Plant Cell Tiss Organ Cult (2010) 102:259–263
DOI 10.1007/s11240-010-9719-3

RESEARCH NOTE

Nuclear DNA content of *Hydrastis canadensis* L. and genome size stability of in vitro regenerated plantlets

Samuel G. Obae · Todd P. West

Received: 3 December 2009 / Accepted: 16 February 2010 / Published online: 4 March 2010

Abstract Knowing the genome size is an important step towards deciding and planning for genome sequencing of a given species. Using flow cytometry, nuclear DNA content of *Hydrastis canadensis* was estimated, and genome size stability of its in vitro regenerated plantlets were assessed. The nuclear DNA content of *H. canadensis* was estimated to be 2.62 ± 0.020 pg/2C. This is the first report to estimate nuclear DNA content of *H. canadensis*; therefore this study provides valuable information that will facilitate genome sequencing and subsequent molecular studies of this economically important medicinal plant. Comparison of genome size between in vitro regenerated (explant source was from wild plants) and wild plants of *H. canadensis* did not reveal any significant difference ($P \leq 0.05$) in nuclear DNA content. This suggests that micropropagation of *H. canadensis*, even after numerous sub-culturing and long-term culture periods produced in vitro plantlets with a stable genome size. These results provide further evidence that micropropagation techniques have the potential to be used as a source of planting stock, along with seeds, for restoring locally threatened *H. canadensis* wild populations and for commercial cultivation to supply the growing herbal market.

Keywords Germplasm conservation · Medicinal plant · Goldenseal · Yellow root · Flow cytometry · Tissue culture

S. G. Obae · T. P. West (✉)
Division of Plant and Soil Sciences, West Virginia University, 1090 Agriculture Sciences Building, Morgantown, WV 26505-6108, USA
e-mail: Todd.West@mail.wvu.edu

Abbreviations

AE	Arumuganathan and Earle's buffer
DTT	Dithiothreitol
FCM	Flow cytometry
mW	Milli watt
PI	Propidium iodide

Introduction

Goldenseal (*Hydrastis canadensis* L.), belongs to Ranunculaceae family and is native to deciduous woodlands of eastern North America (Foster and Duke 2000). It is an economically important medical herb, which in 1999 was ranked fifth among the top thirteen herbal products sold in the USA with sales exceeding $44 million (Blumenthal 1999). In 2004 and 2005, combined cultivated and wild-harvested goldenseal roots that entered the market as dried material was 42,000 and 41,000 kg respectively, an almost 50% increases from 21,000 kg in 2003 (AHPA 2007). Goldenseal preparations have been used traditionally for the treatment of eye infections, bronchitis, pharyngitis, and other digestive disorders (Foster and Duke 2000). Its medicinal properties are attributed to a group of secondary metabolites called alkaloids—mainly berberine and hydrastine. These alkaloids have antibiotic activities and therefore are effective against many bacterial infections (Seazzocchio et al. 2001). Very little is known about the genetics of secondary metabolism, and currently no model species exist for studying medicinal plants or plants with complex secondary metabolite production (Cole et al. 2007), therefore research in medicinal plants especially in areas of gene regulation and function in secondary metabolism has progressed slowly. It is quite evident that research and biotechnology in agriculture and horticulture

fields have benefited tremendously from availability of vast amount of information from fully mapped genomes of plant species such as rice (*Oryza sativa*) and *Arabidopsis* (*Arabidopsis thaliana*). Current plant model species however, have limited applications in research of medicinal plants with complex secondary metabolism. The search for a suitable model species for medicinal plants is ongoing, and it is anticipated that identifying and fully sequencing the genome of such a model species will greatly facilitate research into gene regulation and function, and biotechnology in medicinal plants.

Most of the goldenseal supplied to the market is harvested from the wild (AHPA 2007), and because of increasing demand; wild populations are overharvested and could potentially lead to loss of valuable germplasm. On the other hand, deforestation and habitat fragmentation as a result of land development activities greatly disturbs natural habitats of goldenseal and restricts gene flow among its populations. In 1997 goldenseal was listed as an Appendix II species of the Convention for International Trade and Endangered Species (CITES) of Wild Fauna and Flora (CITES 1997), and is designated as threatened in the US and Canada and therefore granted a high priority for protection (Bannerman 1997). Restoration of goldenseal into the wild is a major concern for plant conservationists, and commercial cultivation is necessary to supply the growing demand and reduce harvest pressure on wild populations. Conventional propagation of goldenseal through seed is slow given high mortality of seedling in early stages, and harvesting of the rhizome for vegetative propagation seems not feasible because the species is endangered and there is always a possibility of losing mother plants during this process (He et al. 2007). Micropropagation has been suggested as a potential technique to rapidly produce goldenseal planting stock for conservation and commercial cultivation, and thus far a number of optimal in vitro propagation protocols have been developed (Hall and Camper 2002; Bedir et al. 2004; Liu et al. 2004; He et al. 2007). However, with in vitro propagation often genetic disturbances do occur and this leads to somaclonal variations (Sliwinska and Thiem 2007). The resulting genetic variability can be consequential; therefore it is imperative to check for genome stability of in vitro produced plants especially if they are to be used as a source of planting stock to restore unique locally threatened wild population germplasm or to produce secondary metabolites with pharmacological activities.

Currently, very little is known about the genetics of secondary metabolism in goldenseal and its genome size is not even known. In this study, we sought to estimate the nuclear DNA content of goldenseal and assess genome size stability of its in vitro regenerated plantlets compared to wild plants using Flow cytometry (FCM). FCM is a fast, relatively inexpensive, and accurate technique for estimating nuclear DNA content, and has been successfully used to assess genome size stability of in vitro regenerated medicinal plants (Sliwinska and Thiem 2007).

Materials and methods

Plant materials

Pea [*Pisum sativum* cv. Ctirad (2C = 9.09 pg, Doležel et al. 1998)] was used as internal reference standard. Pea seedlings were grown in a greenhouse in 150 × 150 mm round pots (Dillen Products, Middlefield, OH, USA) using regular Sunshine Mix 1 (SUNGRO Horticulture, Seba Beach, Canada), under natural light conditions, temperature of 21 ± 3°C, and plants were hand watered daily. Goldenseal plants used in this study were collected from two wild populations in West Virginia (Monongalia County, WV, USA) and from in vitro propagated plantlets initiated from leaf explants of wild plants (Fig. 1a, b).

In vitro plantlet regeneration

In vitro propagated plants were initiated from wild plants through callus phase using a tissue culture protocol for goldenseal as described by Bedir et al. (2004). The cultures were transferred every 4 weeks to fresh medium and regenerated shoots were used for subsequent sub-culturing for shoot multiplication and were maintained in culture for more than 3 years prior to this study.

Nuclei preparation

Nuclei were obtained from young leaves of goldenseal (wild plants or in vitro plantlets) and pea by chopping them with new scalpel blades (No. 10 blade; PhytoTechnology Laboratories, Shawnee Mission, KS, USA) in AE nuclei isolation buffer (Arumuganathan and Earle 1991). The nuclei isolation buffer was two staged, herein referred as buffer A and B. Buffer A comprised of AE buffer supplemented with 50 µg/ml of RNase A (Product Number P4642; Sigma–Aldrich Chemical Company, St. Louis, MO, USA). RNase was heated to 90°C for 15 min to inactivate DNases. Buffer B comprised of AE buffer supplemented with 50 µg/ml propidium iodide (PI) (Product Number P4170; Sigma–Aldrich Chemical Company, St. Louis, MO, USA). All buffers were freshly prepared and stored on ice and in the dark at all times. Nuclei from both sample and

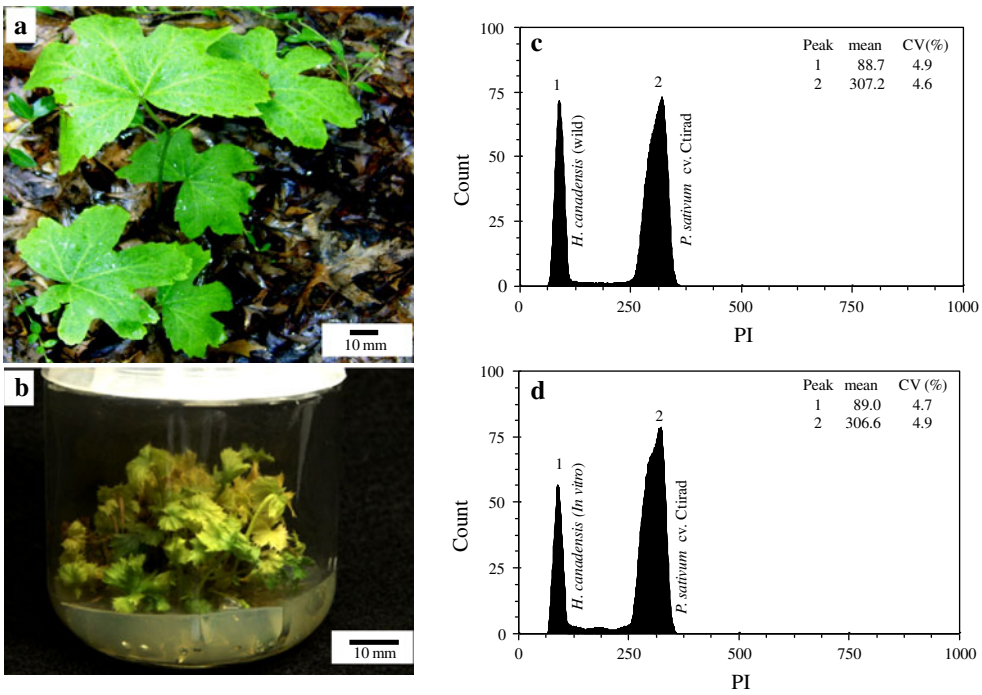

Fig. 1 Wild growing plants (**a**) and In vitro plantlets (**b**) of *H. canadensis*, and histograms of relative fluorescence intensity (**c** and **d**) obtained after analysis of their nuclei isolated simultaneously with that of *Pisum sativum* cv. Ctirad (2C = 9.06 pg, as an internal reference standard). Inset each histogram; the peak number, mean peak value, and coefficient of variation percentage (CV, %) value of each peak is given

reference standard were either separately or simultaneously isolated following a two step protocol; first, 40 mg of fresh young leaves were chopped on ice in a plastic Petri dish containing 1 ml of buffer A, and the resulting slurry was filtered through a 40 µm nylon filter (Product Number. 9086473; BD Falcon, Franklin Lakes, NJ, USA) and centrifuged (Marathon 16 km, Fisher Scientific) at 14,000 rpm for 20 s. The supernatant was discarded and the pellet re-suspended in 400 µl of buffer B. The stained nuclei were allowed to stand at 25 ± 1°C (room temperature) in the dark for 30 min before they were analyzed in a flow cytometer.

Flow cytometry

Stained nuclei were analyzed using a FACS Calibur flow cytometer equipped with a 15 mW 488 nm air-cooled argon-ion laser and Cell Quest Pro software (BD Bioscience, San Jose, CA, USA). Prior to running samples, linearity of the instrument was assessed using the BD DNA QC Particles Kit (Cat. No. 349523; BD Bioscience, San Jose, CA, USA). Doublets, disintegrated nuclei, and other cell debris were eliminated from analysis by gating on the forward and side scatter profiles of the samples. The gate was uniformly maintained across all samples in each run. For each sample 7,000–10,000 events (nuclei) were collected and the resulting histograms were analyzed using FCS Express Version 3 De NOVO software (FCS Research, Los Angeles, CA, USA).

Test for inhibitors

Some plants contain compounds in their cytosol that inhibit PI intercalation/fluorescence therefore compromising the reliability of estimating nuclear DNA contents particularly if detection of small differences in DNA content is desired (Price et al. 2000). One of the key recommendations of the 2003 Plant Genome Size Workshop held at the Royal Botanical Gardens, Kew (London, UK) was that all plant species whose nuclear DNA content is determined by FCM should be tested for the presence PI inhibitors. Presence of inhibitor is confirmed if mean peak position of reference standard is lower in the presence of target sample (Price et al. 2000). In this study we first tested for the presence of such inhibitors in the cytosol of goldenseal, by comparing the peak positions of pea nuclei that were separately processed and those that were simultaneously processed (co-chopped as one sample, with leaves layered on top of each other) with goldenseal.

Data collection and analysis

A total of 12 goldenseal plants were analyzed, 8 were wild grown and 4 were in vitro propagated plantlets. Each plant sample was analyzed in triplicates with each replicate measurement performed on different days. Nuclear DNA content of goldenseal was estimated using the linear relationship according to the following equation (Doležel et al. 2007):

$$\text{Sample 2C value (pg)} = \text{Reference 2C value} \times \frac{\text{Sample 2C mean peak position}}{\text{Reference 2C mean peak position}}$$

Conversion from picograms (pg) to base-pair numbers (bp) was done as follows: 1 pg DNA = 0.978×10^9 bp (Doležel et al. 2003). The results were analyzed using one-way analysis of variance in SAS (SAS Institute, Inc. 2003).

Results and discussion

From FCM analysis, the difference between mean peak position of pea nuclei prepared separately and those prepared by co-chopping with goldenseal was not significant (Fig. 2a, b). This implies that either addition of 0.25% DTT into the isolation buffer was able to minimize the cytosolic effects of goldenseal, or that the secondary compounds (alkaloids) present in goldenseal do not inhibit PI staining of pea DNA. The 2C nuclear DNA content of goldenseal was estimated to be 2.62 ± 0.020 pg. There was no significant difference ($P \leq 0.05$) in nuclear DNA content between wild plants and in vitro plantlets (Fig. 1c, d; Table 1). This implies that the genome size of goldenseal plantlets produced in vitro remained stable even after long-term culture (>3 years) with repeated sub-culturing (every 4 weeks). Variation in genome size has been observed in medicinal plantlets produced through tissue culture (Sliwinska and Thiem 2007). From this study it is inferred that goldenseal plantlets produced in vitro using the protocol developed by Bedir et al. (2004) maintains the same genome size as wild plants, and presents a potential for use in mass production of goldenseal for restoration of threatened wild populations or commercial cultivation.

Based on records from Plant DNA C-values Database (Bennett and Leitch 2005), goldenseal genome size falls in the lower end of C-value distribution of both its family (155 records, ranging from 1.10–50.02 pg/2C) and among angiosperms (total records 4427, ranging from 0.02–254.80 pg/2C). With our estimation, goldenseal genome

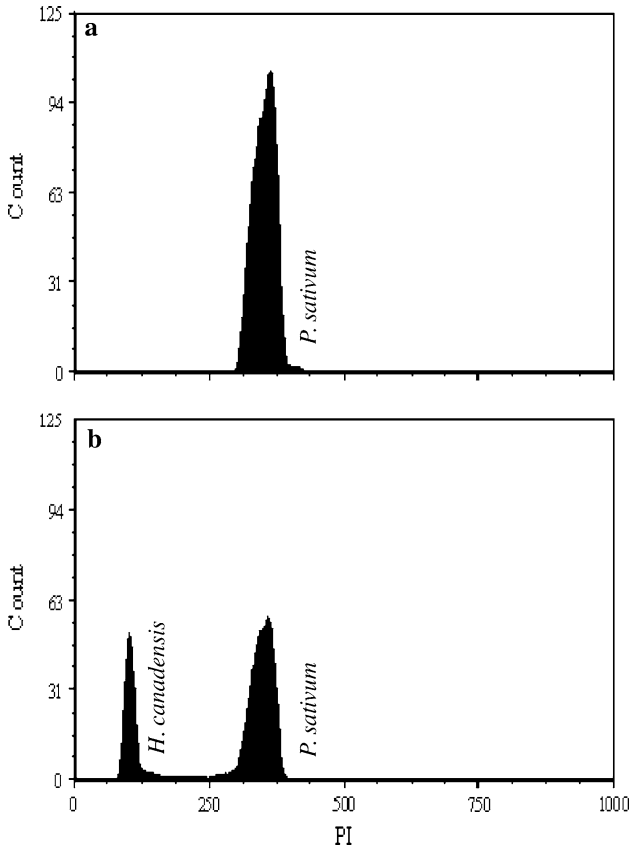

Fig. 2 Histograms of relative fluorescence intensity of *Pisum sativum* cv. Ctirad (internal reference standard) nuclei separately processed (**a**) and simultaneously processed (co-chopped) with *Hydrastis canadensis* (**b**)

Table 1 Nuclear DNA content and genome size (mean ± SD) of *Hydrastis canadensis* estimated with flow cytometry using *P. sativum* cv. Ctirad (2C = 9.09 pg) as an internal reference standard

Plant material	*n* (*R*)	Mean values*		CV (%)
		2C Nuclear DNA content ± SD (pg)	1C Genome size ± SD ($\times 10^9$ bp)**	
Wild plants	8 (3)	2.62 ± 0.022a	1.284 ± 0.011a	4.9
In vitro plantlets	4 (3)	2.62 ± 0.015a	1.283 ± 0.007a	4.7
All plants	12 (3)	2.62 ± 0.020	1.283 ± 0.010	

Sample number (*n*), replicate measurements per sample (*R*) are indicated

* Columns mean values followed by same letter are not significantly different at $P \leq 0.05$ (Tukey's test)

** 1 pg DNA = 0.978×10^9 bp according to Doležel et al. (2003)

size is approximately 8.2 times bigger than Arabidopsis (*Arabidopsis thaliana*, 0.32 pg/2C; Bennett et al. 2003) and 2.6 times that of rice (*Oryza sativa*, 1.0 pg/2C; Bennett and Smith 1991). Determining the genome size is among the initial steps in deciding on species and strategy for genome sequencing (Gregory 2005). This report is the first to estimate nuclear DNA content of goldenseal and therefore provides important information needed to facilitate further genomic and molecular studies of this economically important medicinal species, and for use in identifying a suitable candidate for a model species to study medicinal plants.

Acknowledgments We wish to thank Dr. Jaroslav Doležel of the Institute of Experimental Botany, Olomouc, Czech Republic for kindly providing *P. sativum* cv. Ctirad seeds used as internal reference standard, and Dr. Kathy Brundage of the West Virginia University Flow Cytometry Core Facility for her technical help with Flow Cytometry. The Flow Cytometry Core facility is supported by National Institutes of Health grant # RR016440, whereas this project was supported by the National Research Initiative Competitive Grant no. 2007-35318-18455 from the USDA National Institute of Food and Agriculture.

References

AHPA (2007) Tonnage survey of select North American wild-harvested plants, 2004–2005. American Herbal Products Association, Silver Spring, Maryland. Available via DIALOG. http://www.ahpa.org/Default.aspx?tabid=68#2004tonnagesurvey. Accessed on 18 Nov 2009

Arumuganathan K, Earle ED (1991) Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol Biol Rep 9: 229–241

Bannerman J (1997) Goldenseal in world trade: pressures and potentials. HerbalGram 41:51–52

Bedir E, Lata H, Schaneberg B, Moraes RM (2004) In vitro plant regeneration from leaf-derived callus in *H. canadensis* (*Hydrastis candensis*). Acta Hort 629:277–281

Bennett MD, Leitch IJ (2005) Plant DNA C-values database release 4.0. Royal Botanic Gardens, Kew. Availabbe via DIALOG. http://data.kew.org/cvalues. Accessed on 21 Oct 2009

Bennett MD, Smith JB (1991) Nuclear DNA amounts in angiosperms. Philos Trans R Soc Lond B Biol Sci 334:309–345

Bennett MD, Leitch IJ, Price HJ, Johnston JS (2003) Comparisons with Caenorhabditis (~100 Mb) and Drosophila (~175 Mb) using flow cytometry showgenome size in Arabidopsis to be ~157 Mb and thus ~25% larger than the Arabidopsis genome initiative estimate of ~125 MB. Ann Bot 91:547–557

Blumenthal M (1999) Herb market levels after five years of bloom: 1999 sales in mainstream market up only 11% in first half of 1999 after 55% increase in 1998. HerbalGram 47:64–65

CITES (1997) Proposal for the inclusion of *Hydrastis Canadensis*. Appendix II, CITES

Cole IB, Saxena PK, Murch SJ (2007) Medical biotechnology in the genus *scutellaria*. In Vitro Cell Dev Biol-Plant 43:318–327

Doležel J, Greilhuber J, Lucretti S, Meuster A, Lysák MA, Obermayer R (1998) Plant genome size estimation by flow cytometry: inter-laboratory comparisons. Ann Bot 82(Suppl A):17–26

Doležel J, Bartoš J, Voglmayr H, Greilhuber J (2003) Nuclear DNA content and genome size of trout and human. Cytometry A 51:127–128

Doležel J, Greilhuber J, Suda J (2007) Estimation of nuclear DNA content in plants using flow cytometry. Nature Prot 2(9):2233–2244

Foster S, Duke J (2000) A field guide to medicinal plants and herbs of eastern and central North America, 2nd edn. Houghton Mifflin Company, New York, pp 57–58

Gregory TR (2005) The C-value enigma in plants and animals: a review of parallels and an appeal for partnership. Ann Bot 95:133–146

Hall KC, Camper ND (2002) Tissue culture of goldenseal (*Hydrastis canadensis* L.). In Vitro Cell Dev Biol-Plant 38:293–295

He SS, Liu CZ, Saxena PK (2007) Plant regeneration of an endangered medicinal plant *Hydrasis canadensis* L. Scientia Hort 113:82–86

Liu CZ, Murch SJ, Jain JC, Saxena PK (2004) Goldenseal (*Hydrastis canadensis* L.): In vitro regeneration for germplasm and elimination of heavy metal contamination. In Vitro Cell Dev Biol-Plant 40:75–79

Price HJ, Hodnett G, Johnston JS (2000) Sunflower (*Helianthus annuus*) leaves contain compounds that reduce nuclear propidium iodide fluorescence. Ann Bot 86:929–934

SAS Institute, Inc (2003) SAS proprietary software version 9.1. SAS Inst, Cary, NC

Seazzocchio F, Cometa MF, Tomassini L, Palmery M (2001) Antibacterial activity of *Hydrastis canadensis* extract and its major isolated alkaloids. Planta Med 67:561–564

Sliwinska E, Thiem B (2007) Genome size stability in six medicinal plant species propagated in vitro. Biol Plant 51(3):556–558