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PROTOPLASMA

Polyploidization in embryogenic microspore cultures of *Brassica napus* L. cv. Topas enables the generation of doubled haploid clones by somatic embryogenesis

X. XuHan[1,2], H.-C. Jing[3], X.-F. Cheng[1], A. Iwanowska[1], H. Kieft[1], J. H. W. Bergervoet[3], S. P. C. Groot[3], R. J. Bino[3], and A. A. M. van Lammeren[1,*]

[1] Laboratory of Plant Cytology and Morphology, Wageningen Agricultural University, Wageningen, [2] Institut de la Recherche Interdisciplinaire de Toulouse, Toulouse, and [3] Center for Plant Breeding and Reproduction Research, Agricultural Research Department, Wageningen

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Summary. Embryogenic microspore and pollen culture followed by subculture of microspore-derived plantlets enabled the production of clones of *Brassica napus* cv. Topas. Flow-cytometric analysis revealed that most microspore- and pollen-derived embryos (pEMs) were haploid initially. Spontaneous diploidization occurred at the globular stage of the pEMs, and was expressed as the relative increase of the 2C and 4C nuclear DNA content. Diploidization occurred throughout various organs of the pEMs and resulted in the formation of haploid and doubled haploid chimerics. In some embryos, nearly all cells were doubled haploid. From early cotyledon stage onward, pure haploid embryos were not observed anymore. At late cotyledon and germination stages, pure doubled haploid embryos and plantlets increased in number. Tetraploid pEMs were found occasionally. A culture regime was established to induce somatic embryos on the pEM-derived young plantlets. The ploidy of the somatic embryos varied highly and tended to be the same as that of the tissue at the initiation site on the pEM-plant. The results show that during the embryogenic development of *B. napus* microspores, spontaneous diploidization occurs at globular stage, and increases progressively, resulting in the formation of chimerical haploid and doubled haploid plants as well as pure doubled haploid plants; ploidy neither affects pEM development at embryo developmental stages nor somatic embryogenesis, that starts on young pEM-derived plantlets; doubled haploid somatic embryos can be cloned from single pEM-derived plantlets; and doubled haploid embryos develop to fertile plants.

Keywords: Androgenesis; *Brassica napus*; Ploidy; Pollen; Rapeseed; Somatic embryogenesis.

* Correspondence and reprints: Laboratory of Plant Cytology and Morphology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands.

Introduction

Homozygous seeds and plants are essentially ideal materials for genetics, molecular biology, biotechnology, and ultimately for plant breeding. They provide research and commercial genetic sources of which, within a variety, a largely identical genotype is guaranteed (Jain et al. 1996).

Besides conventional selfing and backcrossing, production of haploids can be a way to produce homozygotes. Various methods have been used, such as ovary and ovule culture (Yang and Zhou 1982), anther culture (Guha and Maheshwari 1964, Lichter 1981), incongruous distant pollination (Lacadena 1974) such as the so-called "bulbosum" technique (Jensen 1977), pollination with irradiated pollen or pollination of irradiated pistils (Gerassimova 1936, Gerlach-Cruse 1970), and culture of microspores and young pollen (Guha and Maheshwari 1966, Lichter 1982). In general, microspore and pollen culture is one of the most frequently used methods to produce haploids in angiosperms. After the doubling of chromosomes, fertile doubled haploid plants can be obtained (Sangwan and Sangwan-Norreel 1990, Ferrie et al. 1995). Thus, microspore embryogenesis provides a shortcut for the production of homozygotes. But the production of clones derived from a single microspore-derived embryo via seeds obtained from the mature microspore-derived plant might still face

self-incompatibility and life-span problems. Somatic embryogenesis is so far the most applicable method to produce large amounts of genotypically identical embryos in vitro (Thorpe 1995). Somatic embryogenesis induced on microspore-derived embryos could give rise to cloned lines. Furthermore, among in vitro propagation techniques, somatic embryogenesis has low somaclonal variation, especially when a callus phase is absent (i.e., direct somatic embryogenesis). Thus, it would be beneficial to combine these two in vitro embryogenesis methods to produce pure clones in both haploids and doubled haploids. *Brassica napus* cv. Topas is especially suited to study the generation of clones derived from microspore-derived plantlets because the induction of embryogenesis in microspores and pollen is relatively easy in the cultivar. It does not require the use of exogenous auxins and leads to high numbers of embryos (Pechan and Keller 1988, Hause et al. 1993, Binarova et al. 1993, Iwanowska et al. 1998). In addition it was found that microspore-derived embryos exhibited spontaneous change in ploidy (Hause et al. 1994).

The development of natural haploids, e.g., gametophytes of mosses, ferns, gymnosperms, and angiosperms, has been widely investigated (Johri 1982). Spontaneous diploidization has not been found to occur at a scale comparable to that observed during microspore embryogenesis (Baldursson and Ahuja 1996). For example, 30% to 40% of pollen- and microspore-derived embryos (pEMs) in *B. napus* acquired increased ploidy because of spontaneous diploidization (Hause et al. 1994, Foisset et al. 1997).

In the present study we aim at combining microspore culture and somatic embryogenesis to obtain clones in *B. napus*. An efficient and simple culture regime is described to produce doubled haploid clones. The spontaneous diploidization occurring in microspore culture is investigated in more detail with flow cytometry to determine the dynamics and embryonal regions of diploidization.

Material and methods

Plant material

Brassica napus L. cv. Topas plants were grown in a phytotron with 16 h light and 8 h dark photoperiods, at 18 ± 0.5 °C and 60% relative humidity. The ploidy levels of the nuclei of the plants were determined by analyzing leaves of 100 plants by flow cytometry. Flower buds, ranging from 3.4 to 3.8 mm in length, were collected for the isolation of microspores and young pollen for in vitro culture.

Microspore embryogenesis

Isolation of microspores and young pollen was according to the method described by Pechan and Keller (1988). Microspore development in planta was determined by staining nuclei with 0.5 µg of 4′,6 diamidino-2-phenylindole (DAPI; Sigma Chemical Co., St. Louis, Mo., U.S.A.) per ml, and only microspores at a late developmental stage and pollen at early bi-nucleate stages were used for the induction of embryogenic development. Microspores and young pollen were cultured in NLN medium (Lichter 1982), pH 6.0, with 13% sucrose but without potato extract. They were cultured at a density of 5×10^4 microspores per ml, in the darkness, at 32 °C for 2 days (i.e., culture under embryogenic condition). Cultures were then transferred to a phytotron, and kept on a rotary shaker at 50 rpm, at 25 ± 0.5 °C in the darkness. After one week, when proembryos had developed, the cultures were exposed to a photo-period of 16 h light and 8 h dark. Cultures were maintained in the NLN medium for 4 weeks until germination stage of the pEMs, i.e., when roots developed. Some plantlets were transferred to soil, and flowering plants were tested for fertility.

Somatic embryogenesis

Germinated pEMs were transferred onto agar-solidified MS-20 medium without growth regulators, pH 5.8, containing MS salts (Murashige and Skoog 1962) and 2% (w/v) sucrose. Culture was at 16 h light and 8 h dark photoperiod and 25 ± 0.5 °C allowing plantlet formation. Somatic embryogenesis occurred spontaneously on the plantlets. Some somatic embryos and the plantlets on which they arose were analyzed for nuclear ploidy levels. Other somatic embryos were allowed to develop to plantlets, which were then transferred to soil to obtain flowering plants within 4 months. Fertility of those plants was tested according to seed set.

Flow cytometric measurement of ploidy levels

Nuclear ploidy levels in microspores, pollen, pEMs, pEM-derived plantlets, somatic embryos, and mature plants were determined by flow cytometry. Sample preparation and measurement of ploidy levels was adopted from Bino et al. (1992). Briefly, nuclei were isolated by chopping the various plant tissues with a sharp razor blade in phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8 mM Na_2HPO_4, 1.47 mM KH_2PO_4) supplemented with 10 mM dithiothreitol, pH 7.3, to isolate nuclei. The nuclei were collected by filtration through a nylon membrane (pore size, 44 µm) and stained with propidium iodide (PI; 10 µg/ml). Measurement was carried out with an XL-MCL flow cytometer (Coulter Corp.). In general 5,000 to 10,000 nuclei were analyzed per measurement (all figures except Fig. 4 A), but for the individual globular and heart-shaped pEMs 500 nuclei were analyzed (Fig. 4 A). Plants to be used for collecting microspores and pollen were tested for their ploidy levels, leaves were measured and used as control.

Microscopy

For light microscopy, microspore-derived embryos and excised somatic embryos were fixed at room temperature for 8 h in a mixture of 4% (w/v) glutaraldehyde and 1% (w/v) paraformaldehyde in PBS. After rinsing in PBS, they were post fixed in 0.5% osmium tetroxide in PBS for 8 h at 4 °C, then rinsed in PBS, and dehydrated in a graded ethanol series. Samples were embedded in a low-viscosity resin (Spurr 1969). Semithin sections with 4 µm thickness were stained with 1% (w/v) Toluidine Blue O.

For scanning electron microscopy (SEM), somatic embryos on microspore-derived plantlets were fixed and dehydrated as

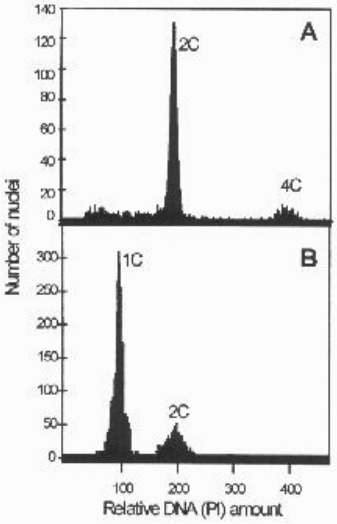

Fig. 1. Flow-cytometric histographs showing **A** ploidy levels of *B. napus* leaves used as diploid control to determine the C-level positions; **B** ploidy state of microspores and pollen

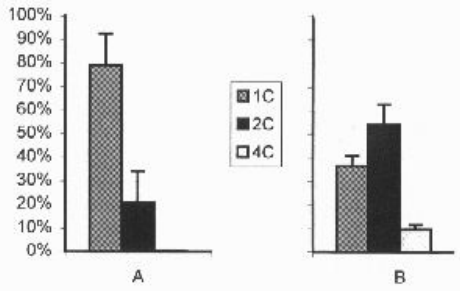

Fig. 2. Distribution of C-levels of **A** microspores and pollen at late uni-nuclear and early bi-nuclear stages used for embryogenesis induction and **B** pEMs at globular stage, showing highly increased 2C and 4C DNA contents in pEM cells. Data based on measurements of populations

described for light microscopy. Samples were then critical-point dried, sputter coated, and observed in a JEOL JSM-5,200 scanning electron microscope.

Results

Ploidy changes in microspore-derived embryos

Plants to be used for collecting microspores and pollen were analyzed by flow cytometry and gave histograms to determine the C-level positions. All plants were diploid showing 2C and 4C levels (Fig. 1 A). Analysis of isolated nuclei confirmed the haploid nature of microspores and pollen, having clear 1C and 2C peaks (Figs. 1 B and 2 A). Culture at embryogenic conditions induced the formation of numerous pEMs. At the globular stage, most pEMs still showed a distinct 1C peak but the 2C and 4C contents had increased, indicating the occurrence of spontaneous diploidization (Fig. 2 B). Within a population of immature pEMs, e.g., at globular to torpedo-shaped stages, three types of pEMs were observed. Some pEMs were still haploid, some were almost doubled haploid, and some were chimeric (Fig. 3 A–C). The almost pure doubled haploid pEMs were first observed at the globular stage. While their number increased with time, the number of haploid pEMs decreased (Fig. 4).

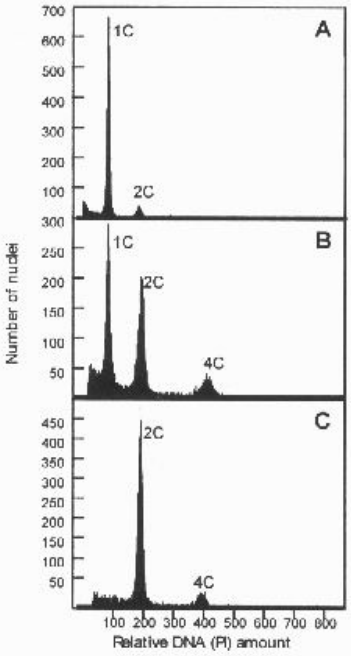

Fig. 3. Flow-cytometric histographs showing C-levels in three types of pEMs: **A** haploid, **B** chimeric, **C** doubled haploid. Data of each graph are based on measurements of an individual but were repeated for at least 50 embryos

Populations of pEM-derived plants also consisted of haploid, doubled haploid, and chimeric individuals, but, in addition, tetraploid plantlets were observed (Fig. 5 A). The doubled haploid plants tended to be

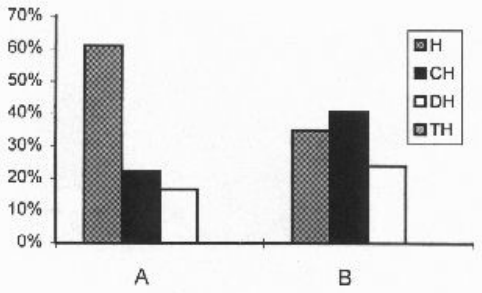

Fig. 4 A, B. Frequency of occurrence of haploid (*H*), chimeric (*CH*), doubled haploid (*DH*) and tetraploid (*TH*) pEMs. **A** globular to torpedo stages (n > 50); **B** cotyledon to mature stages (n > 40). Note the increases of CH and DH populations. TH values are 0. Data based on measurements of individuals

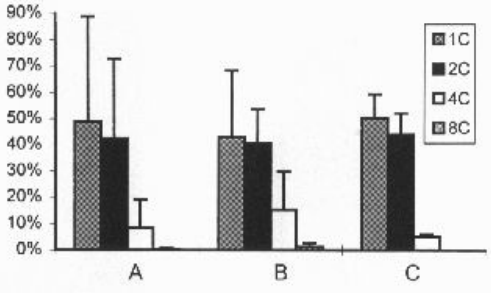

Fig. 6 A–C. Distribution of C-levels in various organs of chimeric pEMs at late cotyledon stage (n = 50) showing that diploidization occurred in all organs. Data based on measurements of individuals. **A** Cotyledon, **B** hypocotyl, **C** root

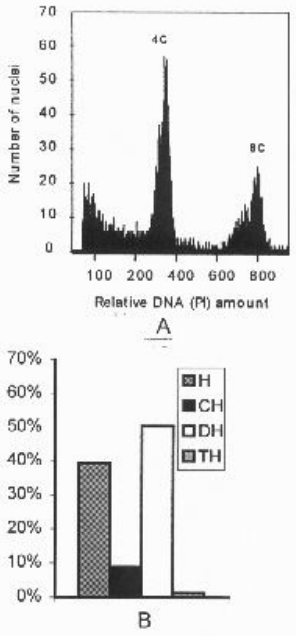

Fig. 5. A Flow-cytometric histograph showing C-levels in a tetraploid pEM (number of embryos analyzed, > 5); **B** frequency of occurrence of haploid (*H*), chimeric (*CH*), doubled haploid (*DH*), and tetraploid (*TH*) pEM-derived plants (n > 200). Data based on individual measurements

stable at diploidy level: only 2% tetraploid plants were formed (Fig. 5 B), and they usually did not develop to healthy plants.

When roots, hypocotyls, and cotyledons of chimeric pEM-derived plantlets were analyzed separately, all organs exhibited diploidization (Fig. 6). When pEm-plantlets were almost pure doubled haploids, they showed diploidy without tissue difference.

Somatic embryogenesis

Somatic embryos developed on the cotyledons as well as hypocotyls of pEM-derived plantlets which were cultured on solid MS-20 medium (Fig. 7 A). More than 60% of the pEM-derived plantlets produced one or more somatic embryos at 18 DIC (days in culture). The number of the somatic-embryo-bearing plantlets increased by on-going culture (Fig. 8 A). The number of somatic embryos per somatic-embryo-bearing plantlet increased in time but varied highly (Fig. 8 B), ranging from 1 to 80 at 22 DIC.

Morphologically, globular, torpedo-shaped, and well developed embryos with cotyledons, hypocotyl, and root were distinguished (Fig. 7 B). Investigating the sites of initiation, it was found that primordia arose at the surface of the plantlets (Fig. 7 B, C), and that only epidermal cells gave rise to somatic embryos (Fig. 7 D). Adjacent subepidermal cells only showed a few cell divisions (Fig. 7 D). Longitudinal sections revealed that the somatic embryos consisted of an axis with root and shoot apices and cotyledons (Fig. 7 E). Some adventitious shoots were also formed on the pEMs-derived plantlets (Fig. 7 F). They were also of epidermal origin.

Analysis of a population of somatic embryos revealed that embryo cells varied in ploidy level from haploid to octaploid (Fig. 9 A) and that classes of embryos could be distinguished ranging from haploid, doubled haploid, tetraploid, and their

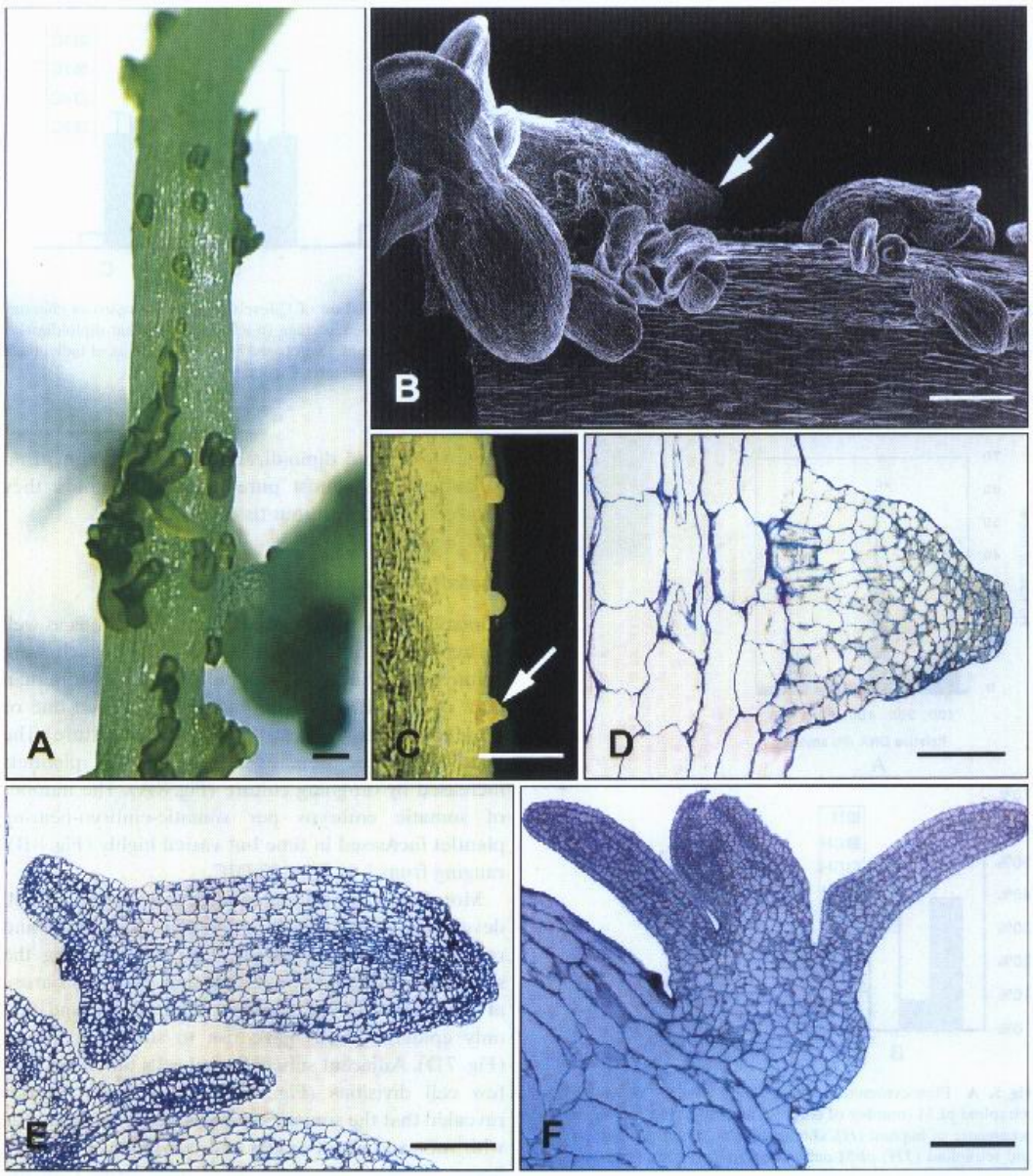

Fig. 7 A–F. Somatic embryo and adventitious shoot formation on pEM-derived plantlets. **A** Overview of morphology of the hypocotyl of a pEM-derived plantlet with developing somatic embryos. Note that the hypocotyl does not show callogenesis. Bar: 1 mm. **B** Scanning electron micrograph showing a close view of somatic embryo initiation on a hypocotyl of a pEM-derived plantlet. Note the asynchronous development of somatic embryos from globular to germinating stages, and the morphology of the hypocotyl that does not show callogenesis. Arrow points to the root of a germinating embryo. Bar: 0.5 mm. **C** Morphology of somatic-embryo primordia on a pEM-derived plantlet. Arrow points to the primordium which is shown in detail in **D**. Bar: 1 mm. **D** Median section through the initiation site of a somatic embryo. Note that only the cells of the epidermis of the hypocotyl formed the primordium, and the adjacent subepidermal cells only showed a few cell divisions and did not contribute to the formation of the pEM primordium. Bar: 0.2 mm. **E** Longitudinal section of a somatic embryo showing a well developed embryo axis with root and shoot apices and cotyledons. Bar: 0.5 mm. **F** Longitudinal section of an adventitious shoot developed on the hypocotyl of a pEM-derived plantlet. Note that it also originates from the epidermal layer. Bar: 0.5 mm

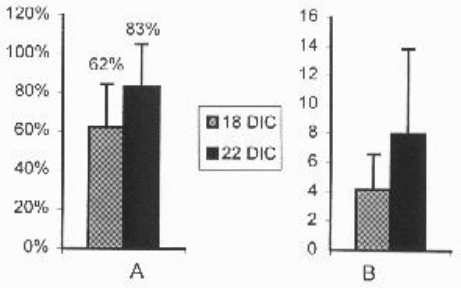

Fig. 8 A, B. Somatic embryo formation in relation to the period of culture of pEM derived plantlets. **A** Percentage of plantlets that formed somatic embryos at 18 and 22 DIC. **B** Number of somatic embryos formed per embryo-bearing plantlet at 18 and 22 DIC. Data from 3 repetitive experiments (100 plants analyzed)

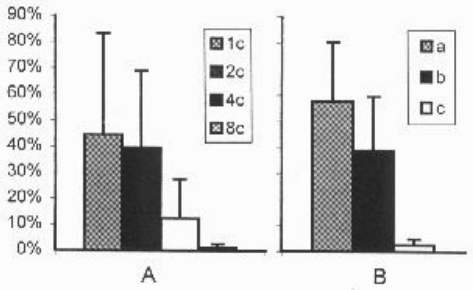

Fig. 9 A, B. Analysis of the ploidy of somatic embryos. Note that at both individual (**A**) and population (**B**) levels, ploidy shows great variations. **A** C-levels in somatic embryos (n = 75); **B** population of haploids and chimerics containing doubled haploid cells (*a*); the population of doubled haploids and chimerics containing tetrahaploid cells (*b*), and the population of tetraploids and chimerics containing octaploid cells (*c*). All data are based on measurements of individuals (n = 75)

chimerics (Fig. 9B). To analyze the mechanism and select doubled haploid somatic embryos for transplantation, the somatic embryos were further measured separately, and different tissue parts of the individual somatic-embryo-bearing plantlets were analyzed. It was found that the ploidy of the somatic embryos tended to be the same as that of the tissue at the initiation site on the pEM-derived plant: when the embryo-bearing tissue was haploid, haploid somatic embryos were developed locally (Fig. 10); when a tissue was chimeric, the local somatic embryos were chimeric (Fig. 11). Doubled haploid plantlets, which were found rather stable and uniform at diploidy level, produced doubled haploid somatic embryos (Fig. 12).

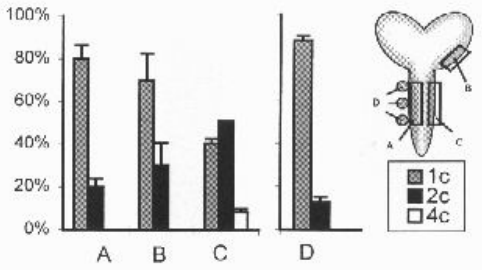

Fig. 10. Distribution of C-levels in three regions of a haploid and doubled-haploid chimeric pEM-derived plantlet (regions A, B, and C are given in the schematic drawing of the plantlet) and some of its somatic embryos (D). Note the haploidy of both the somatic embryos (**D**), and the local tissue (**A**) from which the somatic embryo initiated. Data based on three measurements for each region in an individual (repeated 20 times). Somatic embryos were analyzed in 10 samples of 5 embryos each. **A** Embryogenic hypocotyl tissue part, **B** cotyledon, **C** nonembryogenic hypocotyl tissue part, **D** somatic embryos, all sampled as shown in the scheme

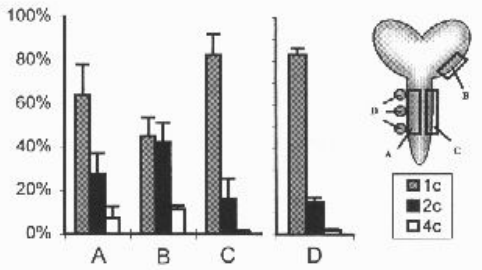

Fig. 11. Distribution of C-levels in three regions of a haploid and doubled-haploid chimeric pEM-derived plantlet (A, B, and C, as indicated in the schematic drawing) and in some of its somatic embryos (D), showing similarity of ploidy between the somatic embryos (**D**) and the local tissue (**A**) on which the somatic embryos initiated. Data based on measurements of individuals. **A** Embryogenic hypocotyl tissue part, **B** cotyledon, **C** nonembryogenic hypocotyl tissue part, **D** somatic embryos, all sampled from region A, as shown in the scheme

Cloning through somatic embryogenesis

After determination of the ploidy level of the embryo-bearing plantlets somatic embryos were used for subculture. From 4 doubled haploid pEM-derived plantlets, 40 well developed somatic embryos were excised and subcultured to allow plant formation. These plants were analyzed by flow cytometry and all appeared doubled haploid (Fig. 13). All these plants formed flowers and showed seed set.

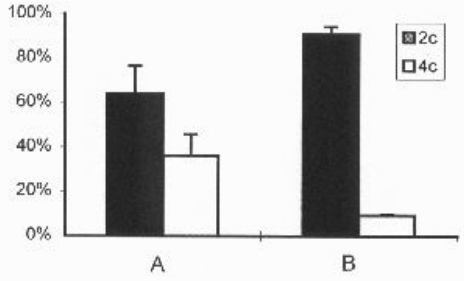

Fig. 12. Distribution of C-levels of **A** pEM-derived doubled haploid plantlets and **B** their somatic embryos. Note that both the embryos and the plantlets from which they arose do exhibit DNA profiles with 2C and 4C peaks. Data based on measurements of individuals (n = 40)

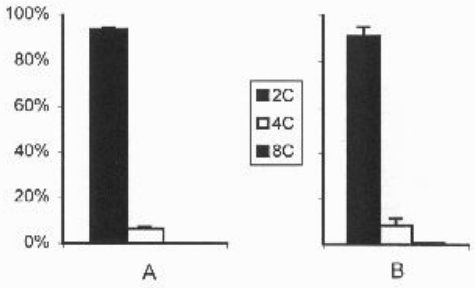

Fig. 13. **A** Distribution of C-levels in leaves of doubled haploid plants (n = 40) derived from doubled haploid somatic embryos. Note the similarity of ploidy between these plants and *B. napus* cv. Topas diploid plants (**B**, n = 100). Data based on measurements of individuals

Discussion

Androgenesis has been successfully induced in about 200 species belonging to more than 50 genera and 25 families of dicots and monocots (for review, see Sangwan and Sangwan-Norreel 1990, Ferrie et al. 1995). One of the goals of androgenesis is to increase significantly the yield of haploids as well as doubled haploids for breeding.

Given haploid embryos are derived from a heterogeneous population of microspores, each pEM probably represents a unique genotype. Thus, theoretically, the conventional methods of microspore embryogenesis will only lead to the formation of a mixture of haploid and doubled haploid embryos and plantlets, with various genetic backgrounds. To increase the quantity of individuals of each genotype, further propagation is required.

To date, somatic embryogenesis has been documented in almost all higher plant species of economic value (Thorpe 1995), but few studies have been addressed to the combination of androgenesis (microspore embryogenesis) and subsequent somatic embryogenesis. In our studies, the microspore embryogenesis is coupled with an amplifying somatic-embryogenesis step. Careful anatomy and flow cytometry confirmed that the somatic embryos develop from a limited number of epidermal cells, and that the ploidy of somatic embryos was highly linked to the ploidy state of the local site where somatic embryos are formed. The combination of androgenesis and somatic embryogenesis complements our previous work on the culture of mixed haploid and doubled haploid protoplasts (Sun et al. 1998). The somatic embryogenesis presented here was carried out by a one-step transfer, in a relatively short term, and avoided the use of auxin and the passage through a callus phase, showing advantages which are known to reduce somaclonal variations (Deverno 1995). Thus, possible genotypic variations in cultures are minimized. Consequently, the coupling of androgenesis and subsequent somatic embryogenesis does improve breeding techniques in terms of quality and quantity and demonstrates the potential for immediate mutation, transformation, and selection.

Diploidity in pEMs may be caused by the following reasons: chemical treatment of haploid microspores and pollen inducing chromosome doubling; the use of tetraploid donor plants; the occurrence of unreduced microspore and young pollen in diploid plants; the introduction of sporophytic diploid cells in the microspore culture; fusion of haploid nuclei; and endomitosis. In our research, diploidization occurred spontaneously, i.e., it was not triggered by the use of mitotic drugs, such as colchicine (Zhao et al. 1996) and trifuralin (Zhao and Simmonds 1995). The donor plants were uniformly diploid, and the microspores and pollen grains haploid. Thus the use of tetraploid donor plants can be excluded. During the isolation of microspores, sporophytic cells might come in the mixture of microspores and pollen and thus give rise to diploid embryos. However, sporophytic cells were removed from the isolation mixture by repetitive centrifugation which was confirmed by microscopy and flow cytometry. Thus, diploidy is not caused by sporophytic cells. Our research reveals that spontaneous diploidization increased progressively during embryo development, and was not caused by nuclear fusion.

because developing pEMs did never show mitosis without cell plate formation. Therefore, we conclude that the diploidy of the doubled haploids is caused by endomitosis. That process can be induced by mitotic drugs that interfere with the microtubular cytoskeleton. The mechanism along which spontaneous diploidization occurred in the present experiments will be further investigated. Tetraploid plants most likely are formed in the same way but, unlike haploid, chimeric, and doubled haploid plants, they were unable to grow to maturity.

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