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Identification of 2n-Pollen Producing Interspecific Hybrids of *Lilium* Using Flow Cytometry

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Many plant species produce 2n-gametes, caused by meiotic polyploidization (Veilleux 1985). This phenomenon is the result of irregularities during the first or second meiotic division. The usually low frequency of 2n-gametes occurring in nature can be increased by environmental conditions like high temperatures but also by selection of suitable genotypes (van Tuyl and Stekelenburg 1988, Wagenvoort 1986). In lily, unreduced gametes are also found in some wide interspecific hybrids (Asano 1982, 1984, van Tuyl *et al.* 1986). Because of the great potential of meiotic polyploidization for breeding programmes (Harlan and de Wet 1975, Hermsen 1984b, Mendiburu and Peloquin 1977), the detection of 2n-gamete producers is of great importance. Conventional screening methods, like pollen measurements, meiotic and crossing analysis are very laborious and time consuming. In this paper we report a rapid method for tracing 2n-pollen producers in *Lilium*, based on flow cytometric measurements of nuclear DNA contents of the vegetative and generative cells of pollen.

Material and methods

Plant material

Table 1 lists the plant material used in this study. Chromosome numbers were assessed by counting chromosomes in root meristem cells. The Oriental hybrid between *L. auratum* (2n=24) and *L. henryi* (2n=24) was kindly provided by Dr. Y. Asano of Hokkaido University (Asano 1980, 1982, 1984). The diploid *L. longiflorum* 'Gelria' was developed by own breeding research. The tetraploid *L. longiflorum* 'Gelria' was obtained after colchicine treatment (Kwakkenbos and van Tuyl 1986, van Tuyl and Kwakkenbos 1986). The Asiatic hybrid plants *L.*×'Enchantment'×*L. pumilum* (Fig. 1) were derived from our interspecific crossing programme (van Tuyl *et al.* 1986). Interspecific hybrids were obtained using the intrastylar pollination technique in combination with embryo culture (Asano and Myodo 1977a, 1977b, Asano 1978, van Tuyl *et al.* 1986).

Flow cytometry

Plants were grown under glasshouse conditions at 15–25°C. Mature pollen was collected just after anthesis and dried in an exsiccator. Leaf and root material was harvested at the same time. Nuclear samples were prepared from pollen, leaves and roots by chopping with a razor blade (Galbraith *et al.* 1983) in an ice-cold commercial Partec buffer (Partec AG, Alesheim, Switzerland) containing 6 μM DAPI (4,6-diamidino-2-phenylindole). After chopping, the suspension was passed through a 20 μm nylon mesh. The relative DNA-content of the nuclei was measured in a flow cytometer ICP-22 (Ortho Diagnostic Systems, Beerle, Belgium) supplied with a high pressure mercury lamp, using the excitation filters UG-1, BG-38, KG-1 and TK-450 together with the BP 440–500 filter in the emission beam. The

flow rate during the analysis was 10–20 nuclei/s. The fluorescent signals, proportional to the quantity of DNA per nucleus, were measured with the multichannel distribution analyzer 2103 (Ortho Diagnostic Systems) and presented as histograms in which nuclear DNA contents were expressed as C-values.

Measurement of pollen size

Pollen size of the *L.* ×'Enchantment'×*L. pumilum* hybrids was measured in random samples of 200 pollen using a light microscope at a magnification of 1000×.

Fig. 1. The flower of the *L.* ×'Enchantment'×*L. pumilum* hybrid.

Table 1. The *Lilium* hybrids and species with corresponding chromosome and IVT*-numbers

Hybrid/species	Chromosome number	IVT-number	Hybrid group**
L. auratum×*L. henryi*	2n=24	82111	O
L. longiflorum 'Gelria'	2n=24	78372-1	S
L. longiflorum 'Gelria' tetraploid	2n=4x=48	84184	S
L. ×'Enchantment'×*L. pumilum*	2n=24	79418-1/6	A

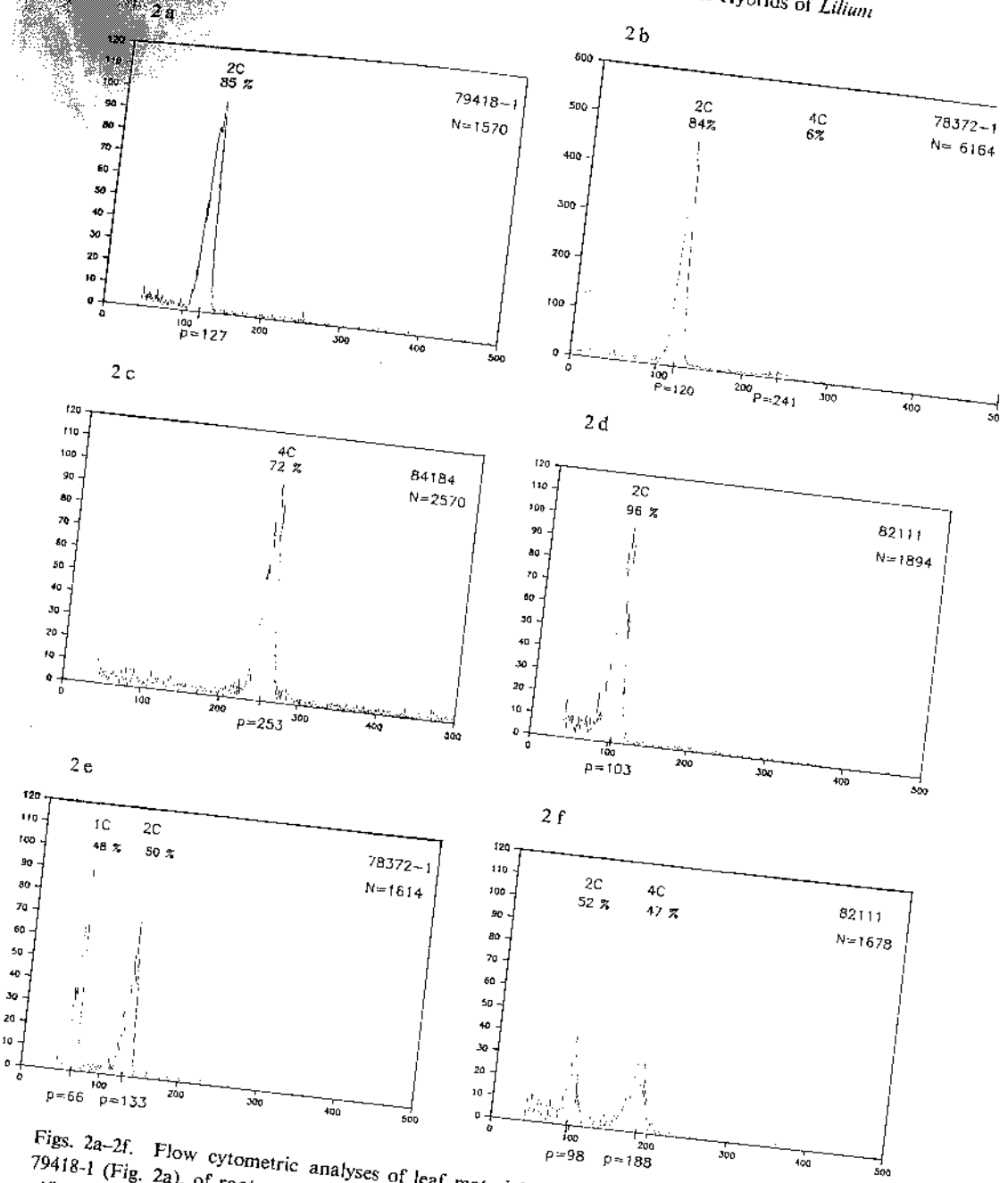
*IVT=Instituut voor de Veredeling van Tuinbouwgewassen.
**A=Asiatic hybrid, O=Oriental hybrid, S=Species.

Results

Flow cytometry—general remarks

Figs. 2a–f and 3a–f show histograms obtained with flow cytometry of nuclei from various tissues of the plants listed in Table 1. On the X-axis the channel numbers of the flow cytometer multichannel distribution analyzer are plotted ranging from 40 to 500, the Y-axis represents the number of fluorescence signals recorded per channel.

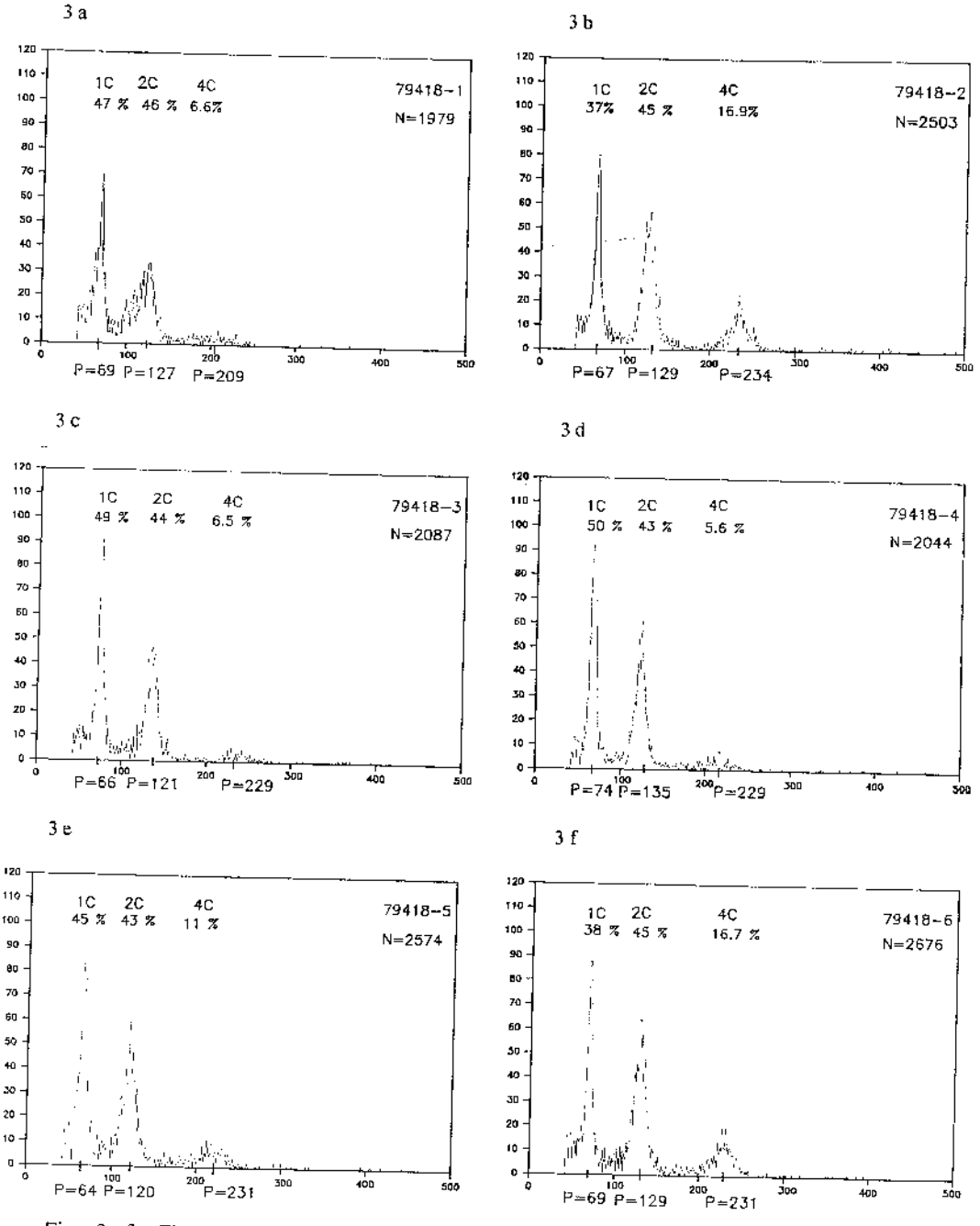
Damaged nuclei as well as cell-wall fragments smaller than 20 μm in size gave rise to background signals which were registered predominantly at lower channel numbers. For this reason it was decided not to record counts below channel 40. Beyond channel 500 no counts were recorded as well. By this the flow cytometer was adapted to register the signals of all intact nuclei. In the histograms of Figs. 2a–f and 3a–f the channel number in which most counts are found, indicated with P, is used to mark a peak's position on the X-axis. Channel numbers approximately halfway in between two P-channels are used to



Figs. 2a–2f. Flow cytometric analyses of leaf material from *L.* × 'Enchantment' × *L. pumilum* 79418-1 (Fig. 2a), of root material from diploid (Fig. 2b) and from tetraploid (Fig. 2c) *L. longiflorum* 'Gelria', of root material from the diploid Oriental hybrid *L. auratum* × *L. henryi* (Fig. 2d), of pollen from diploid *L. longiflorum* 'Gelria' (Fig. 2e) and of pollen from the diploid Oriental hybrid *L. auratum* × *L. henryi* (Fig. 2f).

Figures 2a–f and 3a–f: X-axis: relative DNA amount per nucleus, Y-axis: number of nuclei. N = total number of counts registered in channels 40 to 500; percentages in figures related to N. P = peak position, i. e. channel with highest number of counts in a peak. In Figs. 2d and 2f (*L. auratum* × *L. henryi*) the 2C- and 4C-peaks range from channels 40 to 149 and from channels 150 to 300, respectively. In Figs. 2a, 2b, 2c, 2e and 3a–f (Asiatic hybrid and *L. longiflorum*, which are comparable with respect to nuclear DNA content) the 1C-, 2C- and 4C-peaks are ranging from channels 40 to 89, 90 to 179 and 180 to 300, respectively. See text for further information.

identify the upper and lower limits of peaks. For reasons explained above channel 40 is used as the lowest limit. Channel number 300 is used as upper limit because the number of counts registered beyond this channel was negligible.



Figs. 3a–f. Flow cytometric analyses of pollen from the six diploid Asiatic *L.* × 'Enchantment' × *L. pumilum* hybrids.

Flow cytometry with somatic tissues

Flow cytometric analysis of nuclei from the chopped leaf material of the diploid Asiatic hybrid *L.* × 'Enchantment' × *L. pumilum* 79418-1 (Fig. 2a) shows one large peak at the 2C-

level. The maximum number of counts is found in channel 127. Nuclei isolated from the diploid *L. longiflorum* 'Gelria' give rise to one large peak at the 2C-level (Fig. 2b) with most counts in channel 120 and a small peak at the 4C-level with most counts in channel 241. Nuclei isolated from the tetraploid *L. longiflorum* 'Gelria' give rise to a single large peak at the 4C-level (Fig. 2c) with most counts in channel 253. Therefore the 2C-amount of nuclear DNA of *L. longiflorum* is the same as that of the Asiatic hybrid. Analysis of the diploid Oriental hybrid *L. auratum* × *L. henryi* shws one peak at the 2C-level (Fig. 2d) with the maximum number of counts in channel 103. Apparently, the 2C-DNA amount in nuclei of the Oriental hybrid *L. auratum* × *L. henryi* is about 25% less than in diploid nuclei of both *L. longiflorum* and the Asiatic hybrid *L.* × 'Enchantment' × *L. pumilum*.

Flow cytometry with pollen

Pollen of the diploid *L. longiflorum* 'Gelria' (Fig. 2e) gives rise to two peaks of virtually identical size. When compared with the histogram of nuclei from roots of the diploid and the tetraploid *L. longiflorum* 'Gelria' (Figs. 2b–c) these peaks are located at the 1C- and the

Fig. 4. A microscopic view of a pollen sample from *L.* × 'Enchantment' × *L. pumilum* 79418-2, with small (empty), normal and large pollen grains. ×100.

2C-level respectively. An additional peak at the 4C-level is found when pollen from plants of the diploid interspecific Asiatic hybrid *L.* × 'Enchantment' and *L. pumilum* is analyzed (Figs. 3a–f; cf. Fig. 2a). The percentage of counts covered by the 4C-peak is different for individual hybrid plants, varying from 5.6% (Fig. 3d) to 16.9% (Fig. 3b).

Peaks obtained with nuclei from pollen of the diploid interspecific hybrid between *L. auratum* and *L. henryi* (Fig. 2f) are of identical size and located at the 2C- and 4C-level respectively when compared to Fig. 2d.

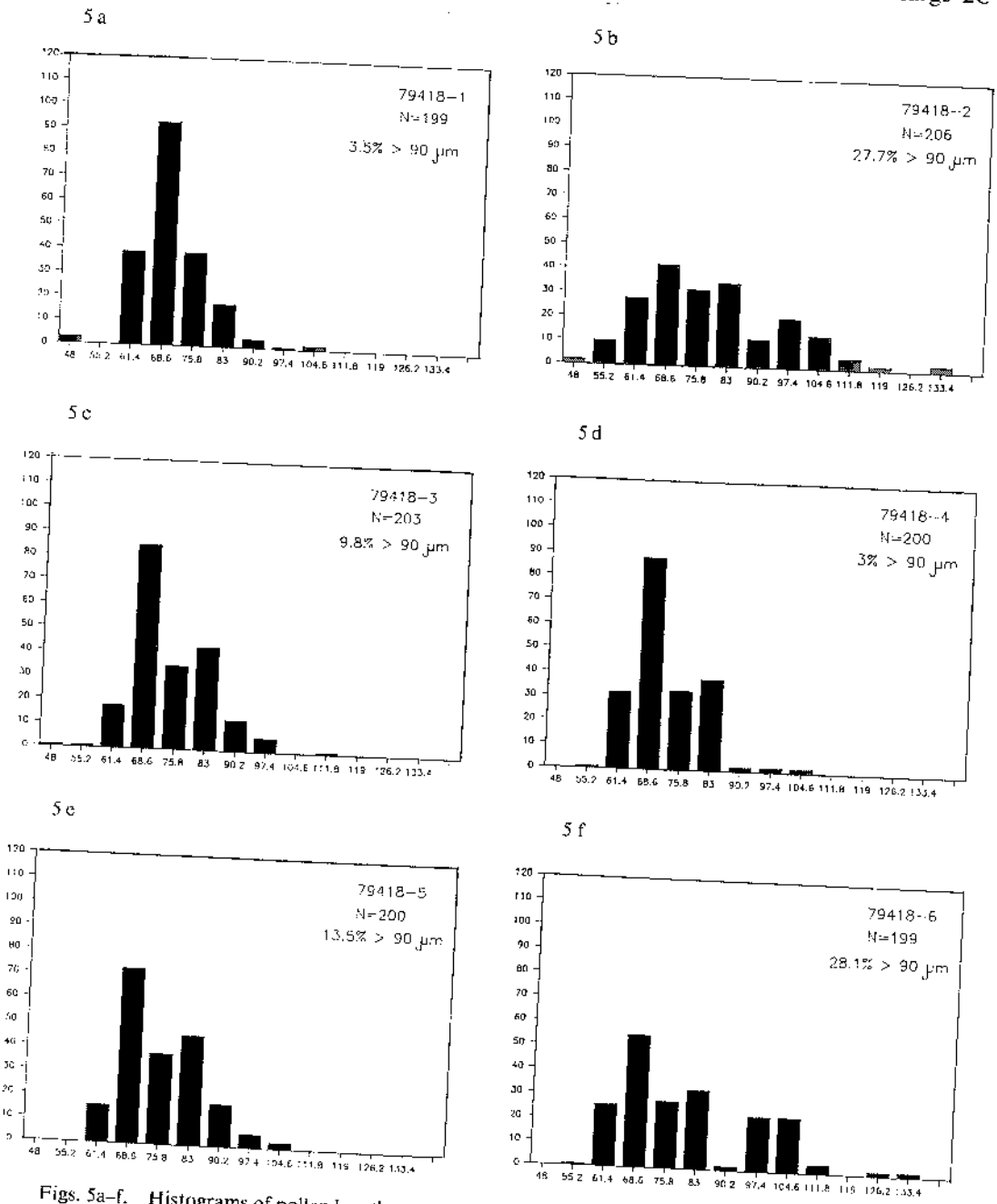
Pollen size of L. × *'Enchantment'* × *L. pumilum*

Microscopic observations of pollen from the *L.* × 'Enchantment' × *L. pumilum* hybrids, showed a large variation in pollen size (Fig. 4). Length of the pollen grains varied between 45 and 137 μm. Several hybrid plants had a relatively high percentage of large pollen (>90 μm). This is shown in Figs. 5a–f representing histograms in which pollen lengths are grouped in classes of 7.2 μm. These data were obtained with the same plants that were used for the flow cytometric determinations. Therefore, Figs. 5a–f correspond with Figs. 3a–f.

Discussion

Flow cytometry with somatic tissues

Differences between diploid and tetraploid genotypes could be detected using flow cytometry with DAPI stained nuclei from roots and leaves. In the diploid genotypes a predominant 2C-peak is visible. As expected nuclei from roots or leaves of tetraploid plants show twice that DNA content as a 4C-peak (Figs. 2a–c). In Fig. 2b besides a large 2C-



Figs. 5a–f. Histograms of pollen lengths grouped in classes of 7.2 µm of the six *L.* × 'Enchantment' × *L. pumilum* hybrids. X-axis: pollen length (µm), Y-axis: number of pollen. N is the total number of pollen measured.

cells were present as dyads. Apparently, the ripening of the remaining tetrads into mature haploid pollen is impeded. Van Tuyl and Kwakkenbos (1986), crossing the hybrid between *L. auratum* and *L. henryi* as the male parent with diploid Oriental cultivars, exclusively found triploids in the progeny which were obtained through embryo culture. This agrees with the observations from the present study as well as those from Asano (1984), indicating that only 2n-pollen took part in fertilization.

Meiotic polyploidization can be the result of First Division Restitution (FDR) or Second Division Restitution (SDR). Distinction between FDR and SDR has important consequences for the extent at which homo- or heterozygosity of the parental genotype is recovered in its 2n-gametes (Hermsen 1984a). With flow cytometry, however, no discrimination can be made between restitution nuclei derived from FDR or SDR, since both types contain the same amounts of DNA. Therefore it was not possible to verify Asano's (Asano 1984) conclusion that the majority of the 2n-pollen produced by the Oriental hybrid *L. auratum* × *L. henryi* are derived from FDR.

Application of meiotic polyploidization is of great importance for the breeding of lilies. Tracing 2n-gamete producers however is very laborious. Although the phenomenon is not uncommon in nature, percentages of producers as well as 2n-gametes produced, are generally small. The low frequency of 2n-gametes can be influenced by environmental conditions like high temperatures and by selection of suitable genotypes (van Tuyl *et al.* 1988, Wagenvoort 1986). To identify potential 2n-pollen producing genotypes large populations of plants have to be screened. For this purpose, a flow cytometer was used in this study. From the results of screening a number of lily genotypes for 2n-pollen production, flow cytometry appeared to be very promising.

Summary

DNA-content of nuclei from pollen was determined using flow cytometry. The pollen from diploid genotypes gave rise to a 1C- and a 2C-peak of identical size, representing the vegetative and the generative nuclei. Pollen of the interspecific hybrid (*L. auratum* × *L. henryi*) presented only 2C- and 4C-peaks, while in the interspecific hybrids (*L.* × 'Enchantment' × *L. pumilum*) 1C-, 2C- and 4C-peaks were distinguished. The different C-levels, representing the DNA-contents of the generative and vegetative nuclei in G1 and G2 phase respectively, can be related to haploid and diploid pollen. Results of a meiotic analysis, a number of crossing experiments and pollen measurements agreed with the outcome of the flow cytometric determinations. The flow cytometry method enables the large scale screening of collections of genotypes for their potential of 2n-pollen production.

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