Plant Cell Tiss Organ Cult (2017) 131:1–14
DOI 10.1007/s11240-017-1256-x

ORIGINAL ARTICLE

Protoplast fusion in the genus *Gentiana*: genomic composition and genetic stability of somatic hybrids between *Gentiana kurroo* Royle and *G. cruciata* L.

Karolina Tomiczak[1] · Elwira Sliwinska[2] · Jan J. Rybczyński[1]

Received: 13 April 2017 / Accepted: 12 June 2017 / Published online: 17 June 2017

Abstract Somatic hybridization by protoplast fusion is used in breeding programs to obtain plant material that has inherited valuable traits from two different species, and in order to broaden plant genetic diversity. Somatic hybrids of the genus *Gentiana* could provide a useful source of new ornamental cultivars and of secondary metabolites. However, in order to evaluate its further usefulness, detailed characterization of the newly created hybrid is essential. Here, genome composition and stability of interspecific gentian somatic hybrids obtained following electrofusion of cell suspension-derived protoplasts of diploid *Gentiana kurroo* Royle with leaf mesophyll-derived protoplasts of tetraploid *G. cruciata* L. were characterized using various molecular markers and flow cytometry. AFLP and ISSR analyses revealed that all 21 hybrid plants and 3 lines of hybrid callus were genetically closer to *G. cruciata* than to *G. kurroo*. According to the results of chloroplast DNA analysis with the use of CAPS markers, all somatic hybrids inherited chloroplasts from "mesophyll" fusion partner *G. cruciata*. Flow cytometry revealed that polyploidization occurred, and probably it took place at the early stage of post-fusion culture. In consequence, gradual elimination of nuclear DNA, mixoploidy, and high genetic instability were observed in most hybrid plants and calli during the subsequent 4 years of in vitro culture.

Keywords AFLP · CAPS · Nuclear DNA content · Gentian · ISSR · Somatic hybridization

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
AFLP	Amplified fragment length polymorphism
BAP	6-Benzylaminopurine
CAPS	Cleaved amplified polymorphic sequence
cpDNA	Chloroplast DNA
DArT	Diversity arrays technology
ISSR	Inter-simple sequence repeat
NAA	α-Naphthaleneacetic acid
RAPD	Random amplified polymorphic DNA
SNP	Single nucleotide polymorphism
SSRs	Simple sequence repeats
TDZ	Thidiazuron (phenyl-*N*′-(1,2,3-thiadiazol-5-yl) urea)

Introduction

Somatic hybridization by protoplast fusion offers several possibilities for increasing genetic variability in higher plants. It is used to overcome sexual incompatibility between species and may lead to the production of novel genetic combinations as a consequence of recombination within either nuclear or cytoplasmic genomes. In a breeding program, somatic cell fusion is usually used for

Communicated by: M. I. Beruto.

Electronic supplementary material The online version of this article (doi:10.1007/s11240-017-1256-x) contains supplementary material, which is available to authorized users.

✉ Karolina Tomiczak
ktomiczak@obpan.pl

[1] Department of Plant Experimental Biology, Polish Academy of Sciences Botanical Garden, Center for Biological Diversity in Powsin, Prawdziwka 2, 02-973 Warsaw, Poland

[2] Laboratory of Molecular Biology and Cytometry, Department of Plant Genetics, Physiology and Biotechnology, UTP University of Science and Technology, Kaliskiego Ave. 7, 85-789 Bydgoszcz, Poland

transferring of polygenic traits and those encoded by organellar genomes (Waara and Glimelius 1995). In the family Gentianaceae, which contains many valuable medicinal and ornamental species, somatic hybrids representing different nucleocytoplasmic combinations would be very useful as new horticultural cultivars and as a valuable source of secondary metabolites (Wang et al. 2011; Tomiczak et al. 2015b).

In contrast to sexual hybridization, following protoplast fusion, all nuclear and cytoplasmic DNA from both parents are united in one individual. However, regeneration of plants from fused protoplasts is often accompanied by polyploidization and/or the elimination of genome parts of either one or both fusion parents (Babiychuk et al. 1992; Oberwalder et al. 1998; Mizuhiro et al. 2001; Guo et al. 2010). Thus, detailed investigation is needed, not only to confirm the hybrid status of regenerants, but also to characterize precisely their genome size and composition, which is essential for their further efficient exploitation (Smyda-Dajmund et al. 2016).

For over 20 years, we have studied the morphogenic abilities of various gentian species in vitro, aimed at conserving and broadening their genetic diversity (Mikuła et al. 1996, 2011; Mikuła and Rybczyński 2001; Fiuk and Rybczyński 2008; Tomiczak et al. 2016). The results of our experiments revealed the tremendous embryogenic potential possessed by *Gentiana kurroo* Royle. This species is an endangered medicinal herb from western and northwestern Himalaya, which has been overexploited in its natural habitats owing to the extensive collection of its leaves, roots, rhizomes, and flower heads, which are highly valued in traditional Indian medicine (Latif et al. 2006; Behera and Raina 2012; Baba and Malik 2014). The embryogenic potential of *G. kurroo* is particularly obvious in cell suspension cultures (Fiuk and Rybczyński 2008) and in cultures of cell suspension-derived protoplasts (Fiuk and Rybczyński 2007), which provide the basis for successful cryopreservation of this species (Mikuła et al. 2011a, b), its genetic transformation (Wójcik and Rybczyński 2015), and its somatic hybridization (Tomiczak et al. 2015b).

In order to determine whether protoplast fusion is a feasible tool for extending the genetic diversity of Gentianaceae, we conducted 28 independent experiments on protoplast fusion between cell suspension-derived protoplasts of *G. kurroo* and leaf mesophyll-derived protoplasts of *Gentiana cruciata* L. and *Gentiana tibetica* King. The last two are tetraploid species of medicinal and ornamental importance (Köhlein 1991) that are easy to cultivate. Although the results of studies conducted so far have indicated, that *G. cruciata* is recalcitrant in cultures of leaf mesophyll-derived protoplasts (Tomiczak et al. 2016), we managed to obtain three somatic hybrid callus lines following electrofusion of their protoplasts with those isolated from cotyledon-derived cell suspension of *G. kurroo* and thereby, regenerated a total of 87 plants via somatic embryogenesis from two of these (Tomiczak et al. 2015b). The aim of the work presented here was to characterize the genomic composition and genetic stability of selected somatic hybrids between *G. kurroo* and *G. cruciata*.

Material and method

Plant material

Interspecific somatic hybrids between *G. kurroo* Royle and *G. cruciata* L., namely three lines of callus (F12A, F12B, and F12C) and a total of 23 plants regenerated via somatic embryogenesis from the first two callus lines (12 regenerants from callus F12A, and 11 regenerants from callus F12B) were used in the experiments (Fig. 1). All calli and plants were obtained following electrofusion of protoplasts isolated from 4-year-old highly embryogenic and cytogenetically stable cotyledon-derived cell suspension culture (K/C) of diploid *G. kurroo* ($2n=2x=26$; Fiuk and Rybczyński 2007) and protoplasts released from leaf mesophyll of in vitro-grown plants of tetraploid *G. cruciata* ($2n=4x=52$; Tomiczak et al. 2016). Their hybrid nature was previously confirmed (Tomiczak et al. 2015b). All somatic hybrid plants were cultured in glass jars on MS medium (Murashige and Skoog 1962) supplemented with 0.5 mg l^{-1} BAP, 0.1 mg l^{-1} NAA, 30 g l^{-1} sucrose, and 8 g l^{-1} agar, and maintained in a phytotron at 21 ± 1 °C with 16-h illumination of 100 µM m^{-2} s^{-1} provided by daylight fluorescent tubes. Plants were subcultured to new medium every 4–6 months.

Three hybrid callus lines, each derived from independent fusion event, were divided after a 2-month-long period of proliferation and cultured simultaneously in darkness at 21 ± 1 °C, on Petri dishes with MS medium containing 30 g l^{-1} sucrose, 8 g l^{-1} agar, and appropriate plant growth regulators. Callus clones marked K_1 were cultured on medium supplemented with 0.5 mg l^{-1} 2,4-D and 1.0 mg l^{-1} kinetin; clones marked K_2 were kept on medium containing 2.0 mg l^{-1} NAA and 0.2 mg l^{-1} TDZ; whereas clones marked K_3 were grown on medium with the addition of 1.0 mg l^{-1} dicamba, 0.1 mg l^{-1} NAA, 2.0 mg l^{-1} BAP, and 80 mg l^{-1} adenine sulfate. All calli were subcultured to new medium every 2–3 months.

Fusion partners, i.e. embryogenic cell suspension of *G. kurroo* and seed-derived in vitro-grown plants of *G. kurroo* and *G. cruciata* constituted the reference plant material in all experiments. Cell suspension of *G. kurroo* was cultured in 250-ml conical flask with 80 ml liquid MS medium containing 30 g l^{-1} sucrose, 1.0 mg l^{-1} DIC, 0.1 mg l^{-1} NAA, 2.0 mg l^{-1} BAP, 80 mg l^{-1} adenine sulfate. The culture was

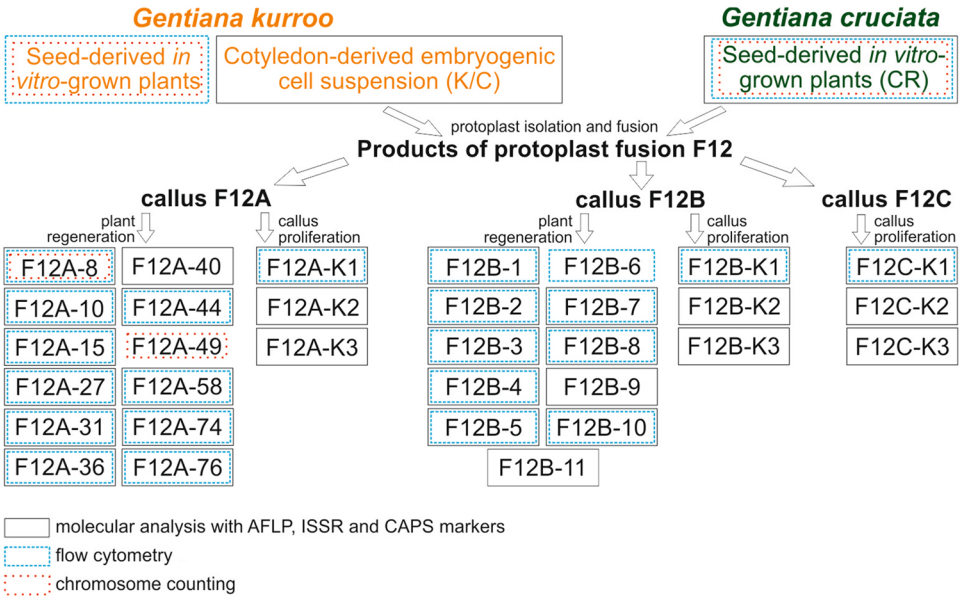

Fig. 1 Scheme showing how plant material (somatic hybrids and parental species) was used for particular molecular, cytometric and cytological analyses

incubated on a rotary shaker at 120 rpm under diffuse light conditions (16-h illumination of 20 µM m^{-2} s^{-1}) and at a temperature of 21 ± 1 °C. Subcultures were set up every 7 days. Plants of *G. kurroo* and *G. cruciata* were cultured in glass jars on MS medium supplemented with 30 g l^{-1} sucrose and 8 g l^{-1} agar, and maintained under the same conditions as hybrid plants.

DNA extraction

Total genomic DNA for all molecular analysis was extracted from six tissue samples of cell suspension of *G. kurroo*, leaves of 6 parental plants of *G. cruciata*, three lines of hybrid callus (clones K_1, K_2 and K_3), 11 hybrid plants regenerated from callus F12A and ten plants regenerated from callus F12B (Fig. 1) using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The quantity of DNA was determined by means of a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The quality of DNA was assessed on 1.0% agarose gel.

AFLP and ISSR analysis

AFLP analysis was performed according to Vos et al. (1995) with minor modifications. DNA digestion with *Eco*RI and *Mse*I restriction enzymes was followed by adaptors ligation and pre-selective PCR reaction. For selective PCR reaction, ten primer combinations were used (Table 1). Products of amplification were analyzed on 6% polyacrylamide gels and visualized by autoradiography.

For ISSR analysis, ten primers of 17 tested, producing clear and reproducible banding patterns, were selected (Table 2). PCR amplifications were performed in a volume of 10 µl 1× Complete NH_4 Reaction Buffer containing 2.5 mM $MgCl_2$ (BIORON GmbH, Ludwigshafen, Germany), 0.2 mM dNTPs, 1.0 µM of primer, 0.5 U SuperHotTaq DNA polymerase (BIORON GmbH, Ludwigshafen, Germany), and 20 ng of template DNA. Initial denaturation at 94 °C for 4 min, was followed by 35 cycles of 1 min denaturation at 94 °C, 30 s annealing at 52–59 °C (depending on the primer) and 1 min 30 s extension at 72 °C, ending in 10 min final extension at 72 °C. The amplified products were separated on a 1.4% (m/v) agarose gel, and bands were detected by ethidium bromide staining.

Table 1 AFLP primers used in selective PCR reactions

No of primer pair	Eco/Mse primers	No of bands	No of polymorphic bands	% polymorphism
I	E-ACG/M-CGC	30	20	66.7
II	E-AGC/M-CAC	45	23	51.1
III	E-AGG/M-CTG	41	25	61.0
IV	E-ACT/M-CCC	40	21	52.5
V	E-ATG/M-CGA	29	11	37.9
VI	E-AAA/M-CCG	37	17	45.9
VII	E-ATC/M-CAA	68	41	60.3
VIII	E-AGA/M-CAG	55	36	65.4
IX	E-ACC/M-CGT	28	14	50.0
X	E-AGT/M-CTC	70	49	70.0
		443	257	58.0

Table 2 Sequences of primers used for ISSR analysis

Primer code	Sequence 5′−>3′	No of bands	No of polymorphic bands	% polymorphism
UBC-818	$(CA)_8G$	15	12	80.0
UBC-835	$(AG)_8YC$	23	14	60.9
UBC-840	$(GA)_8YT$	15	9	60.0
UBC-845	$(CT)_8RG$	23	18	78.3
UBC-846	$(CA)_8RT$	12	8	66.7
UBC-880	$(GGAGA)_3$	14	9	64.3
SBS-808	$(AG)_8C$	12	8	66.7
IS-1	$(AGAC)_4$	14	7	50.0
IS-2	$(GAC)_4RC$	18	9	50.0
IS-813	$(CT)_8T$	5	5	100.0
		151	99	65.6

DNA fingerprinting analysis

The AFLP and ISSR patterns were recorded as 0–1 binary matrices, where "1" indicated the presence and "0" the absence of a given fragment. Measures of genetic uniformity among regenerants and callus samples were determined using the Jaccard similarity coefficient. Excel add-in software XlStat v.2009.2.01 was used to draw the dendrograms (UPGMA, Jaccard). In order to determine the nuclear genome composition of *G. kurroo* (+) *G. cruciata* somatic hybrids, preserved, deleted, and unique markers were described. Preserved markers were markers present in *G. kurroo* or *G. cruciata* parental genome and present also in somatic hybrid genome. Deleted markers were present in one of the parental forms, but absent in the somatic hybrid genome. Unique markers were those absent in *G. kurroo* and *G. cruciata* parental genome, but present in the genome of the somatic hybrid.

cpDNA analysis with CAPS markers

In order to detect polymorphism between the chloroplast genomes of the fusion partners and to analyze the inheritance of chloroplasts by somatic hybrids, PCR amplification of cpDNA regions followed by its restriction digestion was used. Two intergenic cpDNA regions (*trnS-trnG* and *atpB-rbcL*) were amplified with primer pairs designed using Primer3 software (Rozen and Skaletsky 1999; Table 3) on the basis of NCBI data base sequences available for gentians. The PCR reactions were conducted in a Biometra T-Gradient thermocycler in reaction mixtures (15 µl) consisting of 1×reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 µM of each primer, 2 U HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), and 15 ng of sample DNA. The amplification program consisted of polymerase activation step at 95 °C for 15 min, and 30 cycles of 30 s each (denaturing) at 94 °C, 40 s (annealing) at 53–55 °C, 40–60 s (elongation) at 72 °C; and one final cycle of 10 min at 72 °C. Products were separated on 1.5% agarose gels and visualized by staining with ethidium bromide. Amplicons consisting of one clear 400–1000 bp-long band were purified (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany) and sequenced at the DNA Sequencing and Oligonucleotide Synthesis Lab (Institute of Biochemistry and Biophysics PAS, Warsaw). The nucleotide sequences obtained (GenBank accession numbers KY566219-KY566223) were compared by means of BLAST program and mapped for differentiating restriction sites in NEBcutter V2.0 software (Vincze et al. 2003). Subsequently, restriction analyses of cpDNA regions of *G. kurroo, G. cruciata* and their somatic hybrids using in silico-selected endonucleases were performed. A 10 µl aliquots of PCR product was digested with an appropriate endonuclease under the conditions recommended by the manufacturer

Table 3 Primers used for analysis of inheritance of cpDNA

cpDNA region	Primer sequences	Average amplicon size	GenBank accession number
atpB-rbcL	atpBf: 5′-ACCAGAACCGGAAGTAGTCG-3′ rbcLr: 5′-TAGCGCAACCCAATTTTTCT-3′	470 bp	KY566219, KY566220
trnS-trnG	trnSf: 5′-TGGATATGTACACTTTTTAGCAGCA-3′ trnGr: 5′-ACTGGACCTTTTGTCGAACG-3′	470 bp	KY566222, KY566223

(New England Biolabs, Ipswich, USA) and separated on 1.8% agarose gels, followed by visualization by staining with ethidium bromide. Restriction endonuclease cleavage patterns generated for somatic hybrids were compared with those of parental species.

Flow cytometry and chromosome counting

Nuclear DNA content was determined in clones K_1 of three hybrid callus lines, ten hybrid plants regenerated from callus F12A and nine plants regenerated from callus F12B (Fig. 1). Average nuclear DNA content for *G. kurroo* and *G. cruciata* was estimated based on flow cytometric measurements of 24 and 19 seed-derived plants, respectively. Analyses were carried out using Partec CCA flow cytometer according to Tomiczak et al. (2016). *Pisum sativum* L. 'Set' (9.11 pg/2C; Sliwinska et al. 2005) served as an internal standard for the estimation of the nuclear DNA content of *G. kurroo* and all somatic hybrids. Since G_0/G_1 peaks of *G. cruciata* and *P. sativum* overlapped each other, *Petunia hybrida* 'PxPc6' (2.85 pg 2C; Marie and Brown 1993) was used as an internal standard for genome size estimation of *G. cruciata*. For sample preparation, young, fully developed leaves of plants or callus fragments were used. Data was evaluated using DPAC V.2.2 software (Partec, GmbH, Münster, Germany). The nuclear DNA content was calculated using the linear relationship between the ratio of the G_0/G_1 peak positions *Gentiana*/internal standard, on the histogram of fluorescence intensity.

Monitoring of the nuclear DNA content of hybrid calli lines and plants was carried out for 4 years. Data obtained after 1–2 and 3–4 years of culture were compared with those obtained within 2 months from callus proliferation/plant regeneration.

Chromosome numbers were determined for root tips of selected parental and somatic hybrid plants (Fig. 1) following the procedure described by Tomiczak et al. (2016).

Results

Morphology of somatic hybrid calli and plants and their viability in vitro

All somatic hybrid callus lines were viable for the 4-year-long period of in vitro culture. Steady cell proliferation was observed on all tested media. Conversely, somatic hybrid plants showed many changes and disturbances in their morphology during prolonged in vitro culture. Soon after regeneration, all plants displayed dwarf stature with short internodes, small, thick, round or oval leaves and failed to develop roots (Fig. 2a). After several months of culture, differences between selected regenerants and, on occasion, between their clones also became noticeable. While most F12A plants remained underdeveloped and only grew slowly, rapid growth of dark green, wide leaf blades was observed for some F12B plants (Fig. 2b, c). In the case of other F12B regenerants, long, narrow, and ridged leaves developed (Fig. 2d). However, only very few somatic hybrids produced roots. Many plants successively turned brown and finally died.

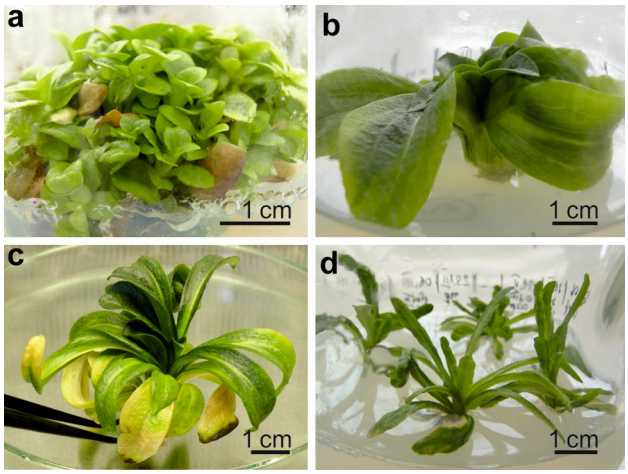

Fig. 2 Various morphology of somatic hybrids *G. kurroo* (+) *G. cruciata* in vitro: **a** dwarf hybrids F12A-10 with small, thick and round leaves, **b** hybrid F12B-2 with very short internodes and wide leaves, **c** strongly growing hybrid F12B-3 with slightly wrinkled *dark green* leaf blades, **d** hybrids F12B-4 with long, narrow, and ridged leaves. (Color figure online)

Nuclear DNA composition of somatic hybrids

AFLP and ISSR analysis revealed that all regenerants and calli tissues possessed specific bands from both parents, which indicated their hybrid nature (Figs. 3, 4). A total of 443 bands was obtained with the use of 10 AFLP selective primer pairs (from 29 to 70 per primer pair), 257 (58.0%) of which were polymorphic (Table 1). From 397 to 424 bands were identified within electrophoretic patterns of somatic hybrids, of which 58–68 (14.4–16.3%) were specific for *G. kurroo*, 145–163 (36.4–39.2%)—specific for *G. cruciata*, and 189–198 (45.7–47.9%)—common to both parental species. From 4 to 22 bands were recognized as deleted from *G. kurroo*, which comprised 5.1–27.8% of all bands common with *G. kurroo* and from 11 to 21 were deleted from *G. cruciata* which was 6.6–12.6% of bands common with this species (Online Resource 1).

With the use of 10 ISSR primers, a total of 151 bands was obtained (from 5 to 23 per primer pair), 99 (65.6%) of which were polymorphic (Table 2). From 125 to 132 bands were identified within electrophoretic patterns of somatic hybrids, of which 24–29 (19–22.1%) were specific for *G.*

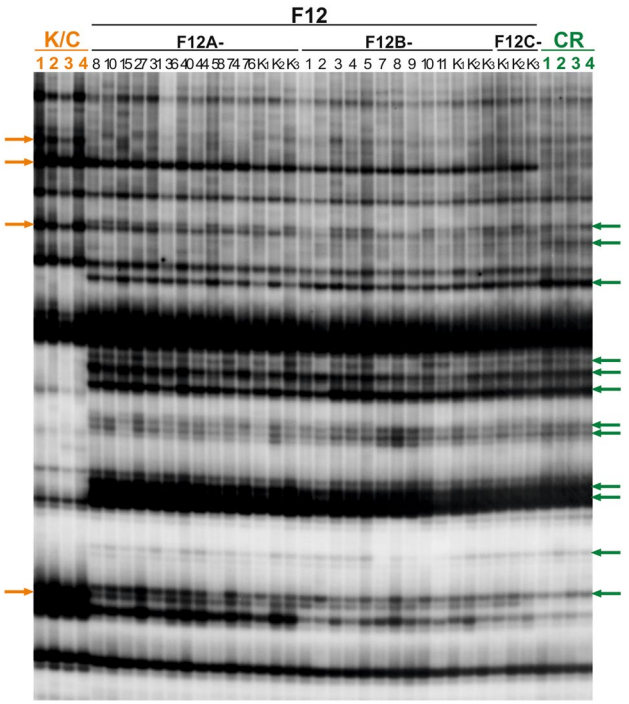

Fig. 3 AFLP electrophoretic pattern obtained for *G. kurroo, G. cruciata* and their somatic hybrids with the use of primer pair IV (E-ACT/M-CCC). *K/C G. kurroo* ("cell suspension" fusion partner), *CR G. cruciata* ("mesophyll" fusion partner), *F12A-F12C* products of three independent fusion events. *Symbols* K_1–K_3 represent particular clones of hybrid callus, cultured on three different media; *numbers 1–76* represent individual regenerants. *Orange arrows* indicate bands specific for "cell suspension" fusion partner; *green arrows* indicate bands specific for "mesophyll" fusion partner. (Color figure online)

kurroo, 47–48 (34.4–37.5%)—specific for *G. cruciata* and 189–198 (45.7–47.9%)—common to both parental species. Some hybrids also possessed 1 or 2 unique, individual bands, which were not observed in ISSR profiles of parental species. From 11 to 16 bands were recognized as deleted from *G. kurroo*, which comprised 27.5–40.0% of all bands common with *G. kurroo*. Also, from 5 to 9 bands were deleted from *G. cruciata* which was 9.4–17.0% of bands common with this species (Online Resource 2).

Combination of AFLP and ISSR data resulted in the recognition of a total of 594 bands, of which 518–544 were present in electrophoretic patterns of somatic hybrids. From 189 to 211 bands were inherited by hybrids from *G. cruciata*, which comprised 85.9–95.9% of all *G. cruciata* bands. From 17 to 29 (7.7–13.2%) bands common with this species were deleted. Consequently, 83–97 (69.7–81.5%) bands were preserved from *G. kurroo* and 19–35 (16.0–29.4%) were deleted (Fig. 5).

UPGMA dendrograms constructed on the basis of AFLP and ISSR analyses showed that Jaccard similarity coefficient for parental species was 0.58 and 0.52, respectively (Online Resources 3, 4). Both molecular markers revealed that all somatic hybrids were closer to *G. cruciata* than to *G. kurroo*. Furthermore, the similarity coefficient calculated for the whole group of somatic hybrids on the basis of both marker systems was comparable, comprising from 0.93 to 0.99 for AFLP and 0.93-1.0 for ISSR. However, hybrids were clustered differently: four and three clusters could be distinguished on the AFLP and ISSR dendrograms, respectively. A dendrogram based on combined AFLP and ISSR data gave a similarity coefficient of 0.56 for parental species. Particular somatic hybrids were clustered as follows: first cluster consisted of all F12B plants and all F12B and F12C calli; secondly—three lines of F12A callus; thirdly—most F12A plants; and fourthly—only the F12A-10 individual (Fig. 6).

Inheritance of chloroplast DNA

Amplification and sequencing of two cpDNA regions were successful for both *Gentiana* species (Table 3). BLAST analysis of *trnS-trnG* region sequences revealed two SNPs and one insertion/deletion polymorphism of a single

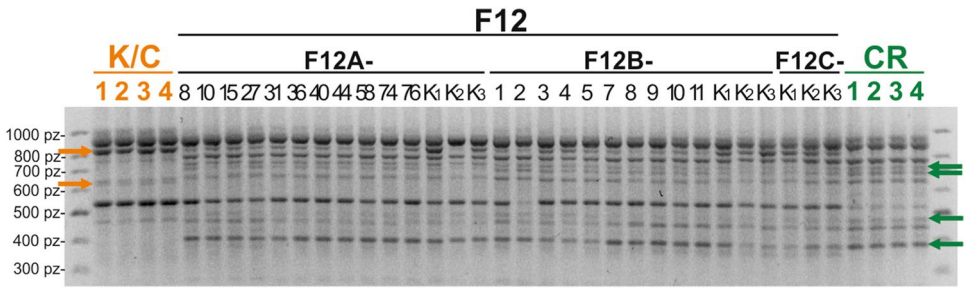

Fig. 4 ISSR electrophoretic pattern obtained for *G. kurroo, G. cruciata* and their somatic hybrids with the use of primer UBC-840. *K/C G. kurroo* ("cell suspension" fusion partner), *CR G. cruciata* ("mesophyll" fusion partner), *F12A-F12C* products of three independent fusion events, *M* DNA size marker (100-bp DNA ladder). *Symbols* K_1–K_3 represent particular clones of hybrid callus, cultured on three different media; *numbers 1–76* represent the individual regenerants. *Orange arrows* indicate bands specific for "cell suspension" fusion partner; *green arrows* indicate bands specific for "mesophyll" fusion partner. (Color figure online)

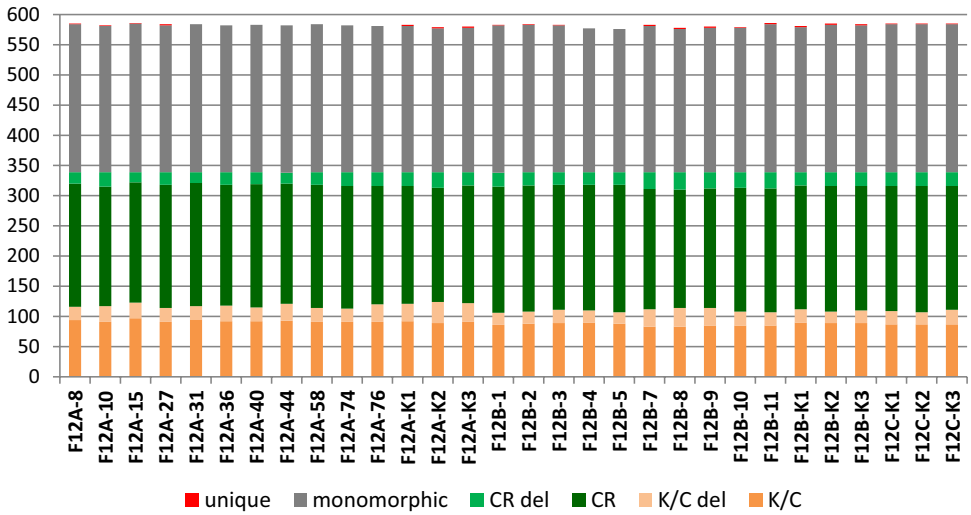

Fig. 5 Composition of all preserved, deleted and unique AFLP and ISSR markers, detected in somatic hybrids *G. kurroo* (+) *G. cruciata*. *K/C* preserved *G. kurroo* markers, *K/C del* deleted *G. kurroo* markers, *CR* preserved *G. cruciata* markers, *CR del* deleted *G. cruciata* markers

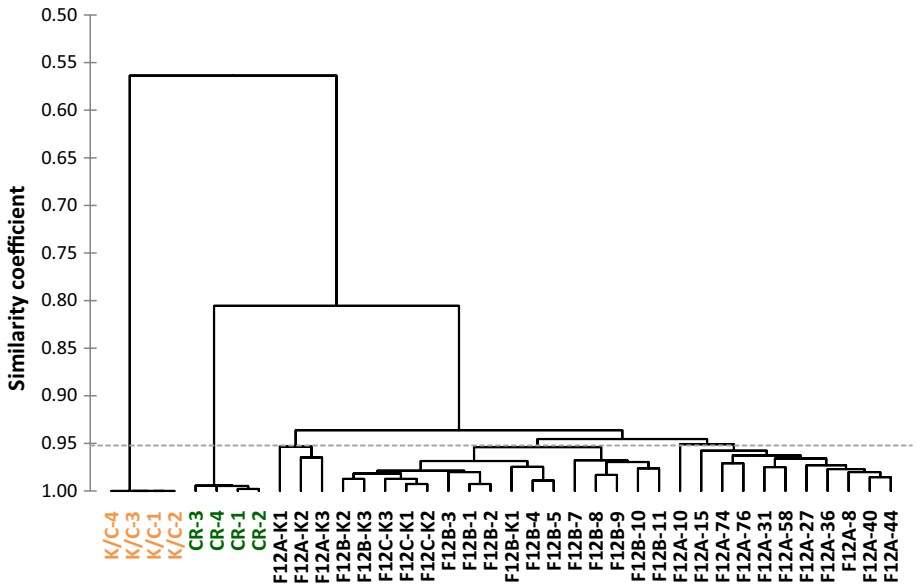

Fig. 6 Dendrogram of genetic similarity between *G. kurroo, G. cruciata* and their somatic hybrids, obtained by UPGMA cluster analysis based on combined AFLP and ISSR molecular markers. *K/C G. kurroo* ("cell suspension" fusion partner), *CR G. cruciata* ("mesophyll" fusion partner), *F12A-F12C* products of three independent fusion events

nucleotide. According to NEB cutter software, one of the SNPs created an additional restriction site for endonuclease *Bfa*I in a sequence of *G. cruciata*. Restriction analysis of *trnS-trnG* amplicons with *Bfa*I resulted in the generation of two 235 bp-long fragments for *G. kurroo* and 3 fragments some 235, 145 and 90 bp in length for *G. cruciata*. All somatic hybrid plants and calli exhibited the same restriction pattern as their mesophyll parent, i.e. *G. cruciata* (Fig. 7).

Nuclear DNA content and chromosome number

Flow cytometry revealed that *G. cruciata* possessed 8.07 ± 0.18 pg DNA/2C, and *G. kurroo* 3.32 ± 0.07 pg DNA/2C. Most somatic hybrids obtained initially from two calli contained 20–23 pg DNA/2C, which is close to or slightly less than twice the total DNA content for both parents (11.39 pg multiplied by 2 would be equal to 22.78 pg; Table 4). Only regenerant F12B-2 possessed significantly less, 16.40 pg DNA/2C. Even so, it was still much more than just the total for both parental species. In two regenerants, F12A-15 and F12B-1, an additional small fraction of nuclei containing much less than 20 pg DNA (15.26 and 12.39 pg, respectively) was detected, speaking for their mixoploidy. By contrast, in another mixoploid, F12B-5, most of the nuclei contained about 15 pg, although in some DNA content exceeded 20 pg.

After about 2 years of culture, essential reduction in nuclear DNA content was observed in most somatic hybrid plants and in the calli F12A-K_1 and F12C-K_1 (Table 4). Only in one of these, F12A-8, however, was it equal to the total of both parental genomes, and in others

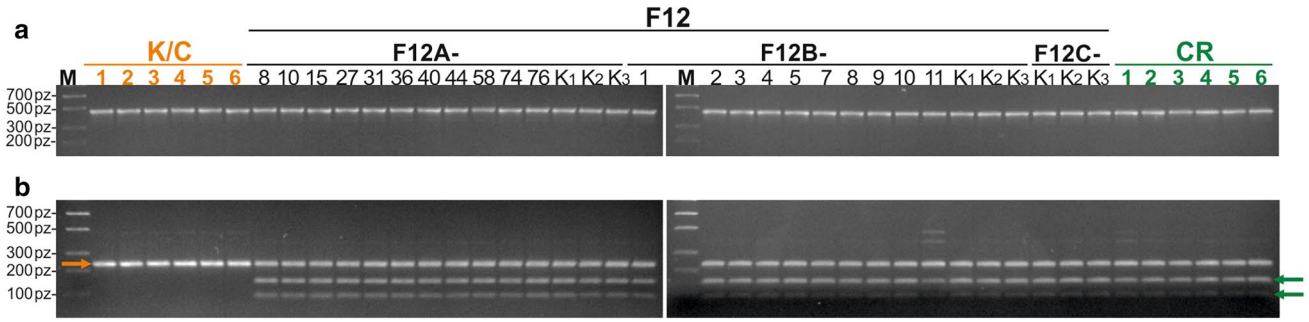

Fig. 7 CAPS analysis of cpDNA inheritance by *G. kurroo* (+) *G. cruciata* somatic hybrids: **a** PCR amplification of an intergenic region *trnS-trnG*, **b** electrophoretic pattern of restriction digestion of PCR product with *Bfa*I. *K/C G. kurroo* ("cell suspension" fusion partner), *CR G. cruciata* ("mesophyll" fusion partner), *F12A-F12C* products of three independent fusion events, *M* DNA size marker (100-bp DNA ladder). *Symbols* K_1–K_3 represent particular clones of hybrid callus, cultured on three different media; *numbers 1–76* represent individual regenerants. *Orange arrows* indicate bands specific for "cell suspension" fusion partner; *green arrows* indicate bands specific for "mesophyll" fusion partner. (Color figure online)

Table 4 Nuclear DNA content of selected somatic hybrids *G. kurroo* (2C = 3.32 pg) (+) *G. cruciata* (2C = 8.07 pg) during 4-year-long culture

Callus line	Symbol of regenerant/ callus	Initial nuclear DNA content (pg)	Nuclear DNA content (pg) after 1–2 years in culture	Nuclear DNA content (pg) after 3–4 years in culture
F12A	F12A-8	19.92	11.30	11.30
	F12A-10	20.16	12.49	12.27
	F12A-15	15.26/**20.56***	15.43	–
	F12A-27	20.23	17.67	–
	F12A-31	20.53	13.30	–
	F12A-36	20.41	11.48/13.85/**20.41**	13.20
	F12A-44	20.77	13.76/17.48	17.48
	F12A-58	20.62	**16.58**/20.58	16.58
	F12A-74	20.81	14.76/**17.49**	–
	F12A-76	20.07	**13.48**/20.22	–
	F12A-K_1	20.07	14.39	13.20
F12B	F12B-1	12.39/**22.77**	**14.39**/22.96	14.24
	F12B-2	16.40	16.40	16.40
	F12B-3	20.36	19.86	**14.69**/20.08
	F12B-4	20.22	19.49	16.92
	F12B-5	**14.83**/20.82	14.03	13.85
	F12B-6	19.71	10.93/**14.76**	14.76
	F12B-7	21.57	20.04	–
	F12B-9	21.83	21.97	–
	F12B-10	21.75	22.05	–
	F12B-K_1	21.94	21.75	20.67
F12C	F12C-K_1	22.71	20.26	20.12

F12A–F12C, products of three independent fusion events; K_1, hybrid callus, cultured on MS medium supplemented with 0.5 mg l^{-1} 2,4-D and 1.0 mg l^{-1} kinetin; 1–76, numbers of individual regenerants

*Values in bold indicate DNA content which was predominant in mixoploid hybrid callus or plant

it varied from 12.5 pg to 22 pg/2C. Of the 24 regenerants, seven were mixoploid, amongst which only one (F12A-36) possessed predominantly nuclei exceeding 20 pg/2C. Three of the hybrids maintained DNA content of about 22 pg, all of them originated from callus F12B.

Following another 2 years of culture, there was further reduction in the DNA content of four regenerants (F12A-36, F12A-K_1, F12B-3, F12B-4), and of the callus F12B-K_1. Only one hybrid (F12B-3) expressed mixoploidy, having a small fraction of nuclei possessing DNA content similar to that detected 2 years earlier; downsizing of genome was evident

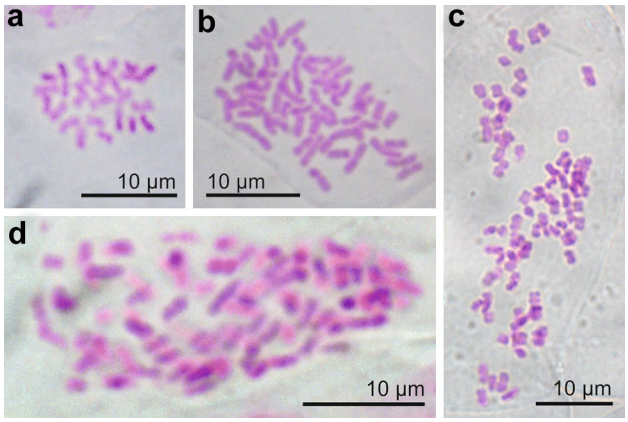

Fig. 8 Chromosome counting of *G. kurroo, G. cruciata* and somatic hybrid plants: **a** 26 mitotic metaphase chromosomes in root-tip cells of a seed-derived parent plant of *G. kurroo*, **b** 52 chromosomes in a root-tip cell of a seed-derived parent plant of *G. cruciata*, **c** 78 chromosomes in a root-tip cell of a somatic hybrid F12A-10, **d** about 82–84 chromosomes in a root-tip cell of a somatic hybrid F12A-49

for most nuclei. Chromosome counting confirmed that parental species, *G. kurroo* and *G. cruciata*, had 26 and 52 chromosomes, respectively (Fig. 8a, b). However, since only a few somatic hybrid plants developed root systems, chromosome number was scored for regenerant F12A-10 in root-tip cells only after 2 years of tissue culture, and this possessed 78 chromosomes (Fig. 8c) whereas cells of regenerant F12A-49 contained about 82–84 chromosomes (Fig. 8d).

Discussion

Somatic hybridization involving fusion of protoplasts isolated from different species, and subsequent plant regeneration from heterokaryons produced, enables one not only to evade the barriers to crossing, but also to create novel, unique combinations of nuclear and cytoplasmic genomes displaying various levels of symmetry/asymmetry and similarity to parental species. The inability to control such interactions between parental nuclear and organellar genomes, and the frequency of genetic instability, however, necessitates the detailed description of the resultant somatic hybrids. Methods for characterizing regenerated somatic hybrid lines include morphological evaluation, cytological and cytogenetic analysis and molecular characterization by DNA markers (Guo et al. 2004).

Nuclear genome composition of somatic hybrids and their genetic similarity to parental species evaluated with the use of AFLP and ISSR markers

Different molecular markers, especially those based on PCR reaction, have been used for the identification and evaluation of somatic hybrids. These markers include the most popular, namely, RAPD (Xu et al. 1993; De Filippis et al. 1996; Szczerbakowa et al. 2003; Guan et al. 2010), microsatellites SSRs (Fu et al. 2003; Cappelle et al. 2007; Liu et al. 2002; Dambier et al. 2011), AFLP (Brewer et al. 1999; Guo et al. 2002; Fu et al. 2004; Tu et al. 2008), ISSR (Scarano et al. 2002; Trabelsi et al. 2005; Xiao et al. 2009) and, more recently, DArT markers (Smyda-Dajmund et al. 2016).

Since various DNA molecular markers target different regions of the genome, the use of more than one molecular technique is recommended so as to exploit as many sources of polymorphism as possible (Palombi and Damiano 2002; Bolibok et al. 2005; Velasco-Ramírez et al. 2014). AFLP allows the generation of a large number of fragments without prior sequence information and the rapid detection of polymorphism (Agarwal et al. 2008; Meudt and Clarke 2007). In the case of *Gentiana* somatic hybrids, ten AFLP primer pairs generated three times more markers than did ten ISSR primers. However, ISSR is still widely used because of its simplicity and higher reproducibility than RAPD (Pradeep Reddy et al. 2002). Furthermore, it revealed higher percentage of polymorphism than did AFLP.

Molecular analysis using both marker systems revealed that all somatic hybrids are genetically closer to *G. cruciata*. On the one hand this may be a consequence of *G. cruciata* tetraploidy. The symmetric hybrid would therefore possess two genomes/sets of chromosomes of *G. kurroo* ($2n=2x=26$; 2C = 3.32 pg) and four of *G. cruciata* ($2n=4x=52$; 2C = 8.07 pg). This was reflected in the total number of specific bands obtained for both parental species, since combination of AFLP and ISSR techniques generated a total of 220 specific bands for *G. cruciata*, but only 119 for *G. kurroo*.

Another factor that affects the final composition of interspecific somatic hybrids is the elimination of nuclear DNA/chromosomes. None of the *G. kurroo* (+) *G. cruciata* somatic hybrids studied here possessed a total number of AFLP and ISSR markers equivalent to the sum of parental species markers, thus indicating that spontaneous partial elimination of parental DNA had occurred. This elimination affected the DNA of *G. kurroo* to a greater degree since a greater percentage of its specific markers was deleted from hybrid genetic material. It is thought that the elimination of nuclear DNA in hybrids may be influenced by genetic similarity between parental species and their ploidy and genome size. In hybrids of closely related species, the co-existence of both parental genomes in a nucleus is more likely, and elimination is random. However, in the case of distantly related species, the elimination of parental DNA is biased (Sundberg and Glimelius 1991; Wang et al. 2008). With regard to the effect of ploidy/genome size, it was shown that preferential elimination of chromosomes

was a feature of species with lower ploidy (Sundberg and Glimelius 1991). Both *G. kurroo* and *G. cruciata* belong to the same section Cruciata, and therefore, their genetic similarity should be high. Thus, the preferential elimination of *G. kurroo* DNA sequences observed in this present study may be the result of its lower ploidy.

Chloroplasts inheritance by Gentiana somatic hybrids

Of the relative small numbers of methods that enable study of chloroplast inheritance in somatic hybrids, CAPS markers, also known as PCR-RFLP, have attained the greatest popularity in the past 15 years (Bastia et al. 2001; Guo et al. 2002; Yamashita et al. 2002). Moreover, cpSSR, if available for species used in fusion experiments, are enthusiastically used (Wang et al. 2008; Fatta Del Bosco et al. 2017).

For this study, the CAPS marker was developed as an easy and quick method for distinguish between the cpDNA of *G. kurroo* and of *G. cruciata*, and for further analysis of their somatic hybrids. Amplification of *trnS-trnG* region, followed by restriction digestion with *Bfa*I allowed us to confirm that all hybrids possessed chloroplasts obtained from "mesophyll" fusion partner *G. cruciata*.

Rapid chloroplast segregation i.e. elimination of chloroplasts inherited from one of the fusion partners in hybrid cells was observed in most somatic hybrids (Walters and Earle 1993). Again, this process can be either random (Scowcroft and Larkin 1981; Lössl et al. 1994; Escalante et al. 1998) or biased (Levi et al. 1988; Bonnema et al. 1992; Buiteveld et al. 1998), whereas the co-existence of chloroplasts from both parental species is very rare (Mohapatra et al. 1998; Moreira et al. 2000). Several factors have been proposed to influence biased chloroplast transmission, along with their possible modus operandi (Rose et al. 1990 and references therein). None of these, however, are completely understood (Walters and Earle 1993). One proposed factors is the type of source cell for protoplasts, leaf mesophyll protoplasts being slightly more effective at transmitting chloroplasts. Li and Sink (1992) showed that *Lycopersicon esculentum* (+) *Solanum lycopersicoides* hybrids possessed chloroplasts from the species that was the source of mesophyll protoplasts. A similar relationship was observed in all *G. kurroo* (+) *G. cruciata* somatic hybrids. However, it should be pointed out, that in other combinations of parental species, cpDNA inheritance can be quite different. For example, it was shown that *Allium ampeloprasum* (+) *A. cepa* and *A. cepa* (+) *A. sativum* hybrids usually inherited plastids from the parental species that was the donor of callus- or cell suspension-derived protoplasts (Buiteveld et al. 1998; Yamashita et al. 2002).

Segregation of chloroplasts may also be affected by genetic similarity and differences in the ploidy level between parental species. It was found that for Brassicaceae the more closely related the fusion partners were, the more random was chloroplast segregation, and chloroplasts from the species, of which genetic material predominated in the nucleus, were favored (Sundberg and Glimelius 1991). Similarly, the plastids in hybrid calli of *Solanum tuberosum* (+) *Nicotiana plumbaginifolia* were contributed by the species that predominated in the nucleus (Wolters et al. 1993). Probably, the genetic divergence between the parental species might, through incompatibility reactions between nuclei and chloroplasts, influence the segregation of chloroplasts. Furthermore, the eliminated chloroplasts were usually derived from the parent of lower ploidy (Sundberg and Glimelius 1991). In general, higher ploidy is accompanied by greater cell volume and a greater number of plastids (Butterfass 1979). As a result, an unequal number of plastids may be donated following fusion to the heterokaryon by parental protoplasts of different ploidy.

The fact that in *G. kurroo* (+) *G. cruciata* somatic hybrids chloroplasts were derived from tetraploid *G. cruciata*, of which DNA predominated in the hybrid nucleus, and not from diploid *G. kurroo*, supports the hypothesis for the unequal input of organelles by protoplasts of differing ploidy and for the occurrence of nucleo-cytoplasmic incompatibility—another plausible explanations of biased chloroplast inheritance.

Nuclear DNA content and chromosome number and its influence on hybrid morphology

Genome rearrangements accompanying sexual hybridization and formation of allopolyploids have been repeatedly described in detail (Comai 2000; Ma and Gustafson 2005; Gao et al. 2011). It seems obvious that similar mechanisms must occur during somatic hybridization, but that they can be intensified by specific conditions of protoplast isolation, pretreatment and fusion, as well as further in vitro culture and, especially, a long-term callus phase, which often precedes plant regeneration. As a result, variation in nuclear DNA content and various chromosomal abnormalities have been observed in many somatic hybrids, including those of *Gentiana* studied here.

Increase in the DNA content was observed in somatic hybrids of *Solanum brevidens* (+) *S. tuberosum* (Puite and Schaart 1993), *Primula malacoides* (+) *P. obconica* (Mizuhiro et al. 2001), and *Diospyros glandulosa* (+) *D. kaki* (Tamura et al. 1998). It was suggested that chromosome doubling occurred during callus proliferation. All the *G. kurroo* (+) *G. cruciata* somatic hybrid plants and calli obtained here initially possessed nuclear DNA content greater than the sum of parental DNA content,

the majority of plants containing about 20–22 pg DNA, which is close to or slightly less than the doubled sum of the parental DNA contents. This could be due to either genome duplication at the early stage of post-fusion cell culture or fusion of four protoplasts. However, taking into consideration the tendency of gentian protoplast-derived cells to undergo spontaneous polyploidization (Fiuk and Rybczyński 2007; Tomiczak et al. 2015a, 2016), the first hypothesis seems more likely. Those highly polyploid forms appeared to be very unstable during the next years of in vitro culture, leading to downsizing of the genome.

Many have reported chromosome loss, mixoploidy and a gradual reduction in DNA content in various somatic hybrids (Fehér et al. 1992; Babiychuk et al. 1992; Oberwalder et al. 1998; Liu and Deng 2002). Some hybrid lines of *P. malacoides* (+) *P. obconica* showed a decrease in DNA content during the plant regeneration process (Mizuhiro et al. 2001). More recently, Guo et al. (2010) reported that somatic hybrids between *S. tuberosum* and *S. chacoense* underwent gradual DNA elimination during 3 years of in vitro culture. This phenomenon was most evident when hybrids had an initial DNA content greater than the sum of parental species DNA, or they were aneuploids and mixoploids. The most stable *Solanum* hybrids were those of ploidy level equal to the sum of parental ploidy. A similar reduction in the nuclear DNA content and temporary mixoploidy were observed in gentian somatic hybrid plants within 4 years from regeneration. Only three somatic hybrids maintained DNA content of about 20 pg/2C at the end of the experiment, and the remainder possessed genomes of various sizes, ranging from 11.3 to 17.5 pg. At that time, only in a very few of them was the genome of the same size as 2 years previously, which suggested that the process of DNA/chromosome elimination was still ongoing. Only in one of the hybrids, F12A-8, had the genome, after 1 year of culture, stabilized, reaching the size that of hexaploid hybrid *G. kurroo* (+) *G. cruciata* (11.3 pg). Chromosome numbers were also estimated for two rooted hybrids to be equal to or slightly greater than the sum of parental chromosome numbers.

Genetic instability and aneuploidy are nearly always associated with sterility and morphological abnormalities (Karp et al. 1989). Somatic hybrids obtained between *G. kurroo* and *G. cruciata* exhibited various morphological aberrations. Dwarf stature of hybrid plants and dark green, wrinkled leaves were the most visible symptoms of high polyploidy. Different changes in morphology, growth, and multiplication rate occurred at the same time as DNA elimination. Similar modifications were also observed in somatic hybrids of *S. tuberosum* (+) *S. brevidens* (Fehér et al. 1992). We assert that vigorous growth of hybrids and rooting were associated with at least temporary genome stabilization, whereas slow growth, lack of roots, browning, and finally death, resulted from high genetic instability.

Conclusion

Several methods including flow cytometry, chromosome counting, and molecular analysis of nuclear and chloroplast DNA were used for describing somatic hybrid calli and plants produced by electrofusion between *G. kurroo* and *G. cruciata* protoplasts. Cytological and cytometric studies provided much evidence of polyploidy and subsequent gradual genome downsizing. According to molecular data, hybrids were slightly asymmetric with a predominance of *G. cruciata* nuclear DNA and *G. cruciata* chloroplasts.

Author Contributions Conceived the experiments: JJR. Designed and performed the experiments: KT. Analyzed the data: KT, ES. Contributed reagents/materials/analysis tools: KT, ES, JJR. Wrote the paper: KT. All authors read and approved the final manuscript. Acknowledgements The authors wish to thank Aleksandra Chomacka for operating the flow cytometer and Magdalena Zaborowska for valuable technical help in ISSR genotyping. This work was supported by the Polish Ministry of Science and Higher Education (Grant No. 3P04C 037 23).

References

Agarwal M, Shrivastava N, Padh H (2008) Advances in molecular marker techniques and their applications in plant sciences. Plant Cell Rep 27:617–631. doi:10.1007/s00299-008-0507-z

Baba SA, Malik SA (2014) Evaluation of antioxidant and antibacterial activity of methanolic extracts of *Gentiana kurroo* Royle. Saudi J Biol Sci 21:493–498. doi:10.1016/j.sjbs.2014.06.004

Babiychuk E, Kushnir S, Gleba YY (1992) Spontaneous extensive chromosome elimination in somatic hybrids between somatically congruent species *Nicotiana tabacum* L. and *Atropa belladonna* L. Theor Appl Genet 84:87–91. doi:10.1007/BF00223985

Bastia T, Scotti N, Cardi T (2001) Organelle DNA analysis of *Solanum* and *Brassica* somatic hybrids by PCR with 'universal primers. Theor Appl Genet 102:1265–1272. doi:10.1007/s001220000508

Behera MC, Raina R (2012) *Gentiana kurroo* Royle: a critically endangered bitter herb. Int J Med Aromat Plants 2:22–29

Bolibok H, Rakoczy-Trojanowska M, Hromada A, Pietrzykowski R (2005) Efficiency of different PCR-based marker systems in assessing genetic diversity among winter rye (*Secale cereale* L.) inbred lines. Euphytica 146:109–116. doi:10.1007/s10681-005-0548-0

Bonnema AB, Melzer JM, Murray LW, O'Connell MA (1992) Non-random inheritance of organellar genomes in symmetric and asymmetric somatic hybrids between *Lycopersicon esculentum*

and *L. pennellii*. Theor Appl Genet 84:435–442. doi:10.1007/BF00229504

Brewer EP, Saunders JA, Angle JS, Chaney RL, McIntosh MS (1999) Somatic hybridization between the zinc accumulator *Thlaspi caerulescens* and *Brassica napus*. Theor Appl Genet 99:761–771. doi:10.1007/s001220051295

Buiteveld J, Kassies W, Geels R, van Lookeren Campagne MM, Jacobsen E, Creemers-Molenaar J (1998) Biased chloroplast and mitochondrial transmission in somatic hybrids of *Allium ampeloprasum* L. and *Allium cepa* L. Plant Sci 131:219–228. doi:10.1016/S0168-9452(97)00252-5

Butterfass T (1979) Patterns of chloroplast reproduction: a developmental approach to protoplasmic plant anatomy. Springer, New York

Cappelle C, Mörchen M, Hilbert JL, Rambaud C (2007) Regeneration and molecular characterization of a male sterile interspecific somatic hybrid between *Cichorium intybus* and *C. endivia*. Plant Sci 172:596–603. doi:10.1016/j.plantsci.2006.11.010

Comai L (2000) Genetic and epigenetic interactions in allopolyploid plants. Plant Mol Biol 43:387–399. doi:10.1023/A:1006480722854

Dambier D, Benyahia H, Pensabene-Bellavia G, Kaçar YA, Froelicher Y, Belfalah Z, Lhou B, Handaji N, Printz B, Morillon R, Yesiloglu T, Navarro L, Ollitrault P (2011) Somatic hybridization for citrus rootstock breeding: An effective tool to solve some important issues of the Mediterranean citrus industry. Plant Cell Rep 30:883–900. doi:10.1007/s00299-010-1000-z

De Filippis L, Hoffmann E, Hampp R (1996) Identification of somatic hybrids of tobacco generated by electrofusion and culture of protoplasts using RAPD-PCR. Plant Sci 121:39–46. doi:10.1016/S0168-9452(96)04505-0

Escalante A, Imanishi S, Hossain M, Ohmido N, Fukui K (1998) RFLP analysis and genomic in situ hybridization (GISH) in somatic hybrids and their progeny between *Lycopersicon esculentum* and *Solanum lycopersicoides*. Theor Appl Genet 96:719–726. doi:10.1007/s001220050794

Fatta Del Bosco S, Napoli E, Mercati F, Abbate L, Carimi F, Ruberto G (2017) Somatic cybridization for *Citrus*: polyphenols distribution in juices and peel essential oil composition of a diploid cybrid from Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) and sour orange (*Citrus aurantium* L.). Genet Resour Crop Evol 64:261–275. doi:10.1007/s10722-015-0348-x

Fehér A, Preiszner J, Litkey Z, Csanádi G, Dudits D (1992) Characterization of chromosome instability in interspecific somatic hybrids obtained by X-ray fusion between potato (*Solanum tuberosum* L.) and *S. brevidens* Phil. Theor Appl Genet 84:880–890. doi:10.1007/BF00227400

Fiuk A, Rybczyński JJ (2007) The effect of several factors on somatic embryogenesis and plant regeneration in protoplast cultures of *Gentiana kurroo* (Royle). Plant Cell Tiss Organ Cult 91:263–271. doi:10.1007/s11240-007-9293-5

Fiuk A, Rybczyński JJ (2008) Factors influencing efficiency of somatic embryogenesis of *Gentiana kurroo* (Royle) cell suspension. Plant Biotechnol Rep 2:33–39. doi:10.1007/s11816-008-0045-8

Fu CH, Guo W-W, Liu J, Deng X-X (2003) Regeneration of *Citrus sinensis* (+) *Clausena lansium* intergeneric triploid and tetraploid somatic hybrids and their identification by molecular markers. In Vitro Cell Dev Biol Plant 39:360–364. doi:10.1079/IVP2002407

Fu CH, Chen CL, Guo WW, Deng XX (2004) GISH, AFLP and PCR-RFLP analysis of an intergeneric somatic hybrid combining Goutou sour orange and *Poncirus trifoliata*. Plant Cell Rep 23:391–396. doi:10.1007/s00299-004-0828-5

Gao J, Zhang Z, Mao L, Cheng Q, Liu L, Lin H, Shen Y, Gao S, Zhao M, Pan G (2011) Advances on research epigenetic change of hybrid and polyploidy in plants. African. J Biotechnol 10:10335–10343. doi:10.5897/AJB10.1893

Guan Q, Guo Y, Wei Y, Meng F, Zhang Z (2010) Regeneration of somatic hybrids of ginger via chemical protoplast fusion. Plant Cell Tiss Organ Cult 102:279–284. doi:10.1007/s11240-010-9730-8

Guo WW, Cheng YJ, Deng XX (2002) Regeneration and molecular characterization of intergeneric somatic hybrids between *Citrus reticulata* and *Poncirus trifoliata*. Plant Cell Rep 20:829–834. doi:10.1007/s00299-001-0399-7

Guo WW, Cai XD, Grosser JW (2004) Somatic cell cybrids and hybrids in plant improvement. In: Daniell H, Chase C (eds) Molecular biology and biotechnology of plant organelles. Springer, Dordrecht, pp 635–659. doi:10.1007/978-1-4020-3166-3_24

Guo X, Xie C, Cai X, Song B, He L, Liu J (2010) Meiotic behavior of pollen mother cells in relation to ploidy level of somatic hybrids between *Solanum tuberosum* and *S. chacoense*. Plant Cell Rep 29:1277–1285. doi:10.1007/s00299-010-0914-9

Karp A, Jones MGK, Foulger D, Fish N, Bright SWJ (1989) Variability in potato tissue culture. Am Potato J 66:669–684. doi:10.1007/BF02853986

Köhlein F (1991) Gentians. Timber Press, Portland

Latif A, Khan TF, Afaq SH (2006) Antiinflammatory activity of flower tops *of Gentiana kurroo* Royale extract. Pharmacology-online 3:575–580.

Levi A, Ridley BL, Sink KC (1988) Biased organelle transmission in somatic hybrids of *Lycopersicon esculentum* and *Solanum lycopersicoides*. Curr Genet 14:177–182. doi:10.1007/BF00569342

Li Y, Sink KC (1992) Cell type determines plastid transmission in tomato intergeneric somatic hybrids. Curr Genet 22:167–171. doi:10.1007/BF00351478

Liu J, Deng X (2002) Regeneration and analysis of citrus interspecific mixoploid hybrid plants from asymmetric somatic hybridization. Euphytica 125:13–20. doi:10.1023/A:1015748411654

Liu JH, Pang XM, Cheng YJ, Meng HJ, Deng XX (2002) Molecular characterization of the nuclear and cytoplasmic genomes of intergeneric diploid plants from cell fusion between *Microcitrus papuana* and Rough lemon. Plant Cell Rep 21:327–332. doi:10.1007/s00299-002-0517-1

Lössl A, Frei U, Wenzel G (1994) Interaction between cytoplasmic composition and yield parameters in somatic hybrids of *S. tuberosum* L. Theor Appl Genet 89:873–878. doi:10.1007/BF00224512

Ma XF, Gustafson JP (2005) Genome evolution of allopolyploids: a process of cytological and genetic diploidization. Cytogenet Genome Res 109:236–249. doi:10.1159/000082406

Marie D, Brown SC (1993) A cytometric exercise in plant DNA histograms, with 2 C values for 70 species. Biol Cell 78:41–51. doi:10.1016/0248-4900(93)90113-S

Meudt HM, Clarke AC (2007) Almost Forgotten or Latest Practice? AFLP applications, analyses and advances. Trends Plant Sci 12:106–117. doi:10.1016/j.tplants.2007.02.001

Mikuła A, Rybczyński JJ (2001) Somatic embryogenesis of *Gentiana* genus I. The effect of the preculture treatment and primary explant origin on somatic embryogenesis of *Gentiana cruciata* (L.), *G. pannonica* (Scop.), and *G. tibetica* (King). Acta Physiol Plant 23:15–25. doi:10.1007/s11738-001-0017-x

Mikuła A, Wesołowska M, Kapusta J, Skrzypczak L, Rybczyński JJ (1996) Cytomorphological studies on somatic embryogenesis of *Gentiana tibetica* (King) and *G. cruciata* (L.). Acta Soc Bot Pol 65:47–51. doi:10.5586/asbp.1996.008

Mikuła A, Tomiczak K, Rybczyński JJ (2011a) Cryopreservation enhances embryogenic capacity of *Gentiana cruciata* (L.) suspension culture and maintains (epi)genetic uniformity

of regenerants. Plant Cell Rep 30:565–574. doi:10.1007/s00299-010-0970-1

Mikuła A, Tomiczak K, Wójcik A, Rybczyński JJ (2011b) Encapsulation-dehydration method elevates embryogenic abilities of *Gentiana kurroo* cell suspension and carrying on genetic stability of its regenerants after cryopreservation. Acta Hortic 908:143–154. doi:10.17660/ActaHortic.2011.908.16

Mizuhiro M, Ito K, Mii M (2001) Production and characterization of interspecific somatic hybrids between *Primula malacoides* and *P. obconica*. Plant Sci 161:489–496. doi:10.1016/S0168-9452(01)00434-4

Mohapatra T, Kirti PB, Kumar VD, Prakash S, Chopra VL (1998) Random chloroplast segregation and mitochondrial genome recombination in somatic hybrid plants of *Diplotaxis catholica* + *Brassica juncea*. Plant Cell Rep 17:814–818. doi:10.1007/s002990050489

Moreira CD, Chase CD, Gmitter FG Jr, Grosser JW (2000) Transmission of organelle genomes in citrus somatic hybrids. Plant Cell Tiss Organ Cult 61:165–168. doi:10.1023/A:1006434221970

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Oberwalder B, Schilde-Rentschler L, Ruoss B, Wittemann S, Ninnemann H (1998) Asymmetric protoplast fusions between wild species and breeding lines of potato: effect of recipients and genome stability. Theor Appl Genet 97:1347–1354. doi:10.1007/s001220051028

Palombi MA, Damiano C (2002) Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (*Actinidia deliciosa* A. Chev). Plant Cell Rep 20:1061–1066. doi:10.1007/s00299-001-0430-z

Pradeep Reddy M, Sarla N, Siddiq EA (2002) Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128:9–17. doi:10.1023/A:1020691618797

Puite KJ, Schaart JG (1993) Nuclear genomic composition of asymmetric fusion products between irradiated transgenic *Solanum brevidens* and *S. tuberosum* : limited elimination of donor chromosomes and polyploidization of the recipient genome. Theor Appl Genet 86:237–244. doi:10.1007/BF00222085

Rose RJ, Thomas MR, Fitter JT (1990) The transfer of cytoplasmic and nuclear genomes by somatic hybridisation. Aust J Plant Physiol 17:303–321

Rozen S, Skaletsky H (1999) Primer3 on the WWW for general users and for biologist programmers. In: Misener S, Krawetz SA (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, pp 365–386. doi:10.1385/1-59259-192-2:365

Scarano MT, Abbate L, Ferrante S, Lucretti S, Tusa N (2002) ISSR-PCR technique: a useful method for characterizing new allotetraploid somatic hybrids of mandarin. Plant Cell Rep 20:1162–1166. doi:10.1007/s00299-002-0450-3

Scowcroft WR, Larkin PJ (1981) Chloroplast DNA assorts randomly in intraspecific somatic hybrids of *Nicotiana debneyi*. Theor Appl Genet 60:179–184. doi:10.1007/BF00264526

Sliwinska E, Zielinska E, Jedrzejczyk I (2005) Are seeds suitable for flow cytometric estimation of plant genome size? Cytom Part A 64A:72–79. doi:10.1002/cyto.a.20122

Smyda-Dajmund P, Śliwka J, Wasilewicz-Flis I, Jakuczun H, Zimnoch-Guzowska E (2016) Genetic composition of interspecific potato somatic hybrids and autofused 4× plants evaluated by DArT and cytoplasmic DNA markers. Plant Cell Rep 35:1345–1358. doi:10.1007/s00299-016-1966-2

Sundberg E, Glimelius K (1991) Effects of parental ploidy level and genetic divergence on chromosome elimination and chloroplast segregation in somatic hybrids within *Brassicaceae*. Theor Appl Genet 83:81–88. doi:10.1007/BF00229229

Szczerbakowa A, Maciejewska U, Zimnoch-Guzowska E, Wielgat B (2003) Somatic hybrids *Solanum nigrum* (+) *S. tuberosum*: morphological assessment and verification of hybridity. Plant Cell Rep 21:577–584. doi:10.1007/s00299-002-0555-8

Tamura M, Tao R, Sugiura A (1998) Production of somatic hybrids between *Diospyros glandulosa* and *D. kaki* by protoplast fusion. Plant Cell Tiss Org Cult 54:85–91. doi:10.1023/A:1006121220306

Tomiczak K, Mikuła A, Sliwinska E, Rybczyński JJ (2015a) Autotetraploid plant regeneration by indirect somatic embryogenesis from leaf mesophyll protoplasts of diploid *Gentiana decumbens* Lf. In Vitro Cell Dev Biol Plant 51:350–359. doi:10.1007/s11627-015-9674-0

Tomiczak K, Mikuła A, Rybczyński JJ (2015b) Protoplast culture and somatic cell hybridization of gentians. In: Rybczyński JJ, Davey MR, Mikuła A (eds) The Gentianaceae: volume 2: biotechnology and applications. Springer, Berlin, pp 163–185. doi:10.1007/978-3-642-54102-5_7

Tomiczak K, Sliwinska E, Rybczyński JJ (2016) Comparison of the morphogenic potential of five *Gentiana* species in leaf mesophyll protoplast culture and ploidy stability of regenerated calli and plants. Plant Cell Tiss Organ Cult 126:319–331. doi:10.1007/s11240-016-1000-y

Trabelsi S, Gargouri-Bouzid R, Vedel F, Nato A, Lakhoua L, Drira N (2005) Somatic hybrids between potato *Solanum tuberosum* and wild species *Solanum vernei* exhibit a recombination in the plastome. Plant Cell Tissue Organ Cult 83:1–11. doi:10.1007/s11240-005-3667-3

Tu Y, Sun J, Liu Y, Ge X, Zhao Z, Yao X, Li Z (2008) Production and characterization of intertribal somatic hybrids of *Raphanus sativus* and *Brassica rapa* with dye and medicinal plant *Isatis indigotica*. Plant Cell Rep 27:873–883. doi:10.1007/s00299-008-0513-1

Velasco-Ramírez AP, Torres-Morán MI, Molina-Moret S, de Jesús Sánchez-González J, Santacruz-Ruvalcaba F (2014) Efficiency of RAPD, ISSR, AFLP and ISTR markers for the detection of polymorphisms and genetic relationships in camote de cerro (*Dioscorea* spp.). Electron J Biotechnol 17:65–71. doi:10.1016/j.ejbt.2014.01.002

Vincze T, Posfai J, Roberts RJ (2003) NEBcutter: A program to cleave DNA with restriction enzymes. Nucleic Acids Res 31:3688–3691. doi:10.1093/nar/gkg526

Vos P, Hogers R, Bleeker M, Reijans M, Vandelee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414. doi:10.1093/nar/23.21.4407

Waara S, Glimelius K (1995) The potential of somatic hybridization in crop breeding. Euphytica 85:217–233. doi:10.1007/BF00023951

Walters TW, Earle ED (1993) Organellar segregation, rearrangement and recombination in protoplast fusion-derived *Brassica oleracea* calli. Theor Appl Genet 85:761–769. doi:10.1007/BF00225017

Wang M, Zhao J, Peng Z, Guo W, Wang Y, Wang L, Xia G (2008) Chromosomes are eliminated in the symmetric fusion between *Arabidopsis thaliana* L. and *Bupleurum scorzonerifolium* Willd. Plant Cell Tiss Organ Cult 92:121–130. doi:10.1007/s11240-007-9308-2

Wang J, Zhao C, Liu C, Xia G, Xiang F (2011) Introgression of *Swertia mussotii* gene into *Bupleurum scorzonerifolium* via somatic hybridization. BMC Plant Biol 11:71. doi:10.1186/1471-2229-11-71

Wójcik AI, Rybczyński JJ (2015) Electroporation and morphogenic potential of *Gentiana kurroo* (Royle) embryogenic cell suspension protoplasts. BioTechnologia 96:19–29. doi:10.5114/bta.2015.54170

Wolters AM, Koornneef M, Gilissen LJ (1993) The chloroplast and mitochondrial DNA type are correlated with the nuclear composition of somatic hybrid calli of *Solanum tuberosum* and *Nicotiana plumbaginifolia*. Curr Genet 24:260–267. doi:10.1007/BF00351801

Xiao W, Huang X, Gong Q, Dai XM, Zhao JT, Wei YR, Huang XL (2009) Somatic hybrids obtained by asymmetric protoplast fusion between *Musa* Silk cv. Guoshanxiang (AAB) and *Musa acuminata* cv. Mas (AA). Plant Cell Tiss Organ Cult 97:313–321. doi:10.1007/s11240-009-9530-1

Xu Y, Clark MS, Pehu E (1993) Use of RAPD markers to screen somatic hybrids between *Solanum tuberosum* and *S. brevidens*. Plant Cell Rep 12:107–109. doi:10.1007/BF00241944

Yamashita K, Hisatsune Y, Sakamoto T, Ishizuka K, Tashiro Y (2002) Chromosome and cytoplasm analyses of somatic hybrids between onion (*Allium cepa* L.) and garlic (*A. sativum* L.). Euphytica 125:163–167. doi:10.1023/A:1015826702550