Plant Cell Tiss Organ Cult (2010) 102:27–33
DOI 10.1007/s11240-010-9700-1

ORIGINAL PAPER

Embryogenesis and doubled haploid production from anther culture in gentian (*Gentiana triflora*)

Hisako Doi · Ryo Takahashi · Takashi Hikage · Yoshihito Takahata

Received: 4 August 2009 / Accepted: 20 January 2010 / Published online: 10 February 2010

Abstract The overall goal of this study is to develop an anther culture system to produce doubled haploid (DH) lines of gentian (*Gentiana triflora*), an ornamental flowering plant, for use in an F1 hybrid breeding program. Embryogenesis was induced from anther cultures incubated on half-strength modified Lichter (NLN) medium containing a high concentration of sucrose (130 g/l) and subjected to heat shock treatment. Among the various parameters investigated, anthers collected from buds 9–12 mm in length induced the highest frequency of androgenesis. Moreover, among three genotypes tested, cvs. Ashiro-no-Aki and Ashiro-no-Natsu produced 21.3 and 3.7 embryos per 100 anthers, respectively, whereas, cv. Lovely-Ashiro failed to produce embryos. Among a total of 427 embryos transferred to a regeneration medium consisting of Murashige and Skoog (MS) medium, 138 plants were regenerated. The ploidy levels of regenerants were determined by flow cytometry and chromosome counts, revealing the presence of 5% haploids, 25% diploids, and 70% triploids. Inter simple sequence repeat (ISSR) analysis using the 6PS line obtained following self-pollination of the diploid plant obtained from anther culture confirmed that the diploid plant was indeed a DH.

Keywords Gentian (*Gentiana triflora*) · Anther culture · Embryogenesis · Doubled haploid · Ploidy level

H. Doi · Y. Takahata (✉)
Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan
e-mail: ytakahata@iwate-u.ac.jp

R. Takahashi · T. Hikage
Hachimantai City Floricultural Research and Development Center, Hachimantai 028-7592, Japan

Introduction

Several species of gentians (*Gentiana* spp.) have been used in the ingredients of medicine and for gardening plants (Kohlein 1991). In Japan, gentians are among the most important plants for the cut- and pot-flower ornamental industry. In particular, both *G. triflora* and *G. scabra* species have been cultivated commercially in Japan (Yoshiike 1992). Since the cultivar Iwate, which is an F1 hybrid between *G. triflora* and *G. scabra*, was bred in 1977, many F1 and clonal cultivars have been developed from intra-specific crosses and inter-specific breeds (Yoshiike 1992; Takahata et al. 1995). In Europe, the cultivation of gentian for cut flower was introduced from Japan in the 1980s, therefore, outside Japan, the gentian is a new ornamental plant as a cut flower (Nishihara et al. 2008).

A problem has been pointed out with F1 hybrid breeding of gentians. Since the F1 hybrid cultivar is usually produced from a cross between two inbred lines or pure lines, the establishment of parental inbred lines is important (Poehlman and Sleper 1995). Inbred lines are generally developed and maintained by self-pollination. However, complete homozygous inbred lines of gentians have not been obtained until now, because gentians show serious inbreeding depression. Thus, parental lines for the gentian F1 varieties have been produced and are maintained by a sib-mating or tissue culture technique (Yoshiike 1992; Takahashi 1997; Tada et al. 2000), therefore, their homozygosity is not complete. The genotype of the parental lines gradually changes during their maintenance, resulting in the breakdown of the F1 variety. In the breeding of high-quality F1 hybrid varieties of gentians, the production of complete homozygous lines is a matter of great importance.

Haploids and doubled haploids (DHs) produced by the in vitro culture of gametophytic cells, particularly male

gametophytes, have great advantages for the production of homozygous lines (Bajaj 1990; Khush and Virmani 1996; Forster et al. 2007), and over 200 varieties have been developed using various DH methods (Thomas et al. 2003). So far, haploid and DH plants derived from gametophytic cells have been reported in about 200 species of angiosperms (Maluszynski et al. 2003). Although several applications of tissue culture such as organogenesis, somatic embryogenesis, and protoplast have been reported in gentians (Skrzypczak et al. 1993; Takahata et al. 1995; Hosokawa et al. 1996), few gametophytic cell cultures for haploid and DH production have been reported in gentians, except for one report by Maruta and Matsumoto (1989), who described the embryogenesis from anther culture of gentian spp.

In this paper, we report embryogenesis and plant regeneration from anther culture of gentians (*G. triflora*). Special attention is paid to several factors influencing androgenesis, such as genotypes, bud sizes, culture conditions, culture media, etc. In addition, genetic homogeneity of the progeny of anther culture-derived plants was confirmed. This is the first report of the production of DH in gentians and contributes to the development of gentian F1 breeding.

Materials and methods

Plant materials

Three genotypes of gentians, cvs. Ashiro-no-Natsu (*G. triflora*), Ashiro-no-Aki (*G. triflora*), and Lovely-Ashiro ([*G. triflora* × unknown] × unknown), were used in this study. These materials were grown in an experimental field of Hachimantai City Floricultural Research and Development Center, Hachimantai, Japan.

Anther culture and plantlet regeneration

Flower buds 7–15 mm in length were harvested. Determination of the microspore developmental stages in the flower buds was performed using 4′,6-diamidino-2-phenylindole (DAPI) staining. The buds were surface-sterilized in 70% ethanol for 30 s, followed by sodium hypochlorite (1.5% active chlorite) for 15 min, and then rinsed three times with sterile distilled water (5 min each time). Five anthers excised from buds 9–15 mm in length were cultured in a 60-mm plastic Petri dish containing 3 ml of 1/2 NLN medium (Huang and Keller 1989) supplemented with 13% sucrose. In some cases, 0.25% gellan gum-solidified 1/2 NLN medium was used. The Petri dishes were incubated at 32.5°C for 1 day prior to maintenance at 25°C. To elucidate the optimum culture conditions, several factors such as sucrose concentrations (10, 13, and 16%), duration of heat shock (0, 1, and 2 days) treatment (32.5°C), number of anthers per Petri dish (5 and 10 anthers), cold pretreatment (4°C) prior to culture (0, 3, 7, and 10 days), and plant growth regulator treatments, including 0, 2.0 mg/l α-naphthaleneacetic acid (NAA) and 0.5 mg/l kinetin, and 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 mg/l 6-benzyladenine (BA) were investigated. There were at least three plates per experiment, and each experiment had at least three independent replicates. For data analysis, the Tukey–Kramer test was performed using the computer program JMP 8.0.

The embryos developed from anthers were transferred to modified gellan gum (0.25%)-solidified Murashige and Skoog (MS) medium (Murashige and Skoog 1962) with the concentration of major salts reduced by 50% and supplemented with 3% sucrose (1/2MS), and incubated at 25°C with a 16 h/day photoperiod. Abnormal embryos were transferred to the 1/2MS medium supplemented with 1 mg/l gibberellic acid (GA_3). Regenerated plants were grown in vermiculate and then transferred to soil in a greenhouse. No method of chromosome duplication was carried out.

Flow cytometry and chromosome counting

The ploidy level of regenerated plantlets was analyzed using a PA-I flow cytometer (Partec). The leaves were chopped with a razor blade in a plastic Petri dish containing the nuclei extraction buffer (solution A of the High Resolution Kit for Plant DNA, Partec). After filtration through a 30-μm nylon sieve, a staining solution of dye 4,6-diamidino-2-phenylindole-2HCl (solution B of the Kit) was added. The nuclei mixture was analyzed using the PA-I flow cytometer. Histograms of DNA content were evaluated using the Partec software package.

Chromosome counts were carried out on root-tip cells pretreated with 0.002 M 8-hydroxyquinoline for 4 h. Roots were fixed in 3:1 ethanol-glacial acetic acid, hydrolyzed in 1 N HCl 60°C for 6 min, and stained with Feulgen solution. Squash preparation was made in acetocarmine.

Inter simple sequence repeat (ISSR) analysis

A diploid plant (P6) derived from anther culture was self-pollinated to produce the S1 progeny (6PS). The homogeneity of the 6PS strain was examined by ISSR analysis (Pradeep Reddy et al. 2002). Total DNAs were extracted from leaves by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). ISSR amplification reactions were carried out in a 20-μl volume containing 10 ng template DNA, 200 μM dNTP, 0.5 U *Taq* DNA polymerase (Takara Bio Inc.), and 0.15 μM primer in 1× reaction buffer containing 10 mM Tris–HCl (pH 9.0),

50 mM KCl, and 1.5 mM $MgCl_2$. The primers used in this analysis were as follows: $(TC)_8A$, $(AG)_8T$, $(GT)_8C$, and $(CA)_8G$. Amplification conditions involved initial denaturation at 94°C for 1 min, and 42 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, followed by 2 min at 72°C. Polymerase chain reaction (PCR) products were electrophoresed on 2% agarose gels and detected by staining with ethidium bromide.

Results

Embryogenesis and plant regeneration from anther cultures

After 2–4 months of culture, embryos could be observed emerging from the yellowish and/or brownish anthers (Fig. 1a). These embryos included various developmental stages from cotyledonary to globular embryos. The embryos at torpedo or cotyledonary stages regenerated to plantlets after being transferred to the regeneration medium (Fig. 1b, c). On the other hand, globular and heart-shaped embryos, which often showed abnormality, failed to regenerate to plantlets after being transferred to the regeneration medium. However, these abnormal embryos were successfully regenerated when they were transferred to a regeneration medium supplemented with GA_3. After acclimatization, the regenerants developed normal plants and flowered in soil (Fig. 1d).

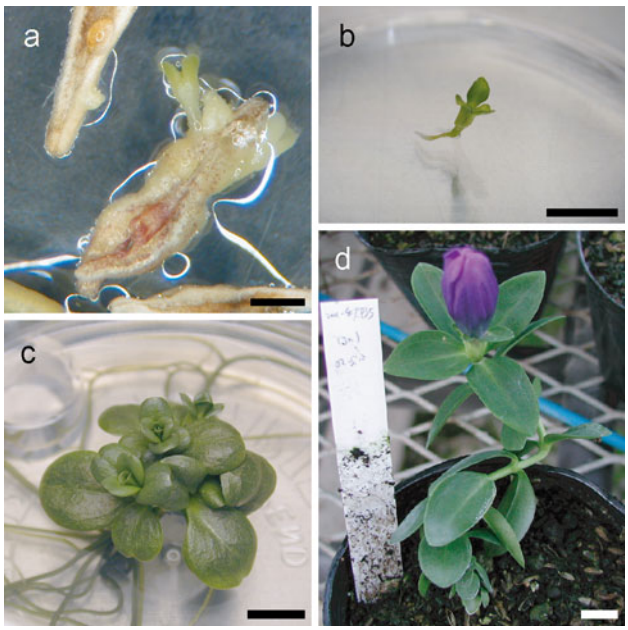

Fig. 1a–d Embryogenesis and plant regeneration in anther culture of gentian (*Gentiana triflora*) cv. Ashiro-no-Aki. **a** Embryo emerged from anther after 2 months of culture. **b** Germination from embryo in regeneration medium. **c** A plantlet regenerated from embryo after 5 months of culture. **d** Regenerated plant grown in soil. The *bars* in **a** = 1 mm, **b–d** = 10 mm

The effects of genotypes and bud sizes on embryogenesis from anther culture are shown in Table 1. Of the three genotypes used, two cvs., Ashiro-no-Aki and Ashiro-no-Natsu, produced the embryos, but Lovely-Ashiro did not produce any embryos. Tukey–Kramer's test showed that a significantly higher yield of embryos was obtained in Ashiro-no-Aki (19.8 embryos per 100 anthers) compared to Ashiro-no-Natsu (0.3 embryos) and Lovely-Ashiro (0 embryos) ($P < 0.05$). The size of the buds from which anthers were excised influenced the embryogenesis. Cytological observation with DAPI showed that buds 7 mm in length included tetrads, buds 8 mm in length included uninucleate microspores, buds 9–15 mm in length included uninucleate and binucleate microspores/pollens with various frequencies, and buds 16 mm in length included binucleate pollens (data not shown). The anthers from all buds between 9 and 13 mm in length produced embryos with a frequency from 2.4 to 40.0 (number of embryos per 100 anthers) in Ashiro-no-Aki. Although the buds 11 mm in length produced an embryo in Ashiro-no-Natsu, the embryos were obtained from the buds 10–12 mm in length in other experiments.

In order to clarify the optimum culture conditions of gentians, several other factors influencing androgenesis were examined. Of these investigations, a significantly higher embryo yield (19.0 embryos per 100 anthers) was obtained when the anthers of Ashiro-no-Natsu were cultured in solidified medium compared to liquid medium (Table 2). However, these embryos could not develop into plantlets because of their vitrification. On the other hand, since stable and significant effects of other factors on embryogenesis were not obtained from each experiment (data not shown), the results obtained from all tests are gathered and summarized in Table 3. The summarized data shows that the genotype had an important effect on the embryo yield of gentian. Among the three genotypes, Ashiro-no-Aki was the most responsive (21.3 embryos per 100 anthers), followed by Ashiro-no-Natsu, which produced a lower number of embryos (3.7 embryos), whereas Lovely-Ashiro showed no response.

After a total of 427 embryos of both Ashiro-no-Aki and Ashiro-no-Natsu were subcultured to the regeneration medium, 42.2 and 8.9% of the embryos regenerated into plantlets in Ashiro-no-Aki and Ashiro-no-Natsu, respectively. As described above, the lower frequency of regeneration in Ashiro-no-Natsu was due to the vitrification of the embryos.

Ploidy level of regenerated plants

The ploidy levels of a total of 105 regenerated plantlets were determined by flow cytometry (Table 4; Fig. 2). Of

Table 1 Effects of genotype and bud size on embryo production in anther culture of gentian

Cultivar	Length of flower bud (mm)	No. of cultured anthers	No. of embryos obtained	No. of embryos per 100 anthers ± SE
Ashiro-no-Aki	9	65	22	33.8 ± 21.3
	10	35	15	42.8 ± 31.2
	11	85	30	35.3 ± 22.9
	12	65	26	40.0 ± 31.5
	13	110	6	5.5 ± 2.4
	14	85	0	0
	15	55	0	0
Ashiro-no-Natsu	9	50	0	0
	10	45	0	0
	11	45	1	2.2 ± 2.2
	12	50	0	0
	13	40	0	0
	14	45	0	0
	15	50	0	0
Lovely-Ashiro	9	30	0	0
	10	20	0	0
	11	40	0	0
	12	55	0	0
	13	30	0	0

Table 2 Effects of medium condition on embryo production in anther culture of gentian cv. Ashiro-no-Natsu

Medium	No. of cultured anthers	No. of embryos obtained	No. of embryos per 100 anthers ± SE*
Liquid	595	1	0.2 ± 0.2b
Solid	605	115	19.0 ± 7.1a

*Mean values with different letters are significantly different using Tukey–Kramer's test ($P < 0.05$)

Table 3 Overall embryo production and plant regeneration from anther culture of three gentian cultivars

Cultivar	No. of cultured anthers	No. of embryos obtained	No. of embryos per 100 anthers	No. of regenerated plants (%)
Ashiro-no-Aki	1,420	303	21.3	128 (42.2)
Ashiro-no-Natsu	3,325	124	3.7	11 (8.9)
Lovely-Ashiro	675	0	0	–

98 plants of Ashiro-no-Aki, the majority (74.5%) of plants were triploid, with 5.1% being haploid and 20.4% diploid. Of seven plants of Ashiro-no-Natsu, six were diploid, and one was triploid. The chromosome number was also observed in the plants determined by flow cytometry analysis. *G. triflora* has a number of 2n = 26 chromosomes. The same chromosome number was confirmed in the diploid plants (Fig. 2). The chromosome number of the haploid (2n = 13) and the triploid (2n = 39) plants also corresponded to the results of the flow cytometric analysis (Fig. 2).

Table 4 Determination of ploidy level of plantlets derived from anther culture of gentian

Cultivar	No. of tested plants	No. of plants (%)		
		n	2n	3n
Ashiro-no-Aki	98	5 (5.1)	20 (20.4)	73 (74.5)
Ashiro-no-Natsu	7	0	6 (85.7)	1 (14.3)

ISSR analysis

In order to confirm whether the diploid plant derived from anther culture is the doubled haploid, ISSR analysis was carried out using the 6PS strain obtained from the self-pollination of the diploid plant (P6). Of four ISSR primers, $(AG)_8T$, $(GT)_8C$, $(TC)_8A$, and $(CA)_8G$, three of them showed polymorphic fragments in the donor plants of Ashiro-no-Aki (Fig. 3). When these three primers were used, ten plants selected randomly from the 6PS strain exhibited no polymorphic fragments (Fig. 3). In addition, the 6PS population grown in the field showed morphological and

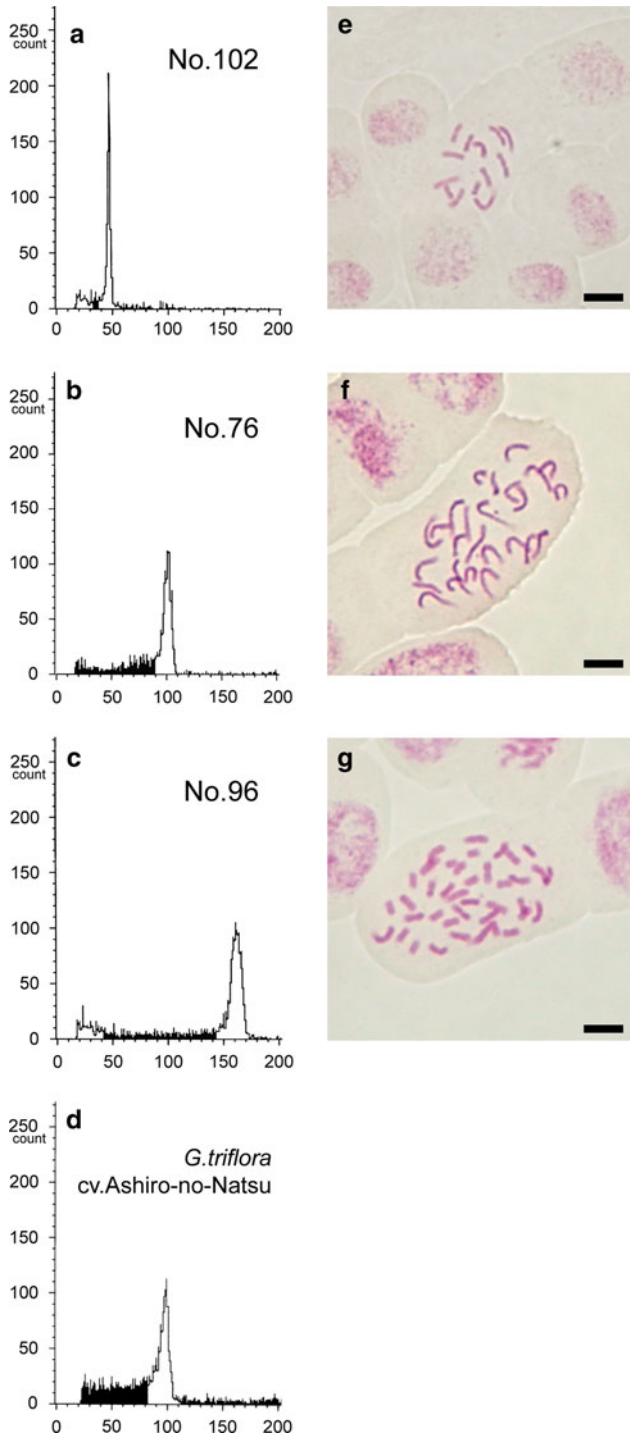

Fig. 2 Determination of the ploidy level of regenerated plants by flow cytometric analysis (**a**–**d**) and chromosome counting (**e**–**g**). **a** and **e** No. 102 plant shows haploid histogram and 2n = x = 13. **b** and **f** No. 76 plant shows diploid histogram and 2n = 2x = 26. **c** and **g** No. 96 plant shows triploid histogram and 2n = 3x = 39. **d** Histogram of cv. Ashiro-no-Natsu used as the diploid control. The *bars* = 10 μm

physiological identity (data not shown). These results revealed that the diploid plant obtained from anther culture of gentian is DH.

Discussion

The production of homozygous inbred lines is essential for breeding the F1 hybrid variety of gentian. However, homozygous inbred lines of gentians have not been obtained until now because of the serious inbreeding depression and difficulty of DH methods. The present study is the first report describing the successful production of DH lines of gentian (*G. triflora*) via anther culture.

Genotypic variation in embryo production was observed. Both responsive genotypes, Ashiro-no-Aki and Ashiro-no-Natsu, are F1 cultivars of *G. triflora*, whereas Lovely-Ashiro, which was not a responsive genotype, is a hybrid between *G. triflora* and unknown parents. From the single-nucleotide polymorphism (SNP) analysis of Lovely-Ashiro, it is speculated that this cultivar includes the *G. scabra* genome (personal communication from Dr. Tsutsumi). Our preliminary experiment using cultivars of *G. scabra* showed that the embryogenesis from anther culture of *G. scabra* was recalcitrant (data not shown). These results indicated that *G. triflora* is more responsive to androgenesis than *G. scabra*. Such an influence of the donor plant genotype on androgenesis has been reported in many species (Sopory and Munshi 1996; Zhang and Takahata 2001).

Bud size, which has been generally used as an index for judging microspore development, influenced the embryo production in gentians. The higher embryogenesis was obtained in buds 9–12 mm in length. Observation using DAPI staining showed that buds 9–15 mm in length included microspores from uninucleate to binucleate stages. Microspores from the late uninucleate to early binucleate stage are reported to be the most responsive in many species (Ferrie et al. 1995; Seguí-Simarro and Nuez 2008). Although our observation did not determine the relationships between bud size and the detailed developmental stages of microspores, it is speculated that buds 9–12 mm in length corresponded to microspores from the late uninucleate to early binucleate stages, and these stages are also responsive in gentians.

In addition to the genotype and developmental stage of microspores, various factors influence androgenesis, including the growing conditions of the donor plants, the pretreatment of anthers, the culture media, and culture conditions (Takahata 1997, Seguí-Simarro and Nuez 2008). Although a number of factors were investigated in this study, we could not find stable and effective conditions enhancing embryogenesis. For example, the effects of plant growth regulators NAA 2.0 and 0.5 mg/l kinetin in anther culture of gentian (Maruta and Matsumoto 1989) and/or of cold pretreatment of anthers before culture in many species (Sopory and Munshi 1996) have been reported. However, our results did not show clear effects of such plant growth

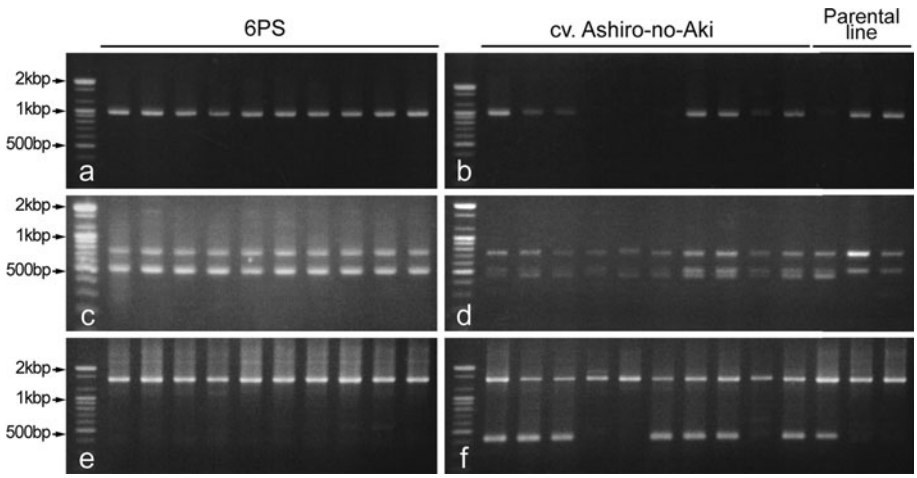

Fig. 3a–f Inter simple sequence repeat (ISSR) profiles in 6PS strains, cv. Ashiro-no-Aki, and the parental line of Ashiro-no-Aki using the ISSR primers, $(TC)_8A$ (**a** and **b**), $(AG)_8T$ (**c** and **d**), and $(CA)_8G$ (**e** and **f**)

regulators and cold pretreatment (data not shown). On the other hand, solidified medium was more effective on embryogenesis than liquid medium, though most embryos could not regenerate plantlets due to their vitrification. Our results contradicted those of Ferrie et al. (1995), who indicated that the flotation of anthers on liquid is generally more effective than plating on solid medium. More detailed studies are needed to develop a more efficient anther culture system in gentians.

In this study, more than 100 plants were obtained from anther culture of gentian. These regenerated plants consisted of haploids, diploids, and triploids. In particular, triploids reached a level of 70%. In addition to haploids, the production of diploids and polyploids has been reported in microspore-derived plants of many species, and such diploidization and polyploidization is considered to be caused by nuclear fusion, endomitosis, endoreduplication, and functioning of unreduced microspores (Keller et al. 1987; Rao and Suprasanna 1996; Shim et al. 2006). In addition, species and/or genotype are also known to influence such phenomenon, as shown in our results. Of regenerated plants, 20% in Ashiro-no-Aki and 86% in Ashiro-no-Natsu were diploid. If these diploid plants are homozygous, such spontaneous diploidization would be advantageous for haploid breeding, as it omits the need for doubling treatment. ISSR analysis using the 6PS strain obtained by the self-pollination of the diploid plant from anther culture of Ashiro-no-Aki revealed that the diploid plant is DH. This is supported by the allelic test of the *W14* and *W15* genes, which are allelic and code the proteins related to the α/β hydrolase fold superfamily (Hikage et al. 2007). They reported that a donor cultivar Ashiro-no-Aki carried both alleles *W14*/*W15*, whereas 6PS plants carried homozygous *W15* (*W15*/*W15*).

In this study, many DH plants were established in gentian (*G. triflora*). Additionally, a part of the DH plants could produce S1 generation by self-pollination. Not only breeding programs and genetic studies using DH lines obtained in this study, but also the establishment of more effective DH methods in gentian, including *G. scabra*, are currently being carried out.

Acknowledgments We greatly appreciate Dr. J. Soejima of the Apple Research Station, National Institute of Fruit Tree Science, Japan, for his help in providing the PA-I flow cytometer (Partec).

References

Bajaj YPS (1990) In vitro production of haploids and their use in cell genetics and plant breeding. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 12. Springer-Verlag, Berlin, pp 3–44

Ferrie AMR, Palmer CE, Keller WA (1995) Haploid embryogenesis. In: Thorpe TA (ed) In vitro embryogenesis in plants. Kluwer Academic Publishers, Dordrecht, pp 309–344

Forster BP, Heberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12:368–375

Hikage T, Saitoh Y, Tanaka-Saito C, Hagami H, Satou F, Shimotai Y, Nakano Y, Takahashi M, Takahata Y, Tsutsumi K (2007) Structure and allele-specific expression variation of novel α/β hydrolase fold proteins in gentian plants. Mol Genet Genomics 278:95–104

Hosokawa K, Nakano M, Oikawa Y, Yamamura S (1996) Adventitious shoot regeneration from leaf, stem and root explants of commercial cultivars of *Gentiana*. Plant Cell Rep 15:578–581

Huang B, Keller WA (1989) Microspore culture technology. J Tissue Cult Methods 12:171–178

Keller WA, Arnison PG, Cardy BJ (1987) Haploids from gametophytic cells: recent developments and future prospects. In: Green CE, Somers DA, Hackett WP, Biesboer DD (eds) Plant tissue and cell culture. Alan R. Liss, New York, pp 223–241

Khush GS, Virmani SS (1996) Haploids in plant breeding. In: Mohan Jain S, Sopory SK, Veilleux RE (eds) In vitro haploid production in higher plants, vol 1. Kluwer Academic Publishers, Dordrecht, pp 11–33

Kohlein F (1991) Gentians. Christopher Helm, London, p 183

Maluszynski M, Kasha K, Szarejko I (2003) Published doubled haploid protocols in plant species. In: Maluszynski M, Kasha K, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants: a manual. Kluwer Academic Publishers, Dordrecht, pp 309–335

Maruta I, Matsumoto E (1989) Induction of haploid plants from anther culture in *Gentiana* sp. Bull Nagano Veg Ornam Crops Exp Sta Japan 5:51–55 (in Japanese)

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

Nishihara M, Nakatsuka T, Mizutani-Fukuchi M, Tanaka Y, Yamamura S (2008) Gentians: from gene cloning to molecular breeding. In: Teixeira da Silva JA (ed) Floriculture, ornamental and plant biotechnology V. Global Science Books, London, pp 57–67

Poehlman JM, Sleper DA (1995) Breeding field crops. Iowa State University Press, Ames, pp 200–215

Pradeep Reddy M, Sarla N, Siddiq EA (2002) Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128:9–17

Rao PS, Suprasanna P (1996) Methods to double haploid chromosome number. In: Mohan Jain S, Sopory SK, Veilleux RE (eds) In vitro haploid production in higher plants, vol 1. Kluwer Academic Publishers, Dordrecht, pp 317–339

Seguí-Simarro JM, Nuez F (2008) How microspores transform into haploid embryos: changes associated with embryogenesis induction and microspore-derived embryogenesis. Physiol Plant 134:1–12

Shim YS, Kasha KJ, Simion E, Letarte J (2006) The relationship between induction of embryogenesis and chromosome doubling in microspore cultures. Protoplasma 228:79–86

Skrzypczak L, Wesilowska M, Skrzypczak E (1993) *Gentiana* species: in vitro culture, regeneration, and production of secoiridoid glucosides. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 21. Medical and aromatic plants IV. Springer, Berlin, pp 172–186

Sopory SK, Munshi M (1996) Anther culture. In: Mohan Jain S, Sopory SK, Veilleux RE (eds) In vitro haploid production in higher plants, vol 1. Kluwer Academic Publishers, Dordrecht, pp 145–176

Tada T, Hoshi N, Nakatani F (2000) Adventitious shoot induction and plant regeneration from leaf explant of *Gentiana triflora*. Breed Res 2(Suppl 1):183 (in Japanese)

Takahashi T (1997) Development of gentian cultivars for valuable characters in Iwate prefecture. Tohoku Agric Res Extra Issue 10:65–73 (in Japanese)

Takahata Y (1997) Microspore culture. In: Kalia HR, Gupta SK (eds) Recent advances in oilseed brassicas. Kalyani Publishers, New Delhi, pp 162–181

Takahata Y, Jomori H, Miyano S, Kunitake H, Mii M (1995) Regeneration of plants from protoplasts of *Gentiana* species (Gentian). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 34. Plant protoplasts and genetic engineering VI. Springer, Berlin, pp 55–62

Thomas WTB, Forster BP, Gertsson B (2003) Doubled haploids in breeding. In: Maluszynski M, Kasha K, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants: a manual. Kluwer Academic Publishers, Dordrecht, pp 337–349

Yoshiike T (1992) Rindou (*Gentiana*). Seibundo Shinkosha, Tokyo, p 177 (in Japanese)

Zhang FL, Takahata Y (2001) Inheritance of microspore embryogenic ability in *Brassica* crops. Theor Appl Genet 103:254–258