BIOLOGIA PLANTARUM 50 (3): 457-460, 2006

BRIEF COMMUNICATION

A modified method of flow cytometric seed screen simplifies the quantification of progeny classes with different ploidy levels

A. KRAHULCOVÁ*[1] and J. SUDA*,**

*Institute of Botany, Academy of Sciences of the Czech Republic, CZ-252 43 Průhonice, Czech Republic**
*Department of Botany, Charles University, Benátská 2, CZ-128 01 Prague, Czech Republic***

Abstract

Flow cytometric analysis of ten bulked seeds is proposed to quantify particular embryo ploidy classes in *Hieracium*. The method is recommended *1*) for the detection and quantification of residual sexuality in facultative apomicts, which can generate progeny from heteroploid crosses, *2*) for the quantitative screening of pollen donors with different ploidy levels, based on the fertilization success of the maternal plant, and *3*) for the screening of parents producing a high proportion of polyhaploids.

Additional key words: facultative apomixis, polyhaploids, reproduction routes, seed progeny screening.

The flow cytometric seed screen method (FCSS) introduced by Matzk *et al*. (2000) exploits differences in the embryo/endosperm ploidy ratio of the seed progeny generated by various reproduction pathways (sexual reproduction *versus* apomixis, parthenogenesis, pseudogamy *versus* autonomous apomixis). Using FCSS, the reproduction mode of the maternal species can be detected, that is, whether it is obligatory or facultative with respect to particular pathways. This purpose is usually served by analysis of bulked samples of 50 fresh seeds. Obligatory sexuals or apomicts yield two peaks corresponding to the embryo and endosperm nuclei, respectively. When the non-obligatory reproduction mode occurs in the maternal plant, seeds originating via two or more pathways may be combined within one bulked sample. Consequently, several peaks will be displayed on the flow cytometric screen (Matzk *et al*. 2000). The method described has brought an important technical advance that can be used to good effect for the detection of distinct reproduction pathways, especially in facultative apomicts, *e.g.* in *Hypericum* (Matzk *et al*. 2001, Koperdáková *et al*. 2004), *Paspalum* (Cáceres *et al*. 2001), and *Coprosma* (Heenan *et al*. 2003).

However, exact quantification of particular seed categories (originating via distinct reproduction routes), is not feasible when bulked samples of 50 fresh seeds are analysed (Matzk *et al*. 2001). In this case, the different number of events in each peak does not reflect the proportion of particular seed categories with sufficient accuracy. Moreover, very rare seed categories are likely to remain undetected. For this reason, additional flow cytometric analyses of single seeds must be performed to determine the frequency of particular reproduction pathways in facultative apomicts (Cáceres *et al*. 2001, Matzk *et al*. 2001, Heenan *et al*. 2003, Koperdáková *et al*. 2004).

We tested the FCSS method as part of a project focused on investigating reproduction mode variation in a facultatively apomictic group of *Hieracium* subgen. *Pilosella*. Our model species, hexaploid *H. rubrum*, forms seed progeny via three distinct breeding systems (autonomous apospory – the prevailing mode, sexual reproduction, and haploid parthenogenesis). After crossing the maternal plant with tetraploid *H. pilosella*, five progeny classes were obtained, each with a distinct ploidy level corresponding to the particular reproduction

Received 29 October 2004, *accepted* 10 May 2005.
Abbreviations: FCSS - flow cytometric seed screen; DAPI - 4‘-6-diamidino-2-phenylindole; CV - coefficient of variation.
Acknowledgements: This work was supported by the Grant Agency of the Academy of Sciences of the Czech Republic (grant No. IAA6005203), by the Academy of Sciences of the Czech Republic (institutional research proposal No. AV0Z60050516), and partly by the Ministry of Education (project MSM0021620828). Lenka Mártonfiová (Botanic Garden of P.J. Šafárik University, Košice, Slovakia) is acknowledged for advice with the method applied. We are also grateful to Professor David Short (University of London, England) for the language revision.
[1] Corresponding author; fax: (+420) 267 750 031, e-mail: krahulcova@ibot.cas.cz

route (origin of embryo 2n + 0, n + 0, n + n, 2n + n, and n + 2n, according to the nomenclature of Harlan and De Wet 1975). Thus, reproduction pathways were here not inferred directly from the different proportional embryo/endosperm ploidy (as in Matzk *et al*. 2000), but indirectly, from the ploidy level of progenies arising subsequent to heteroploid hybridization. Previously, these five progeny classes were quantified by flow cytometry using bulked leaves of established seedlings together with a matroclinal individual as internal standard (Krahulcová *et al*. 2004). Our next intention was to adopt a like procedure for the investigation of seed progenies. Unlike ploidy screening in young seedlings, any re-analysis is not feasible for the bulked seed material. Accurate quantification of ploidy categories is thus a point of crucial importance.

To test the accuracy of flow cytometric acquisitions, two obligatory apomictic (diplosporous) species of *Hieracium* subgen. *Hieracium*, namely the tetraploid *H. nigritum* (2n = 4x = 36) and triploid *H. cochleare* (2n = 3x = 27), were selected. The seeds originated from emasculated maternal plants to ensure the homogeneity of embryo ploidy level. After harvesting, they were kept dry for two years. This storage still allowed of reliable identification of embryo peaks, without the cytometric analyses being biased by double-ploid endosperm peaks. As in other *Asteraceae*, a low amount of endosperm is typical of fresh seeds of *Hieracium*, and almost total endosperm loss occurs during embryo ripening and seed storage (Cronquist 1977, Hegi 1979, Erdelská 1981). Each bulked seed sample consisted of 10 seeds mixed in definite ratios, changing gradually from 1 triploid + 9 tetraploids to 9 triploids + 1 tetraploid (Table 1).

The flow cytometric analyses followed the protocol of Matzk *et al*. (2001) with certain modifications. The seeds were finely chopped with a new razor blade in a Petri dish containing 1 cm^3 of ice cold nuclei extracting and staining buffer (see below). The suspension was filtered through nylon tissue of 42 µm mesh-width into a tube and incubated for 30 min in the refrigerator (at 4 °C). Before measurement, the DAPI-stained nuclei were resuspended by gentle shaking of the tube. Their fluorescence intensity was determined by *Ploidy Analyser PA II* (*Partec GmbH*, Münster, Germany), equipped with HBO high pressure mercury lamp for UV excitation. The extracting and staining buffer (Matzk *et al*. 2001, modified) was prepared by dissolution of 0.107 g $MgCl_2 \cdot 6\ H_2O$, 0.5 g NaCl, 0.1 cm^3 Triton-100 and 1.211 g Tris (Trisma-Base) in deionized water. The volume was adjusted to 200 cm^3 and pH to 7.5, using 1 M HCl. This solution was stored at 4 °C in the dark. Before use, 4'-6-diamidino-2-phenylindole (DAPI) was added to the final concentration of 4 µg cm^{-3}.

The fluorescence of 5 000 nuclei was recorded for each sample, and the mean coefficients of variation (CVs) were 3.71 % and 3.30 % for the triploid and tetraploid peaks, respectively. Each set of 9 samples was re-analysed three times on different days (Table 1). The histograms were evaluated using *Flomax* software (*Partec GmbH*). The ratio between the number of particles in two resulting peaks was recorded, rounded to the order of tens, and compared with the expected distribution.

Table 1. Relative numbers of nuclei in peaks of *Hieracium cochleare* (2n = 3x) and *H. nigritum* (2n = 4x) obtained after flow cytometric analysis of 10 bulked seeds mixed in different proportions. Acquisitions yielding ratios different from the values expected (rounded to the order of tens) are denoted by *. Significant improvement was achieved after the subtraction and addition of the correction coefficient of 3 % to the observed percentages of triploid and tetraploid nuclei, respectively.

Number of seeds		Nuclei proportion [%] expected	observed	corrected	observed	corrected	observed	corrected
3x	1	10	12.9	9.9	13.3	10.3	10.8	7.8
4x	9	90	87.1	90.1	86.7	89.7	89.2	92.2
3x	2	20	18.8	15.8	23.9	20.9	21.1	18.1
4x	8	80	81.2	84.2	76.1	79.1	78.9	81.9
3x	3	30	29.1	26.1	32.2	29.2	34.8	31.8
4x	7	70	70.9	73.9	67.8	70.8	65.2	68.2
3x	4	40	42.2	39.2	48.5*	45.5*	47.5*	44.5
4x	6	60	57.8	60.8	51.5*	54.5*	52.5*	55.5
3x	5	50	54.2	51.2	54.1	51.1	53.0	50.0
4x	5	50	45.8	48.8	45.9	48.9	47.0	50.0
3x	6	60	62.1	59.1	58.1	55.1	61.7	58.7
4x	4	40	37.9	40.9	41.9	44.9	38.3	41.3
3x	7	70	69.3	66.3	76.0*	73.0	73.4	70.4
4x	3	30	30.7	33.7	24.0*	27.0	26.6	29.6
3x	8	80	84.8	81.8	83.8	80.8	80.8	77.8
4x	2	20	15.2	18.2	16.2	19.2	19.2	22.2
3x	9	90	91.5	88.5	91.5	88.5	91.4	88.4
4x	1	10	8.5	11.5	8.5	11.5	8.6	11.6

In 24 out of 27 runs (88.9 %), the proportion of particular seed categories inferred from the histogram corresponded to the expected ratio (Table 1). Generally, the frequency of lower ploidy (3x) was overestimated (in 23 runs, mean difference 3.28 %, range 0.81 - 8.53 %), a degree of underestimation occurred only in 4 runs (mean difference 1.21 %, range 0.70 - 1.89 %). This disproportion might be explained by the lower number of nuclei in tetraploid seeds (due to larger cell size), or more difficult nuclei release during preparation of the sample. To reduce the discrepancy between observed and expected values, we used a correction coefficient of 3 % that was added to and subtracted from the percentages of higher (4x) and lower (3x) ploidy, respectively. This modification led to the proper estimation of particular seed proportions in all but one run (Table 1). We found the modified method for quantification of particular ploidy classes in bulked seed samples to be very accurate. In comparison with flow cytometric analysis of a single seed (Matzk *et al*. 2001) and progeny quantification using established seedlings (Krahulcová *et al*. 2004), our new approach has several advantages particularly apparent in studies focused on the investigation of reproduction mode variation in facultative apomicts. Firstly, if the apomictic pathway that maintains the original ploidy of the maternal plant prevails, this seed category would be presented in each bulked sample and can thus serve as an internal standard. Consequently, the ploidy level of other progeny categories within the sample will be estimated more precisely than using FCSS on a single seed (lacking any internal standardization). Secondly, the method is less time-consuming than acquiring each seed separately, permitting the analysis of a considerably larger quantity of samples. Even very rare events can therefore be detected, and the frequency of minority ploidy classes can be assessed with higher accuracy. In addition, progenies with different ploidy levels may vary in germination capacity and/or seedling survival ability that may bias results based on the ploidy examination of established plants (less vital cytotypes are bound to be underestimated). This limitation is easily overcome by the proposed ploidy screening in intact stored seeds.

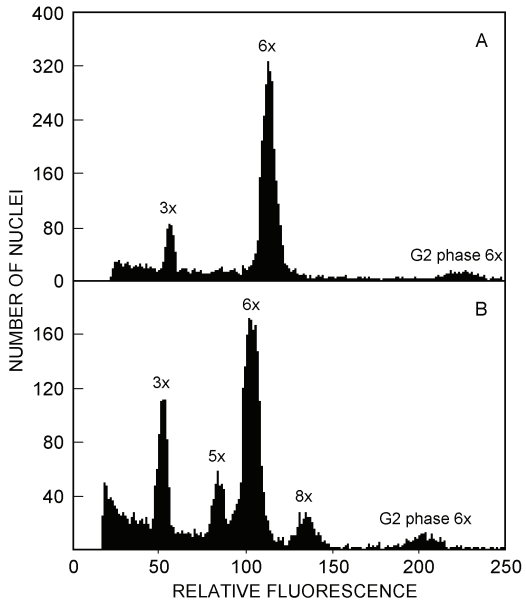

Fig. 1. Flow cytometric histograms demonstrating relative fluorescence of DAPI-stained embryonic nuclei isolated from seeds of facultatively apomictic *Hieracium rubrum* (2n = 6x). The bulked samples (10 seeds) contained either progeny originated autonomously after emasculation [*A*; 1 triploid (haploid parthenogenesis) + 9 hexaploids (apospory)], or progeny generated from crosses with tetraploid (2n = 4x) *H. pilosella* [*B*; 2 triploids (haploid parthenogenesis, n + 0 gametes) + 1 pentaploid (hybrid, n + n gametes) + 6 hexaploids (apospory, 2n + 0 gametes) + 1 octoploid (hybrid, 2n + n gametes)]. The prominent peak corresponding to hexaploid nuclei in G_1 phase of the cell cycle was used as an internal standard to determine the ploidy level of other embryo categories. The relative proportion of events in particular peaks was analysed using *Flomax* software.

Flow cytometric histograms showing relative fluorescence of embryonic nuclei isolated from ten bulked seeds of *Hieracium* subgen. *Pilosella* are presented in Fig. 1. If more than two ploidy categories were detected within the sample (Fig. 1*B*), the correction coefficient was applied only to the lowest and the highest ploidy. The described modified FCSS method is recommended as a beneficial tool for the detection and quantification of residual sexuality in facultative apomicts subjected to heteroploid crosses. Additional applications cover the quantitative screening of pollen donors with different ploidy levels according to their compatibility with the maternal genotype (useful in plant breeding), and the screening of parental plants that produce a high proportion of polyhaploids.

References

Cáceres, M.E., Matzk, F., Busti, A., Pupilli, F., Arcioni, S.: Apomixis and sexuality in *Paspalum simplex*: characterization of the mode of reproduction in segregating progenies by different methods. - Sex. Plant Reprod. **14**: 201-206, 2001.

Cronquist, A.: The Compositae revised. - Brittonia **29**: 137-153, 1977.

Erdelská, O.: Embryológia Krytosemenných Rastlín. [Embryology of Angiosperms]. - Veda, Bratislava 1981.

Harlan, J.R., De Wet, J.M.J.: On O. Winge and a prayer: the origins of polyploidy. - Bot. Rev. **41**: 361-390, 1975.

Heenan, P.B., Molloy, B.P.J., Bicknell, R.A., Luo, C.: Levels of apomictic and amphimictic seed formation in a natural population of *Coprosma robusta* (Rubiaceae) in Riccarton Bush, Christchurch, New Zealand. - New Zeal. J. Bot. **41**: 287-291, 2003.

Hegi, G. (ed.): Flora von Mitteleuropa VI/3. Pp. X - XLIV. - Paul Parey Verlag, Berlin - Hamburg 1979.

Koperdáková, J., Brutovská, R., Čellárová, E.: Reproduction pathway analysis of several *Hypericum perforatum* somaclonal families. - Hereditas **140**: 34-41, 2004.

Krahulcová, A., Papoušková, S., Krahulec, F.: Reproduction mode in the allopolyploid facultatively apomictic hawkweed *Hieracium rubrum* (Asteraceae, *H*. subgen. *Pilosella*). - Hereditas **141**: 19-30, 2004.

Matzk, F., Meister, A., Brutovská, R., Schubert, I.: Reconstruction of reproductive diversity in *Hypericum perforatum* L. opens novel strategies to manage apomixis. - Plant J. **26**: 275-282, 2001.

Matzk, F., Meister, A., Schubert, I.: An efficient screen for reproductive pathways using mature seeds of monocots and dicots. - Plant J. **21**: 97-108, 2000.