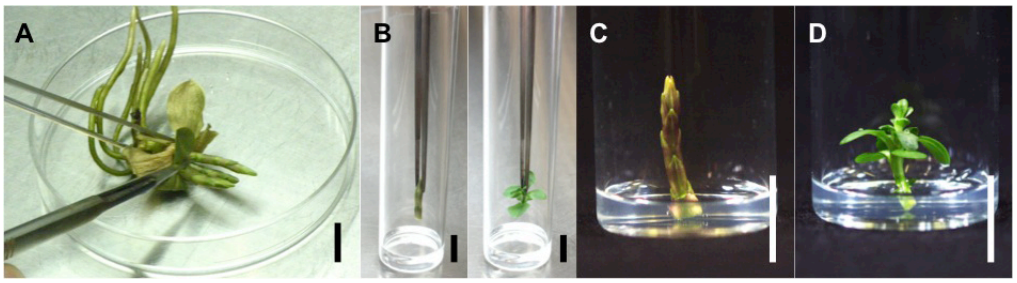

Figure 2. Gentiobiose feeding into gentian IOWB and plantlet. A. Harvest of produced IOWB. B. Placement of IOWB and shoot tip on gentiobiose medium. C. IOWB cultured on gentiobiose medium. D. Shoot tip cultured on gentiobiose medium. Bar: 1 cm

6. Culture at 20 °C under a 16/8 h light/dark photoperiod for appropriate period (Figure 2 C-D).
7. Harvest parts of the samples not in contact with the medium.

B. Detection of gentiobiose in IOWBs or plantlets
1. Freeze-dry and pulverize IOWBs or plantlets in a ball-miller.
2. Homogenize 10 mg dry weight of samples with 500 µl of 50% (v/v) methanol.
3. Centrifuge the homogenates at 20,000 *x g* for 5 min and filtrate the supernatants through a cutoff filter.
4. Subject the filtrates to thin layer chromatography (Figure 3) or other analyses to detect gentiobiose.

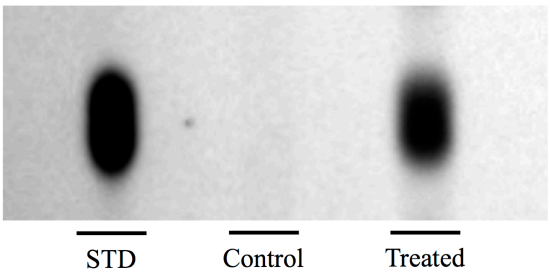

Figure 3. Example analysis of gentiobiose incorporated in gentian plantlets. Extracts from leaves of plantlets cultured on normal MS medium (Control) or MS medium containing 0.05% gentiobiose labeled with rhodamine (Treated) for 3 days were subjected to TLC analysis. STD, 0.01% gentiobiose labeled with rhodamine.

Notes

1. Propagation and feeding steps should be aseptically performed using a clean bench.
2. Labeled gentiobiose should be used only for confirmation of incorporated gentiobiose.
3. For details about gentian IOWBs and plantlets, see Imamura *et al*. (2014).
4. For details about TLC analysis, see Takahashi *et al.* (2014).

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5. For gentiobiose labeling, see the following publications, Hase *et al.* (1978) and Fry (1997).

Recipes

1. MS vitamins (100 ml)
   10 g myo-inositol
   50 mg nicotinic acid
   50 mg pyridxine hydrochloride
   10 mg thiamine hydrochloride
   200 mg glycine
2. Propagation medium (1L)
   4.6 g MS salt
   1 ml MS vitamins
   30 g sucrose
   2 g gellan gum
   Adjust pH 5.7 with KOH and autoclave for 15 min
3. IOWB induction medium (1L)
   4.6 g MS salt
   1 ml MS vitamins
   60 g sucrose
   2 g gellan gum
   Adjust pH 5.7 with KOH and autoclave for 15 min
4. Gentiobiose medium (1L)
   4.6 g MS salt
   1 ml MS vitamins
   10 g gentiobiose or labeled gentiobiose*
   2 g gellan gum
   Adjust pH 5.7 with KOH and autoclave for 15 min
   *Add to autoclaved MS medium after decrease in temperature to approximately 60 °C
   *Labeled gentiobiose is used for confirmation of incorporated gentiobiose

Acknowledgements

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