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REGENERATION OF DIPLOID AND TETRAPLOID PLANTS OF *ARABIDOPSIS THALIANA* VIA CALLUS

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Different explants from diploid and tetraploid plants of *Arabidopsis thaliana* ecotype Wilna were cultured in vitro on the same medium in the same conditions. The mode and capacity for regeneration and the ploidy level of regenerants were examined in eight callus lines. The explant responses to in vitro conditions depended on the ploidy level of donor plants and on the type of explant. Callus derived from leaves of tetraploid plants showed the highest ability to regenerate. Histological investigation showed that plants regenerated mostly through organogenesis and occasionally through embryogenesis. Somatic embryos were observed more frequently in callus derived from tetraploid plants. Regenerated plants were diploids, triploids and tetraploids. The majority of regenerated plants from callus of diploid origin were diploid, but diploids were also observed quite frequently among regenerants from callus of tetraploid origin.

Key words: *Arabidopsis thaliana*, regeneration, ploidy, organogenesis, polyploidization, somatic embryogenesis.

INTRODUCTION

Polyploids play an important role in evolution and plant breeding programs. Most angiosperms are of polyploid origin (Soltis and Soltis, 1999). Because of their significant role, polyploid species have been the subjects of many studies including in vitro culture. Polyploids can occur among regenerated plants from in vitro culture or during the transformation process. The mode of regeneration and regeneration capacity of polyploid tissues cultured in vitro is especially interesting. So far, efficient regeneration systems among polyploids have been developed mainly for potato and rapeseed (Garcia and Martinez, 1995; Ono et al., 1994).

Arabidopsis thaliana, a model plant in classical genetics and molecular biology, has been used extensively in biotechnology. However, tissue culture techniques effective in other plant species have not yet been satisfactorily applied to *Arabidospsis*, especially in regard to regeneration. The most important problem of in vitro culture is to maintain the original genotype. The well-known unwanted phenomenon of somaclonal variation occurs in culture in vitro, particularly when the plant is regenerated indirectly via callus (Larkin and Scowcroft, 1981). Several factors have been found to influence somaclonal variation, including ploidy level, genotype, and type of explant (Karp, 1988).

This paper presents a cytological analysis of callus obtained from diploid or tetraploid *A. thaliana* plants. The relations between the regeneration capacity and polyploidization of callus and between the mode of regeneration and ploidy level of the donor plant were examined. Also discussed is the chromosome number of regenerated plants in relation to donor plant genotype.

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MATERIALS AND METHODS

PLANT MATERIAL

Diploid seedlings and plants of *Arabidopsis thaliana* (L.) Heynh. (2n = 2x = 10) and its autotetraploid line (2n = 4x = 20), ecotype Wilna (Maluszynska et al., 1990; Karcz et al., 2000), were the sources of explants. The plants were grown in test tubes on hormone-free MS medium (Murashige and Skoog, 1962) enriched with 3% sucrose and 0.8% agar, with a 16 h photoperiod and stable 70% humidity at 21˚C.

IN VITRO CULTURE

Three types of non-meristematic explants (cotyledons, hypocotyls, leaves) and a meristematic explant (root tips) were used for callus induction. Cotyledons, hypocotyls and root tips were excised from three-day-old seedlings; leaves were isolated from rosettes of flowering plants. Fifty explants of each type were cultured in Petri dishes (9 cm dia.) on B5 medium (Gamborg et al., 1968) containing 0.5 mg/l 2,4-D (dichlorophenoxyacetic acid), 0.05 mg/l kinetin and 2% sucrose, solidified with 0.8% agar. After primary culture of 28 days, four lines (cotyledon callus, hypocotyl callus, leaf callus, root tip callus) of diploid origin (called 2n callus here) and four lines (cotyledon callus, hypocotyl callus, leaf callus, root tip callus) of tetraploid origin (called 4n callus here) were maintained by subculture every four weeks for five months. In each subculture, five separate callus lumps (~5 mm dia.) of each line were cultured in ten glass jars. Five-month-old callus lines were transferred to B5 regeneration medium supplemented with 5 mg/l 2iP [N^6 (2-isopenthenyl) adenin] and 0.15 mg/l IAA (3-indole-acetic acid) (Valvekens et al., 1988). Regenerated rosettes and shoots obtained from four-week culture on regeneration medium were transferred to new B5 medium supplemented with 0.15 mg/l NAA (1-naphthalene acetic acid) for rooting. Roots regenerated only occasionally from all investigated callus lines, and with very low frequency, so the regeneration capacity of callus lines was estimated as the number of callus lumps with shoots or embryos per 50 cultured callus lumps of each line. Then whole and fertile regenerants were obtained. The explants, callus and regenerated plants were grown under the same experimental conditions.

CHROMOSOME ANALYSIS

Mitotic chromosomes were analyzed in callus cells of each line throughout the culture and also in cells from flower buds of all regenerants. To estimate the ploidy level of callus cells, callus lumps were taken for analysis on the seventh day of each subculture. Small pieces of callus and flower buds from regenerated plants were pretreated with 0.002 M 8-hydroxyquinoline for 2 h at room temperature and for 2 h at 4˚C and fixed in a mixture of methanol, chloroform and propionic acid (6:3:2 v/v). For chromosome preparation, fixed material was rinsed in 0.01 M citrate buffer and digested with 1% pectinase (Sigma No. P5146, from *Aspergillus niger*) and 2% cellulase (Calbiochem No. 21947) for 40 min at 37˚C, then rinsed in the same buffer again and finally squashed in a drop of 45% acetic acid. The cover slips were removed and the slides were air-dried, stained with DAPI (4',6-diamidino-2-phenylindole; 2 μg ml^{-1}) and mounted in Citifluor antifade buffer (AFI, Pelco Int. Redding, U.S.A.). For each callus sample at least five preparations were made and ~40 metaphases were analyzed. Aneuploid cells observed in callus were included in the group of cells at the corresponding ploidy level. Analyses were made with an epifluorescence microscope (Olympus) with appropriate filters.

HISTOLOGY

To analyze the origin of shoots and somatic embryos, the callus lumps were fixed after 7, 10, 14, 21 and 28 days of culture on regeneration medium in 3% solution of glutaraldehyde in sodium phosphate buffer (pH 7.2) for 4 h. After three rinses in 0.1 M phosphate buffer, callus lumps were dehydrated in a series of alcohol and propylene oxide. Finally the material was embedded in Epon resin. Semithin sections (~1 μm) were cut with a glass knife on an ultramicrotome (Tesla) and stained in a 1:1 mixture of azure B and methylene blue. The sections were analyzed with a light microscope (Olympus).

RESULTS

POLYPLOIDIZATION PATTERN OF CALLUS

Cytogenetic study of eight callus lines showed a high level of polyploidization of all lines during the period of culture. The polyploidization pattern during primary culture – the frequency of polyploid metaphases and their ploidy level – depended on the polyploidy of the donor plant and on the type of explant. After one week of primary culture the frequency of cells with ploidy level above that of the donor plant was high in all callus lines. During the

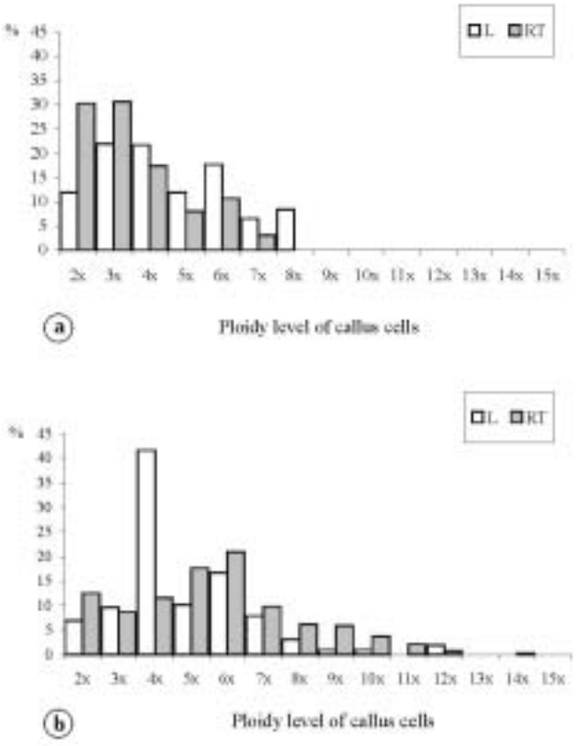


Fig. 1. *Arabidopsis thaliana* (L.) Heynh. Frequency of cells with various chromosome numbers in callus obtained from leaf (L) and root tip (RT) explants. (**a**) Diploid, (**b**) Tetraploid plants. The results are average frequencies observed in callus during five months of culture.

subsequent five-month culture, the polyploidization pattern differed slightly between types of callus. Generally the chromosome numbers ranged from 10 to 40 (2x–8x) in 2n callus and between 20 and 70 (4x–14x) in 4n callus (Fig. 1). Diploid and tetraploid cells were predominant in 2n callus, and tetraploid and hexaploid cells dominated in 4n callus. Interestingly, diploid and triploid cells were often found in callus of tetraploid origin.

There were differences in the extent of chromosome variation between callus derived from meristematic (root tips) and from non-meristematic explants. Diploid cells were more frequent in root tip callus than in leaf callus of diploid origin. In contrast, leaf callus of tetraploid origin was much more stable, with 40% of the cells having an unchanged chromosome number, while in root tip callus of the same origin only 10% of the cells were tetraploid.

REGENERATION

The five-month-old callus lines exhibited various regeneration potentials. Shoots were observed on callus of all lines. Somatic embryos were detected only on both leaf callus lines. The shoot regeneration potential depended mainly on the type of explant (Fig. 2). Analogous callus lines, that is, those derived from the same type of explant of diploid or tetraploid plants, showed very similar regeneration ability. Leaf callus exhibited the highest regeneration ability, 100% for 2n callus and 80% for 4n callus. Two lines of callus derived from root tips showed reasonable regeneration capacity (~50%). Hypocotyl and cotyledon callus derived from tetraploid plants exhibited the lowest regeneration potential, less than 40%.

Histological examination revealed the presence of meristematic cell centers in all callus lines after one week of culture on regeneration medium (Fig. 3a). These centers consisted of small cells distinctly different from neighboring cells, with nuclei intensively stained by the azure B and methylene blue mixture. Sometimes cells located externally in these centers were arranged in one or two distinct layers (Fig. 3b). Cells with numerous starch grains were present adjacent to these centers.

Apart from the above-mentioned structures in the leaf callus after one-week culture on regeneration medium, structures resembling embryos at globular stage were observed (Fig. 3c). Histological analysis confirmed these observations; globular embryos were present in the callus, separated from the callus tissue and from each other (Fig. 3e). After two weeks of callus culture on regeneration medium, structures similar to somatic embryos at heart stage were present on the surface of leaf callus of tetraploid origin (Fig. 3d,f). Somatic embryos were not very frequent, but were observed more often in callus derived from tetraploid plants (8%) than in callus of diploid origin (3%). Somatic embryogenesis was not only more frequent but also more advanced in development in 4n callus.

Some places on the callus surface turned green, and small shoots with a leaf, single leaflets or small roots could be observed (Fig. 3g,h,i). Shoots and rosettes of normal dimensions but without roots appeared in all callus lines after three or four weeks of culture on regeneration medium. Transferring regenerated rosettes and shoots without roots to new rooting medium produced whole, fertile plants (Fig. 3j).

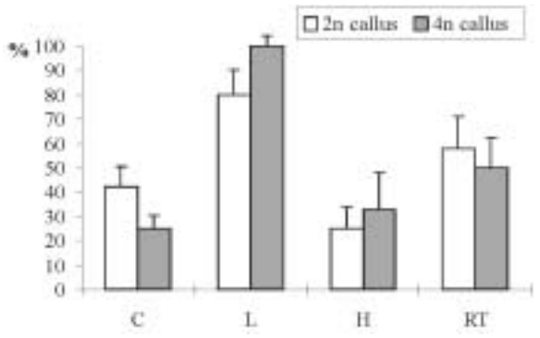


Fig. 2. *Arabidopsis thaliana* (L.) Heynh. Shoot regeneration capacity of callus derived from diploid plants (2n callus) and from tetraploid plants (4n callus). C – cotyledon callus; L – leaf callus; H – hypocotyl callus; RT – root tip callus.

REGENERANTS

Diploid, triploid and tetraploid plants were regenerated from the investigated callus lines. The majority of regenerants from all callus lines of diploid origin were plants with the same chromosome number as the donor plant (Tab. 1). Tetraploids were observed with a frequency of 18% and triploids with 7%.

Surprisingly, most of the regenerants from all callus lines of tetraploid origin were diploids. The frequency of plants with the chromosome number of the donor plants was ~20%, and the frequency of triploid regenerants was similar. The ploidy level of regenerants correlated with the type of explant. Only diploid plants regenerated from cotyledon callus of both diploid and tetraploid origin (Fig. 4a). Diploids were most frequent among the regenerants from leaf callus lines, but polyploids (3n, 4n) also appeared with low frequency (Fig. 4b). The plants regenerated from the two hypocotyl callus lines were diploids and triploids (Fig. 4c). Regenerants with the original donor plant chromosome number dominated only in root tip callus lines (Fig. 4d).

TABLE 1. Frequency of regenerated plants *Arabidopsis thaliana* at different ploidy level from all types of callus lines of diploid (2n callus) and tetraploid (4n callus) origin

Regenerants	2n callus	4n callus
Diploid	75%	58%
Triploid	7%	21%
Tetraploid	18%	21%

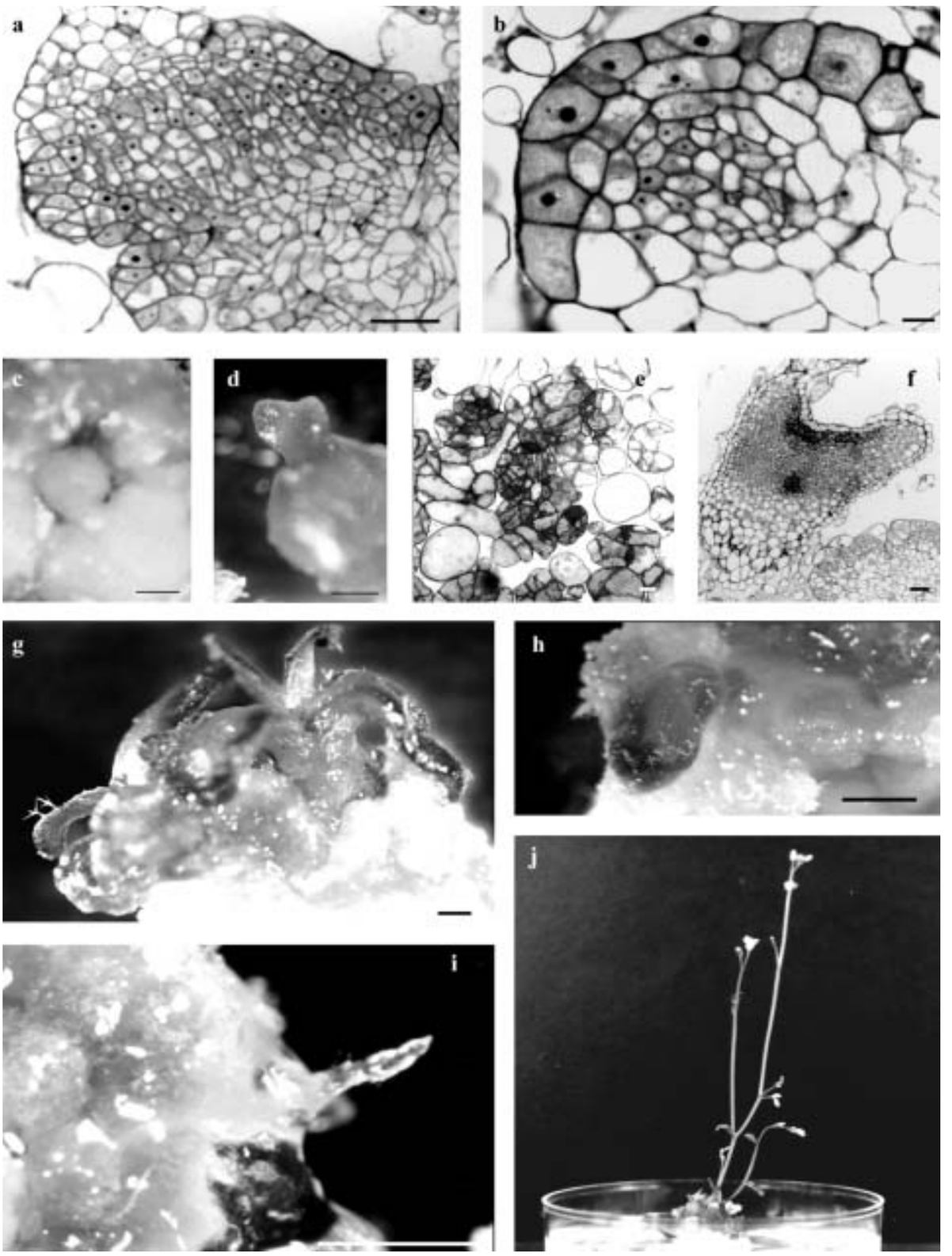
DISCUSSION

All callus lines exhibited a high level of polyploidization during callogenesis and in the subsequent culture, irrespective of their diploid or tetraploid origin (Fraś, 2001). This may be explained by the mixoploidy of *A. thaliana* tissue, a result of endoreduplication during differentiation of somatic cells (Galbraith et al., 1991). Thus the non-meristematic explants consisted of cells differing not only in structure and physiology but also in nuclear DNA amount; this was probably the reason for the presence of polyploid cells in callus from the beginning of culture (Jacq et al., 1992). In contrast, the callus derived from a non-polysomatic plant such as *Crepis capillaris* was stable in chromosome number during almost a year of culture (Maluszynska, 1990).

All analyzed *Arabidopsis* callus lines were capable of regeneration despite the high level of polyploidization. Similar results have been obtained in other plant species (Kovacs, 1985; Brutovská et al., 1998). There was no evidence that regeneration ability was related to the ploidy level of the donor plant, but it did correlate with the source of explants. Patton and Meinke (1988) also noted differences in shoot regeneration capacity between cotyledon and hypocotyl explants of *A. thaliana* cultured in vitro.

Histological investigation of regenerating *A. thaliana* callus revealed two types of regeneration. Most regenerants were obtained through orga-

Fig. 3. *Arabidopsis thaliana* (L.) Heynh. Regeneration via callus – organogenesis and somatic embryogenesis. (**a**) Meristematic center in hypocotyl callus of tetraploid origin after one week of culture on regeneration medium. Bar = 10 µm, (**b**) Meristematic center with callus cells arranged in two distinct layers in leaf callus of tetraploid origin after one week of culture on regeneration medium. Bar = 5 µm, (**c**) Morphology of somatic embryos at globular stage regenerated on leaf callus of diploid origin after one week of culture. Bar = 100 µm, (**d**) Morphology of somatic embryos at heart stage regenerated on leaf callus of tetraploid origin after two weeks of culture. Bar = 100 µm, (**e**) Cell groups in leaf callus of diploid origin after one week of culture on regeneration medium. Bar = 10 µm, (**f**) Somatic embryos regenerated from leaf callus of tetraploid origin after two weeks of culture on regeneration medium. Bar = 10 µm, (**g**) Regenerated shoot on leaf callus of diploid origin after three weeks of culture on regeneration medium. Bar = 1mm, (**h**) Small leaflets regenerated on hypocotyl callus of diploid origin after two months of culture on regeneration medium, (**i**) Small roots regenerated on hypocotyl callus of diploid origin after two weeks of culture on regeneration medium. Bar in (h) and (i) = 500 µm, (**j**) Regenerated plant on cotyledon callus of diploid origin after two months of culture on regeneration medium.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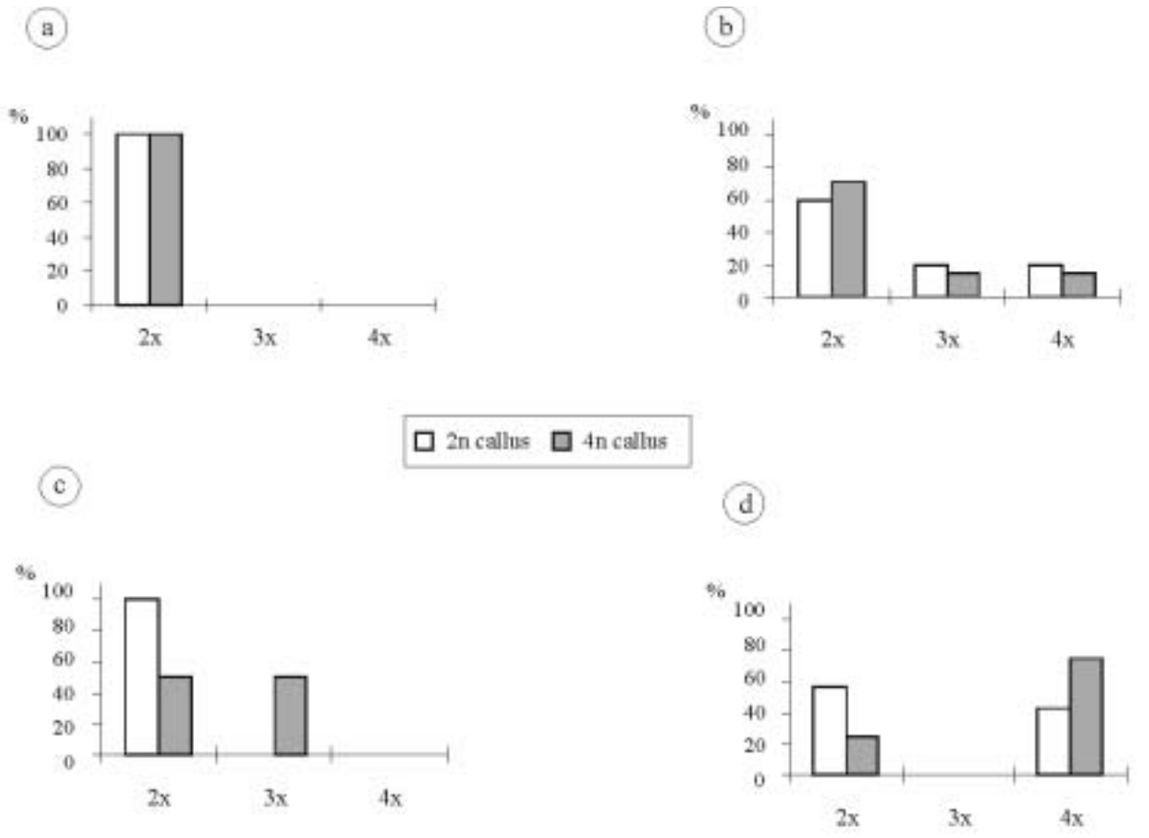

Fig. 4. *Arabidopsis thaliana* (L.) Heynh. Frequency of regenerants at different ploidy levels obtained from (**a**) Cotyledon callus, (**b**) Leaf callus, (**c**) Hypocotyl callus, and (**d**) Root tip callus of diploid and tetraploid origin.

nogenesis, while some plants regenerated via embryogenesis from leaf callus. Somatic embryos appeared on leaf callus of tetraploid origin more frequently than on 2n callus, and the development of these embryos was more advanced. As has been reported by many authors, somatic embryogenesis in *A. thaliana* is not so frequent (Wu et al., 1992; O'Neill and Mathias, 1993; Pillon et al., 1996). Direct somatic embryogenesis was obtained with high frequency when immature zygotic embryos were used as explants and cultured in vitro for a short time (Gaj, 2001). Regeneration via embryogenesis has been reported for several diploid and tetraploid species, but there has not been much evidence on the relationship between somatic embryogenic ability and the ploidy level of the donor plant. Kunitake et al. (1998) studied such a relationship in *Asparagus officinalis*. They cultured in vitro four callus lines established from haploid, diploid, triploid and tetraploid plants, and concluded that the frequency of somatic embryogenesis was highest in callus of tetraploid origin. Fraś et al. (2002) compared callus regeneration in *Crepis capillaris* and *A. thaliana*; in both species, more advanced somatic embryogenesis, with higher frequency, was observed in tetraploid than in diploid origin callus. Numerous authors have emphasized that embryogenic potential depends on the genotype of donor plants (Rani et al., 2000; Imani et al., 2001).

Independent of the ploidy level of 2n callus, the majority of regenerants were diploids, perhaps indicating the advantage of diploid cells in regeneration. Similar observations were reported for hypocotyl callus cultures of another polysomatic plant, *Beta vulgaris*, where a large number of diploid regenerants was obtained (Jacq et al., 1992). It is difficult to explain the large number of diploid plants regenerated from 4n callus. Tetraploid callus was obtained

from autotetraploid *Arabidopsis* plants induced by colchicine treatment, and presumably the dedifferentiated callus cells underwent mitotic abnormalities including amitosis and multipolar spindles, leading to their diploidization (D'Amato, 1986); these cells participated preferentially in plant regeneration. The frequency of diploid regeneration was highest in both lines of cotyledon callus and lowest in callus of meristematic (root tip) origin. Several authors have reported that artificial tetraploids of *Pisum sativum* induced by colchicine or X-rays sometimes produced in vivo plants with the chromosome number reduced to the diploid level (Mercy Kutty and Kumar, 1983; Kasperek, 1986; Gottschalk, 1988).

Chromosomal variation in cultured and regenerating cells are a source of the somaclonal variation that so frequently occurs in many regenerated plants (Larkin and Scowcroft, 1981; Lee and Phillips, 1988). Polyploid plants among regenerants are a form of somaclonal variation. We found some correlation between the source of the explant and the ploidy level of the regenerants. True-to-type regenerants were obtained with a frequency above 50% only from root tip callus. In the other callus lines, most of the regenerated plants were diploids, while tetraploids were very rare. In tomato, the percentage of polyploid cells present in explant material in vivo was found to correlate with the frequency of polyploids regenerated from leaf, cotyledon and hypocotyl explants (van den Bulk et al., 1990).

All these results suggest that leaves are convenient explants for callus culture and regeneration. They are easy to obtain in unlimited numbers, and leaf-origin callus has a high regeneration potential. Leaves are most frequently used for transformation and cell suspension or protoplast culture in vitro. It should be noted that regeneration of diploid plants with high frequency from this callus is essential for DNA transformation. It has been shown that the polyploidy of *Arabidopsis* transgenic plants can modify epigenetic silencing of transgenes (Mittelsten Scheid et al., 1996). Leaf callus is also capable of somatic embryogenesis, but this requires further work to increase the frequency of regeneration. So far, somatic embryogenesis with a reasonable frequency has been reported only for immature zygotic embryos (Gaj, 2001). In studies where regenerants with the exact ploidy level of the donor plant are required, a meristematic type of explant is recommended.

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