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Short Communication

Induction, identification and characterization of polyploidy in *Stevia rebaudiana* Bertoni

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Abstract *Stevia rebaudiana* Bertoni produces sweet steviol glycosides; these are regarded as the most promising substitute for sucrose and have a wide range of applications with high economic value. To improve steviol glycosides yields, we established an efficient method for inducing polyploidy in *S. rebaudiana*. Treatment with 0.05% colchicine for 48 h or with 0.1% colchicine for 24 h efficiently induced polyploidy in germinating seeds and polyploidy could be accurately identified using flow cytometry. Most of the tetraploid *S. rebaudiana* plants exhibited apparent variations in cytological and morphological characteristics, and had higher contents of the two main steviol glycosides, stevioside and rebaudioside A, than the diploid controls. These tetraploid plants may be further selected for breeding purposes or micropropagated for commercial production.

Key words: colchicine, flow cytometry, polyploidy, *Stevia rebaudiana*.

Stevia rebaudiana Bertoni is an herbaceous perennial plant of the Asteraceae family. It is native to the Amambay region of northeastern Paraguay and neighboring countries Brazil and Argentina (Madan et al. 2010; Yadav et al. 2011). Its leaf and stem tissues accumulate a mixture of more than 20 different glycosides derived from the tetracyclic diterpene steviol (Ohta et al. 2010). The main steviol glycosides are stevioside and rebaudioside A, which are the sweetest compounds that are 250–300 and 350–450 times sweeter than sucrose, respectively (Crammer and Ikan 1986). Because the steviol glycosides are naturally occurring compounds with high sweetness and little to no calorific value, they have been regarded as the most promising substitutes for sucrose and have a wide range of applications and high economic value (Soejarto et al. 1983; Yadav et al. 2011). Applications include dietary, diabetic, and dental consumer markets, and health-promoting uses as antitumor, antihypertensive, antihyperglycemic and antioxidant agents (Singh et al. 2015). *S. rebaudiana* has been introduced and is grown as a crop in a number of countries, including Japan, China, Korea, Indonesia, Tanzania, Mexico, the United States and Canada (Madan et al. 2010).

To improve steviol glycosides yields in *S. rebaudiana*, polyploidy breeding may be a promising approach. Polyploidy is widely acknowledged as a major mechanism of adaptation and speciation in the evolution of higher plants (Ramsey and Schemske 1998). Polyploid plants can arise spontaneously in nature by several mechanisms, including meiotic or mitotic failures and fusion of unreduced (2n) gametes (Comai 2005). In general, polyploid plants are large, healthy and vigorous. Some chemicals induce polyploidy in plants and cell cultures; the best known of these chemicals is colchicine, which frequently causes chromosome doubling (Blakeslee and Avery 1937). Polyploidy has been used to develop new crop cultivars; including wheat, cotton, potato and sugarcane.

The primary objectives of this study were to establish a protocol for induction of polyploidy in *S. rebaudiana*. Seeds of *S. rebaudiana* cultivar Zhongshan No. 2 were germinated by placing them on paper towels soaked with distilled water in a petri dish. With 2 day of soaking, the seeds expanded and small white apices began to emerge from the seed coat. These germinating seeds were considered to be most suitable for colchicine treatment. The colchicine treatment was performed according to our preliminary experiment with some modifications. Briefly, 100 germinating seeds per treatment, were imbibed in 0.05% or 0.1% (w/v) colchicine solution (035-03853, Wako, Japan) supplemented with 2% (w/v)

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dimethyl sulphoxide for 24 h or 48 h in darkness at room temperature. Germinating seeds were imbibed in distilled water as a control. Following imbibition, seeds were rinsed four times with distilled water and then sowed in pots containing mixed soil (2 parts red gravel: 1 part pearlite: 1 part peat moss: 1 part barnyard manure) and germinated in a culture room at 25±2°C with a 16-h photoperiod (50 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density). The germination rate of the control treatment was over 80%, while those of the 24 h and 48 h 0.05% colchicine treatments were 82% and 75.33% respectively. The germination rates of the 0.1% colchicine treatments were reduced, particularly for the 48 h treatment (Table 1).

After one month culture, approximately 50 mg of fresh apical leaves were excised from the control and all the experimental seedlings (one-month old) derived from the different colchicine treatments. Nuclei were extracted and analyzed for screening polyploidy using flow cytometry (CyFlow® Cube8, Partec, Germany). The excised leaves were chopped with a razor blade in a petri dish containing 400 μl of nuclei extraction buffer (CyStain® UV Precise P 05-5002, Partec), after which the homogenate was filtered through a 30-nm mesh. The filtrate containing released nuclei was stained with 1600 μl 4′,6-diamidino-2-phenylindole (DAPI) staining buffer (CyStain® UV Precise P 05-5002, Partec) for 1 min. All extraction steps were carried out on ice. Fluorescence emission was measured using a 365 nm long pass filter. In comparison with the diploid control sample (2×), for which the fluorescent intensity peak position was adjusted to channel 100 (Figure 1A), the peak of the tetraploid sample (4×) was expected to appear at channel 200 (Figure 1B). The result of flow cytometry screening showed that tetraploidy was detected in seedlings derived from the germinating seeds that were imbibed either for 24 h in 0.1% colchicine or for 48 h in 0.05% colchicine (induced 10–20% tetraploids, respectively; Table 1). No tetraploidy was detected in seedlings that were derived from the germinating seeds imbibed for 24 h in 0.05% colchicine or for 48 h in 0.1% colchicine (Table 1). This result suggests that 24 h of 0.05% colchicine treatment was insufficient to induce cell polyploidization, whereas 48 h of 0.1% colchicine treatment probably had some toxic effects, as suggested by the reduced germination rate and very low survivability after germinating of these seeds. Some mixoploids were also detected, especially in the seedlings derived from seeds imbibed for 48 h in 0.5% colchicine (Table 1). However, the mixoploid seedlings had poor ploidy stability and deformity, almost died after germinating.

The seedlings with putative polyploidy were continually grown in the culture room and some of their shoots were asexually propagated by cuttage. Mature plants and asexually propagated plants (three-months old) were analyzed again by flow cytometry. The results were similar in mature and propagated plants, indicating that their polyploidy were able to maintain after growth or asexual propagation.

Subsequently, 10 three-months old controls and putative polyploid plants that were analyzed by flow cytometry were selected for chromosome verification, cytological and morphological characteristics. To verify the flow cytometry results, root tips (each plant selected 3 roots from different positions) were excised

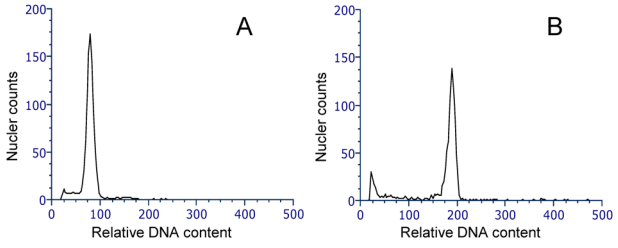

Figure 1. Ploidy identification by flow cytometric analysis. A: diploid control ($2n$=2×=22); B: tetraploid ($2n$=4×=44).

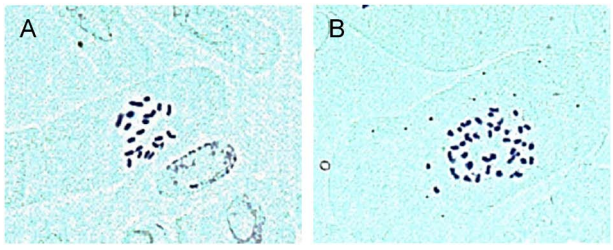

Figure 2. Verification of ploidy by microscope observation of chromosomes. A: diploid control ($2n$=2×=22); B: tetraploid ($2n$=4×=44).

Table 1. Effects of colchicine treatments on polyploidy induction.

Colchicine treatment		Germination rate (%)	Ploidy levels (%)		
Concentration (%)	Duration (h)		2×	2×+4×	4×
Control (0)	24	80.00±3.61	100	0	0
	48	91.33±4.73	100	0	0
0.05	24	82.00±2.65	100	0	0
	48	75.33±4.16	50.00±13.75	40.00±11.36	10.00±6.24
0.1	24	80.33±3.79	70.00±11.14	10.00±7.00	20.00±8.66
	48	60.67±4.51	100	0	0

Data represents means±standard deviations of 3 independent replications (n=100).

and pretreated in distilled water for 24 h at 4°C, then fixed with Carnoy's solution for 24 h. They were rinsed with distilled water, hydrolyzed in 1 N HCl for 10 min at 60°C, squashed under glass cover slips and stained with improved phenol fuchsin. The chromosome number of each sample was counted using a microscope at 40×magnification (DM2500, Leica, Germany). Cells from all the putative tetraploid plants had $2n=4\times=44$ chromosomes, which was twice the diploid control number of $2n=2\times=22$ chromosomes (Figure 2). This indicated that flow cytometry can be used for identification of polyploidy in *S. rebaudiana*.

Cytological and morphological characterization of the tetraploid plants focused on the number and size of the stomata, the types and densities of the trichomes, the chlorophyll content, and some vegetative (leaf, stem and root) features. Fully-expanded leaves from the 10 three-months old controls and verified polyploid plants were excised at the third leaf of the primary branch (from the apex down). Using forceps, an epidermal peel was taken along the vein of the abaxial surface of each leaf, stained with iodine, and then mounted on a microscope slide in a drop of distilled water under a coverslip. Stoma size and frequency were measured using a microscope at 10×20 magnification. The Fully-expanded leaves were also used for trichomes microexamination and chlorophyll measurement. The leaves were thoroughly washed with distilled water to remove dust particles and were cut to small pieces (2 mm^2). The pieces were dehydrated for 3 h at −20°C with a vacuum using tertiary butyl alcohol freeze-drying method (Sato and Tanaka 1990), and then were coated with gold by sputter coating unit at 10 Pascal vacuum for 10 s. Images were captured by a scanning electron microscope (SEM) (EVO18, Zeiss, Germany) using 5.0 kV acceleration voltage. The trichome densities were calculated by taking an average of three different microscopic fields at 60×magnification. The chlorophyll content index was measured using a portable chlorophyll meter (CCM-200 Plus, Apogee, USA). Vegetative features (leaf, stem and root) of the 10 three-months old controls and verified polyploid plants were investigated, together with measurements of their leaf size and dry weight. There was a remarkable difference in stoma size and density, with tetraploid plants having larger stomata, especially in terms of length. However, tetraploid plants only had about half the number of stomata of the diploid controls ($p<0.05$, Table 2 and Figure 3). Stoma size is associated with CO_2 gain and water discharge in plant photosynthetic and transpiration processes, and can be an indicator of ploidy levels (Moghbel et al. 2015). Three types of trichomes, including glandular trichomes (subspherical shape) and two types of non-glandular trichomes (long hair and short hair), were found on both adaxial and abaxial leaf surfaces that the former bore more trichomes than the latter (Figure 4).

Table 2. Stomata sizes and densities of diploid and tetraploid plants.

Strain	Stoma length (μm)	Stoma width (μm)	Stoma number (10×20 field)
Control (diploid)	15.63±0.03[a]	14.27±0.09[a]	9.00±0.81[b]
Tetraploid	27.16±0.10[b]	19.71±007[b]	4.30±0.47[a]

Data represents means±standard deviations of 3 independent replications ($n=10$). Different letters indicate significant differences detected using a two-sample *t*-test ($p<0.05$).

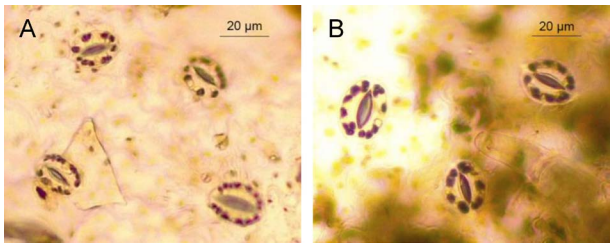

Figure 3. Stomata characteristics in diploid and tetraploid plants. A: diploid control; B: tetraploid. Stomata were obtained from the same part of the leaves in all treatments.

However, contrary to earlier report (Yadav et al. 2013), the tetraploid plants had approximately 2–4 times more glands than the diploid controls ($p<0.05$, Table 3). The glandular trichomes had been demonstrated that their apical part of the gland exposed above the leaf surface had subcuticular cavity which can be filled with secrete, and a positive correlation between glands' density and steviol glycosides content (Bondarev et al. 2010). Tetraploid plants also had a significantly higher chlorophyll content index than the diploid controls ($p<0.05$, Table 4), indicating that the former have higher photosynthetic capacity. Moreover, Most of the tetraploid plants were more vigorous than the controls, with large, thick leaves, stouter stems and shorter internodes ($p<0.05$, Table 5 and Figure 5) that contributed to the increase of biomass in the aerial part, but had not difference in roots. Both tetraploid and diploid plants began to put forth their blossoms after four-months culture, indicating that polyploidy had not been obviously influenced in the following anthesis. Some tetraploid plants were bagged before blossom, and self or cross (with the pollen grains from tetraploid or diploid plants) pollinations were conducted. Unfortunately, any type of viable pollinated seeds could not be obtained due to their self-incompatibility and producing infertile seed (Singh and Rao 2005; Yadav et al. 2013).

In order to distinguished the genetic variation of diploid and polyploidy, inter-simple sequence repeat (ISSR) technique was performed as described previously (Li 2015), which reported that 5 primers (screened out of 60 random primers) could produce polymorphic and separated well for diploids and tetraploids. The total genomic DNA were extracted from the leaves of the 10 three-months old controls and verified polyploid

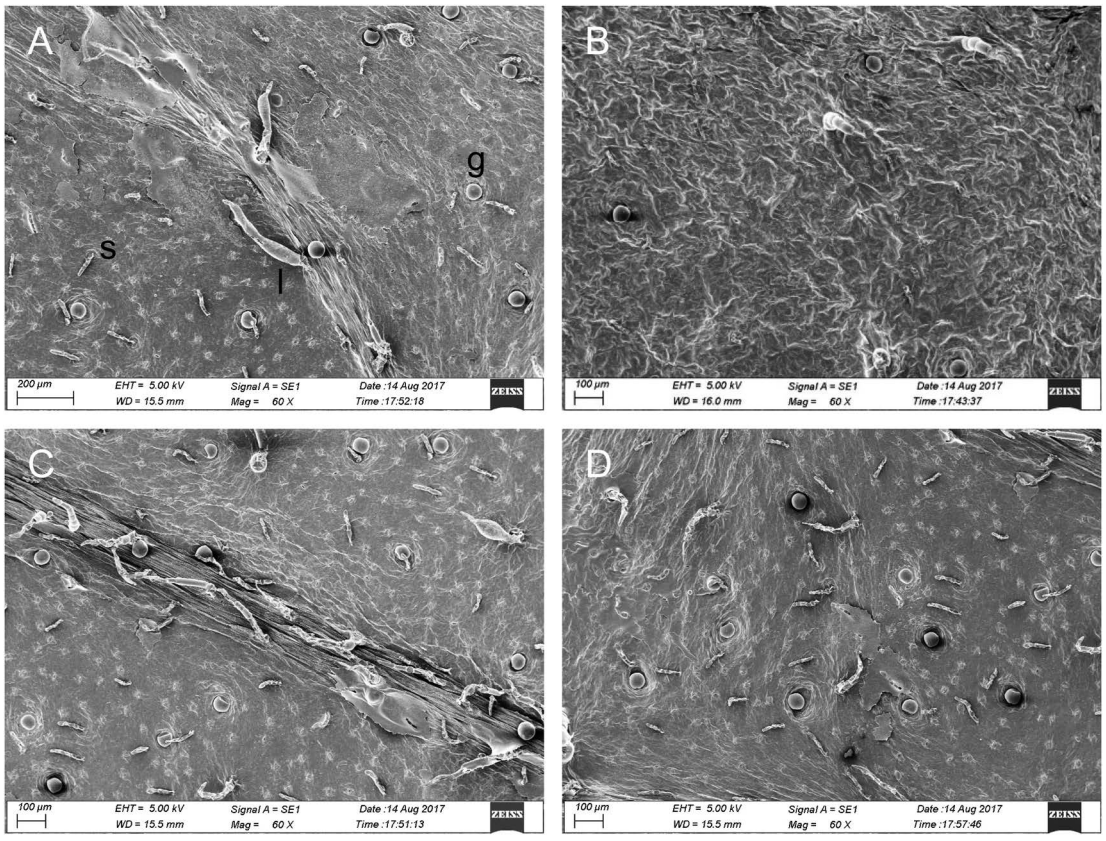

Figure 4. Trichome characteristics in diploid and tetraploid plants. A: diploid control (adaxial leaf surface); B: diploid control (abaxial leaf surface); C: tetraploid (adaxial leaf surface); D: tetraploid (abaxial leaf surface). g: glandular trichome (subspherical shape); l: non-glandular trichome (long hair); s: non-glandular trichome (short hair).

Table 3. Trichome densities of diploid and tetraploid plants.

Strain	Leaf surface	Glandular trichome (number/60×field) subspherical shape	Non-glandular trichome (number/60×field)	
			Long hair	Short hair
Control (diploid)	Adaxial	5.67±2.51[a]	4.67±2.51[a]	25.33±2.52[a]
	Abaxial	4.00±1.00[a]	3.67±1.15[a]	3.67±1.53[a]
Tetraploid	Adaxial	23.67±0.58[b]	16.67±6.80[b]	49.33±1.53[b]
	Abaxial	9.67±0.58[b]	8.33±1.15[b]	34.00±12.49[b]

Data represents means±standard deviations of 3 independent replications (n=10). Different letters indicate significant differences detected using a two-sample t-test (p<0.05).

Table 4. Chlorophyll content indices of diploid and tetraploid plants.

Strain	Chlorophyll content index
Controll (diploid)	29.00±2.66[a]
Tetraploid	41.03±2.86[b]

Data represents means±standard deviations of 3 independent replications (n=10). Different letters indicate significant differences detected using a two-sample t-test (p<0.05).

Table 5. Leaf characteristics of diploid and tetraploid plants.

Strain	Leaf length (cm)	Leaf width (cm)	Dry weight/leaf (mg)
Control (diploid)	4.45±0.21[a]	1.85±0.17[a]	4.50±0.50[a]
Tetraploid	5.45±0.37[b]	2.55±0.23[b]	6.20±0.31[b]

Data represents means±standard deviations of 3 independent replications (n=10). Different letters indicate significant differences detected using a two-sample t-test (p<0.05).

plants using the Plant Genomic DNA Kit (DP305, Tiangen, China). The ISSR PCR exhibited that the number of polymorphic fragments changed after diploid chromosome doubling (Figure 6) when using a P24 primer ($(GT)_8YG$).

The fully expanded leaves from 10 one-month, three-months (before flowering) and five-months (after flowering) old controls and verified polyploid plants were collected for analysis of steviol glycosides using a modified FAO JECFA Monographs 10 (2010) protocol. For each sample, 500 mg of dried and powdered leaves

Table 6. Steviol glycoside contents of diploid and tetraploid plants.

Period	Strain	Stevioside (%)	Rebaudioside A (%)
1-moth	Control (diploid)	2.01± 0.39[a]	3.76± 0.54[a]
	Tetraploid	4.89± 1.15[b]	6.91± 1.53[b]
3-moths	Control (diploid)	3.26±0.75[a]	7.10±0.86[a]
(before flowering)	Tetraploid	8.25±0.25[b]	10.10±0.63[b]
5-moths	Control (diploid)	2.18± 0.49[a]	4.26± 1.00[a]
(after flowering)	Tetraploid	5.83± 0.58[b]	7.28± 1.17[b]

Data represents means±standard deviations of 3 independent replications (n=10). Different letters indicate significant differences detected using a two-sample t-test (p<0.05).

Figure 5. Morphological characteristics of diploid and tetraploid plants. A: diploid control; B: tetraploid (with large, thick leaves, stouter stems and shorter internodes).

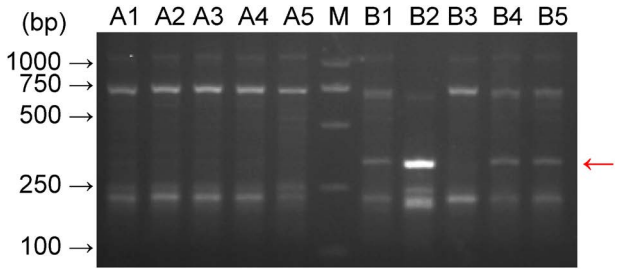

Figure 6. ISSR amplification products of diploid and tetraploid plants. A1, A2, A3, A4, A5: diploid controls; B1, B2, B3, B4, B5: tetraploids; M: DNA marker; Right red arrow indicates polymorphic fragments.

were dissolved with water : acetonitrile (70 : 30, v/v) for 60 min at 35±5°C, and made up to 50 ml. Stevioside and rebaudioside-A standards (Wako) were separately prepared by dissolving 10 mg of each in 10 ml with water : acetonitrile (70 : 30, v/v), and then diluted to 0.02, 0.04, 0.06, 0.08, 0.10, 0.15 and 0.20 mg/ml standard solutions. High performance liquid chromatography (HPLC) was performed using a gradient chromatographic system (LC-10A, Shimadzu, Japan) equipped with a NH_2 column (4.6×250 mm, Kromasil, Sweden) with the Mode SPD-10A variable wavelength UV-VIS detector at 210 nm. The column was equilibrated with water : acetonitrile (20 : 80, v/v), after which 10 μl of sample solution was injected and elution was performed using a linear gradient of water : acetonitrile (20 : 80, v/v) with a 0.8 ml/min flow rate over 60 min at 40°C (Ohta et al. 2010). HPLC analysis showed that abundances of the two main steviol glycosides, stevioside and rebaudioside A in both tetraploid and diploid plants, increased along with growth (1–3 months) until flowering (5 months). The results are in conformity with findings in previous reports (Kang and Lee 1981; Singh and Rao 2005; Yadav et al. 2011). Nevertheless, in whole growth stage the levels of stevioside and rebaudioside A in tetraploid plants were about 2.5- and 1.5-fold higher than those of the diploid controls (p<0.05, Table 6).

All data in this study were obtained from three independent replicates. Data were analyzed using Statistical Package for the Social Sciences 16.00 (SPSS, USA). Significant differences were evaluated using a Duncan's multiple range test.

This study shows that artificial polyploidy can be induced in *S. rebaudiana* using colchicine treatment. The concentration of colchicine and the duration of seed imbibition influenced the level of polyploidization. Treatments of germinating seeds with 0.05% colchicine for 48 h or with 0.1% colchicine for 24 h were most effective for inducing tetraploidy. Although polyploid *S. rebaudiana* plants had been achieved by treating a number of explants such as seeds (Ghonema et al. 2015) or imbibed seeds (Yadav et al. 2013), buds (Ghonema et al. 2015) or axillary buds (Chavan et al. 2014; Hegde et al. 2015; Mahadi 2012) by imbibing or spraying with different concentrations of colchicine, we found that using 2-day germinating seeds as starting material gave good results according to our preliminary experiment (data not shown), probably because the germinating seeds were at the time point that began to differentiate the apical meristem, and thus colchicine could effectively arrest mitosis that conduced to chromosome doubling.

Flow cytometry is very accurate for evaluating plant polyploidy as it sensitively measures the plant nuclear DNA content within seconds (Arumuganathan and Earle 1991). Our flow cytometry results were supported by chromosome counting and morphological characterizations, confirming that flow cytometry is also a powerful method for identification of polyploidy in *S. rebaudiana*.

Most of the tetraploid *S. rebaudiana* plants in this study exhibited apparent variations in cytological and morphological phenotypes compared with the diploid controls. The tetraploid plants had significantly larger stomata and higher chlorophyll content indices,

suggesting that the tetraploid plants may have higher photosynthetic and transpiration capacities. The tetraploid plants were vigorous with large, thick leaves, stouter stems, shorter internodes and had higher glandular trichome density. The remarkable differences in leaf morphology and ultrastructure in the tetraploid plants may explain their elevated levels of steviol glycosides, because *S. rebaudiana* leaves are the main tissue for both synthesis and primary accumulation of steviol glycosides (Yadav et al. 2011, 2013). In *S. rebaudiana*, the early steps of the steviol glycoside biosynthesis pathway resemble those of gibberellic acid biosynthesis. Briefly, geranylgeranyl diphosphate in the chloroplast is converted to (−)-kaurene, which is then converted into the tetracyclic diterpene steviol in the endoplasmic reticulum. Steviol then proceeds through a multi-step glycosylation pathway in the cytoplasm, to produce the various steviol glycosides that accumulate in the vacuole (Humphrey et al. 2006) or glandular trichomes (Bondarev et al. 2010). We found that the levels of the two main steviol glycosides, stevioside and rebaudioside A, were higher in the tetraploid plants than in the diploid controls.

The tetraploid *S. rebaudiana* plants generated in this study have improved characteristics, including vigorous growth, increased leaf biomass, high yields of steviol glycosides. The ISSR marker suggests that these characteristics may result from DNA polymorphisms changed after diploid chromosome doubling. Their polyploidy and characteristics are able to maintain after growth or asexual propagation. Since the seed germination of *S. rebaudiana* is commonly very poor due to infertile seed (Singh and Rao 2005; Yadav et al. 2013), these plants may be further selected for asexual propagation and distributed to local nurseries for commercial production.

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