Theor Appl Genet (2001) 102:104–110

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Flow cytometric evidence for endopolyploidy in seedlings of some *Brassica* species

Received: 17 March 2000 / Accepted: 17 April 2000

Abstract Flow cytometric analysis of the nuclear DNA contents of somatic tissues from seedlings of *Brassica rapa* L. and *B. oleracea* L. revealed extensive endoreduplication, resulting in tissues that contain cells with multiple ploidy levels (also called 'endopolyploidy'). Multiples of the haploid nuclear genome complement (1C) corresponding to 2C, 4C, 8C, 16C, 32C and 64C were observed in *Brassica rapa*, while *B. oleracea* exhibited a mixture of cells with five ploidy levels, 2C, 4C, 8C, 16C and 32C. The distribution of cells with the different ploidy levels was tissue-specific and characteristic of the stage of development. Multiploidy was not found in the embryos of dry seeds. Rapid endoreduplication occurred during seedling development. It is most probable that multiploidy is, if not a general feature, at least very common in *Brassica* species. The physiological and genetic implications of this original feature are discussed.

Keywords *Brassica oleracea* · *Brassica rapa* · Endopolyploidy · Endoreduplication · Flow cytometry

Introduction

Endoreduplication – amplification of the genome in the absence of mitosis – is a common process in eukaryotes (Nagl 1978). It is a naturally occurring disruption of the mitotic cell cycle, resulting in cells with multiple ploidy levels (also called 'endopolyploidy'). Endoreduplication is very frequent in arthropods, where the highest values of endopolyploidy occur in the Malpighian tubules and salivary glands of various dipters (Sauer et al. 1995). In mammals, endopolyploidy is rare and evident only in the trophoblast, decidua and myocardial cells (Barlow and Sherman 1972; Conlon and Raff 1999). In contrast to the situation in animals, endopolyploidy is widespread but poorly understood in plants, particularly in angiosperms (D'Amato 1952; Nagl 1976). Most examples of endopolyploidy have been restricted to specific cell types that are highly specialized and usually large, such as raphide crystal idioblasts (Kausch and Horner 1984), root hairs (Dosier and Riopel 1978), embryo suspensor cells (Nagl 1974), anther trichomes (Barlow 1975) and endosperm (Kowles et al. 1990). Besides local endoreduplication, systemic somatic endopolyploidy has recently been reported in succulent *Mesembryanthemum crystallinum* (De Rocher et al. 1990), *Arabidopsis thaliana* (Galbraith et al. 1991), cucumber (Gilissen et al. 1993) and tomato (Smulders et al. 1994). In *M. crystallium*, the distribution of nuclei with multiple ploidy levels was found to be organ-specific and, in fully expanded leaves, up to four rounds of endoreduplication had taken place, giving rise to 2C, 4C, 8C, 16C and 32C nuclei, where C is the haploid DNA content per nucleus. In the somatic cells of *A. thaliana*, three rounds of endoreduplication were consistently observed. In cucumber, the first endoreduplication occurred in the transition tissue between the hypocotyl and radicle during germination, and additional rounds of endoreduplication took place in the cotyledons and flowers. Both the leaves and roots of cucumber maintained the initial patterns of endopolyploidy. In tomato, the first round of endoreduplication occurred in the cotyledons and hypocotyl during germination. In tomato leaves, C-values increase during plant development from 2 C at the very young seedling stage to a mixture of cells with DNA amounts ranging from 2 C to 128 C at senescence.

Detailed patterns of endopolyploidy in *Arabidopsis thaliana* have also been described for leaf and stem epidermal cells as well as for the hypocotyl cells (Melaragno et al. 1993; Gendreau et al. 1997, 1998). These investigators suggest that increases in ploidy levels are related to increases in nuclear volume or cell size and environmental conditions.

Communicated by K. Glimelius

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In view of the intensive use of molecular and cellular techniques in the field of plant breeding, increased knowledge of the degree of endopolyploidy in the explant tissue source will be highly valuable for the maintenance of the original ploidy level and the genotype of the crop species. However, very little information on endopolyploidy of in *Brassica* species is available.

We report here that most of the somatic tissues of seedlings of *B. rapa* and *B. oleracea* are multiploid and that this phenomenon is developmentally regulated.

Materials and methods

Plant materials

DNA contents were separately assessed from nuclei isolated from various tissues of the *Brassica* species listed in Table 1. Seeds were surface-sterilized for 15 min in 1% (v/v) sodium hypochlorite solution and washed three times with sterile distilled water. Two seeds were plated on MS medium (Murashige and Skoog 1962) containing 30 g/l (w/v) sucrose, solidified with 2.5 g/l Gelrite in a 300-ml plastic bottle; the pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. The culture was maintained at 25°C and 16-h daylight under cool-white fluorescent lights.

Flow cytometry

Intact embryos and various tissues from seedlings were analyzed by flow cytometry. Embryos were carefully excised from seeds with a fine dissecting needle under a dissecting binocular microscope. An individual seedling at a specific developmental stage was divided into several parts: shoot tip (including immature leaves), leaf (the entire first developing leaf, including petiole), cotyledons (a pair of whole cotyledons, including petiole) and hypocotyl. The samples were individually chopped with a sharp razor blade in nuclei extraction buffer (solution A of the High Resolution Kit for Plant DNA, Partec, Münster, Germany). After filtration through a 30-µm nylon sieve, a staining solution containing the dye 4,6-diamidino-2-phenylindole-2HCl (DAPI, solution B of the kit) was added. The analyses were performed with a PAS flow cytometer (Partec). The mean and coefficient of variation of the fluorescence peaks were estimated with WINMDI software (version 2.8, copyright® 93–99 Joseph Trotter). Within one sample, a minimum of 3000 particles (total count) were analyzed. Measurements of nuclear DNA content were carried out with at least six replications originating from different embryos and seedlings. To determine the standard peak position of 2 C cells, we analyzed the 2C peak from nuclei of embryos at stage 0 (Fig. 1) at least twice for each measurement. The data were plotted on a semi-logarithmic scale, so that the histogram peaks from 2C to 64C were evenly distributed along the abscissa. The data are presented as percentages of the total amount of nuclei in all peaks of the histogram.

Results

Flow cytometry

Flow cytometry is a simple and robust method for measuring nuclear DNA contents: as few as six tissue samples suffice for a reproducible measurement. The peak positions showed some variation between the samples from different parts of individual seedlings. This variation may be due to instrumental fluctuations between analyses, for example in the excitation of the lamp, the warming of the optical filter system and in the sample flow (Partec, personal communication). However, this variation did not interfere with the assignment of peaks. Another problem was the presence of background signals. This noise was predominantly located in the lower channel numbers and was found to depend on the tissue types.

With respect to the evaluation of the quality of the histogram, the coefficient of variation (CV) of the peaks ranged from 1.8% to 6.8%, depending on the tissue types.

Developmental stages from seed to seedling in vitro

A description of the developmental stages of the *Brassica* species is given in Fig. 1. Four developmental stages were morphologically distinguishable between the dry

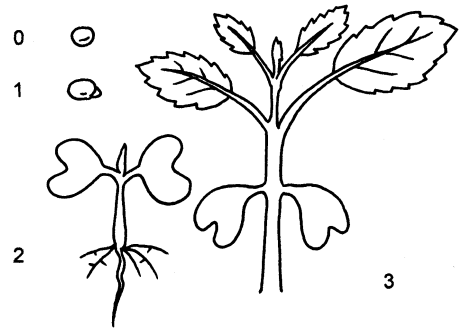

Fig. 1 Stages of seed germination and seedling development of the *Brassica* species tested. *Stage 0* dry seed, *1* emergence of the radicle from the seed coat; *2* opening of the cotyledons and development of shoot tip, *3* development of the third, fourth, and fifth leaf, full expansion of the first and second leaf, and wilting of the cotyledons

Table 1 Names, codes and origins of the *Brassica* species used in this study

Species	Name	Code[a]	Origin[b]
Brassica rapa L. ssp. *chinensis*	Pakchoi 'Sei-Tei'	S	J
Brassica rapa L. ssp. *pekinensis*	Chinese cabbage 'Muso'	T	J
Brassica rapa L. ssp. *rapa*	Turnip 'Haku-Taka'	M	J
Brassica oleracea L. var. acephala	Kale 'Ko-Ra-Do'	S	J
Brassica oleracea L. var. capitata	Cabbage 'Tuma-Midori'	K	J
Brassica oleracea L. var. italica	Broccoli 'Ryoku-Rei'	S	J
Brassica oleracea L. var. botrytis	Cauliflower 'Sira-Giku'	N	J

[a] M, Musasino Seeds Co; S, Sakata Seeds Co; T, Takii Seeds Co; K, Kaneko Seeds Co; N, Nozaki Seeds Co
[b] J, Japan

Table 2 Ploidy patterns in *B. rapa*

Name	Stage	Tissue type	Ploidy patterns (% of nuclei populations)[a]					
			2C	4C	8C	16C	32C	64C
Pakchoi	0	Embryo	100.0±0					
	1	Embryo	82.0±2.4	18.0±2.4				
	2	Shoot tip	82.0±2.8	18.0±2.8				
		Cotyledons	32.8±1.9	48.2±2.2	15.3±2.0	3.7±1.6		
		Hypocotyl	21.0±1.8	37.3±1.8	20.0±4.5	17.8±1.2	3.8±1.2	
	3	Shoot tip	77.2±3.1	22.8±3.1				
		Leaf	42.7±4.3	43.3±3.4	9.5±1.4	4.5±1.7		
		Cotyledons	23.2±1.5	50.8±3.1	16.5±0.7	9.5±1.7		
		Hypocotyl	22.0±1.8	35.3±2.4	11.7±1.9	13.5±2.1	12.5±0.7	5.0±1.5
Chinese cabbage	0	Embryo	100.0±0					
	1	Embryo	76.7±2.3	23.3±2.3				
	2	Shoot tip	79.7±2.5	20.3±2.5				
		Cotyledons	33.8±2.2	48.3±2.2	16.0±1.7	1.8±1.0		
		Hypocotyl	20.2±1.6	35.0±2.4	19.3±3.3	18.5±2.1	6.2±2.0	
	3	Shoot tip	78.7±3.6	21.3±3.6				
		Leaf	48.8±3.6	39.3±3.0	8.0±1.7	3.8±1.5		
		Cotyledons	20.7±0.6	50.3±2.5	19.3±1.3	9.7±1.6		
		Hypocotyl	20.8±2.1	36.7±2.5	10.0±0.5	11.8±1.9	12.5±1.0	8.0±1.7
Turnip	0	Embryo	100.0±0					
	1	Embryo	80.3±2.2	19.7±2.2				
	2	Shoot tip	81.2±1.8	18.3±1.8				
		Cotyledons	35.2±3.3	47.0±2.5	13.2±3.5	4.7±1.3		
		Hypocotyl	22.2±1.9	37.5±2.1	19.8±5.3	18.5±1.8	2.0±1.4	
	3	Shoot tip	80.5±2.2	19.5±2.2				
		Leaf	45.8±3.8	42.5±3.3	9.7±1.3	2.5±0.8		
		Cotyledons	20.3±0.8	48.7±2.7	19.7±1.3	11.3±2.1		
		Hypocotyl	23.0±2.9	37.7±4.6	11.2±1.5	12.5±2.2	11.7±0.7	4.2±0.7

[a] For each value: mean±standard deviation

seed (stage 0) and the seedling (stage 3). Germination of seeds occurred within 1 day. After imbibition of the seed, the outgrowth of the radicle was observed (stage 1). Stage 2 was reached after 6–8 days. Seedlings developed (stage 3) within 20–30 days.

Endopolyploidy of *Brassica rapa* seedlings

Table 2 illustrates the ploidy patterns observed during seedling development of *B. rapa* crops.

In dry seeds (stage 0) of pakchoi, embryos showed a large amount of 2C signals. In addition to 2C signals, 4C values were found in embryos at stage 1. The 4C values indicate the G_2/M phase of the diploid cell cycle. During imbibition, the elevated water content of the seeds may trigger cell cycle activity and induce the germination of the seed.

Endoreduplication had already taken place in cotyledons and hypocotyl tissues at stage 2. At this stage, apart from the 2C and 4C peaks, cotyledons and hypocotyl contained 8C and 16C nuclei and 8C, 16C and 32C nuclei, respectively. Shoot tips contained 2C and 4C nuclei, indicating that these tissues maintain the diploid level.

Further endoreduplication was also detected at stage 3; the hypocotyl tissues displayed a mixture of multiploid nuclei ranging from 2C to 64C (Fig. 2D). The fifth round of DNA replication was specific to the hypocotyl tissues. In cotyledons, the patterns of endopolyploidy at stage 3 were very similar to those at stage 2 (Table 2, Fig. 2C). The first true leaves contained nuclei with four ploidy levels, corresponding to 2C, 4C, 8C and 16C (Fig. 2B). Stability of the diploid level was observed in the shoot tips (Fig. 2A). Values indicating DNA contents higher than 64C were never observed in any tissues.

Endopolyploidy was also observed in Chinese cabbage and turnip. These crops showed patterns of endopolyploidy similar to those of pakchoi (Table 2).

Endopolyploidy of *Brassica oleracea* seedlings

Table 3 shows the ploidy patterns observed during seedling development of *B. oleracea* crops.

In embryos of kale at stage 0, only 2C signals were detected. Besides the 2C signals, 4C nuclei were found at stage 1. At stage 2, apart from the 2C and 4C peaks, 8C peaks were found in cotyledons and hypocotyl samples gave 8C and 16C histograms. Shoot tips showed a diploid level.

At stage 3, the hypocotyl contained nuclei of five ploidy levels (2C–32C, Fig. 2H). In addition to the 2C, 4C and 8C signals, 16C peaks were found in the cotyledons (Fig. 2G). The first true leaf produced three histo-

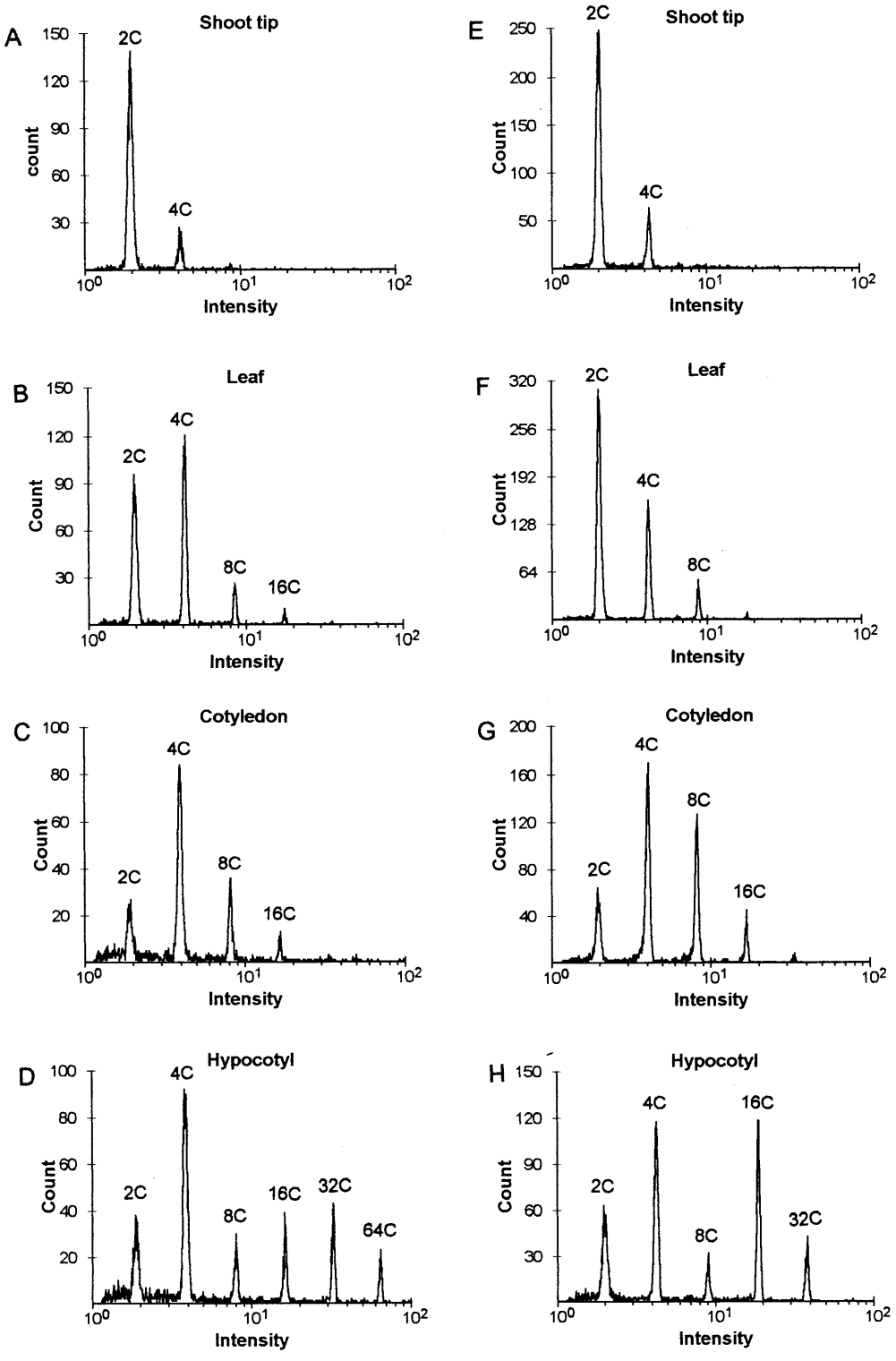

Fig. 2 Characteristic histograms of nuclei distribution at stage 3 in pakchoi (**A**–**D**) and kale (**E**–**H**): **A**, **E** shoot tip, **B**, **F** leaf, **C**, **G** cotyledons, **D**, **H** hypocotyl

grams, corresponding to 2C, 4C and 8C (Fig. 2F). The diploid level was maintained in shoot tips (Fig. 2E). *B. oleracea* seedlings contained no nuclei having DNA contents higher than the 34C level, indicating a repression of the fifth endoreduplication round.

Similar patterns of endopolyploidy were observed in other *B. oleracea* crop types (Table 3).

Discussion

Our results demonstrated that in all of the *Brassica* species tested, most of the somatic cells of seedlings underwent several rounds of endoreduplication, resulting in cells with multiple ploidy levels and that this phenomenon was developmentally regulated. In *B. rapa*, multi-

Table 3 Ploidy patterns in *B. oleracea*

Name	Stage	Tissue type	Ploidy patterns (% of nuclei populations[a])				
			2C	4C	8C	16C	32C
Kale	0	Embryo	100.0±0				
	1	Embryo	81.3±2.1	18.7±2.1			
	2	Shoot tip	79.8±3.2	20.2±3.2			
		Cotyledons	20.7±1.3	49.8±3.7	29.5±4.9		
		Hypocotyl	27.0±2.8	33.5±3.2	22.8×2.8	16.7±2.7	
	3	Shoot tip	81.7±2.6	18.3±2.6			
		Leaf	57.0±6.7	36.3±7.8	6.7±1.9		
		Cotyledons	18.8±0.9	41.7±0.8	29.7±1.5	9.8±2.7	
		Hypocotyl	19.3±2.6	37.5±3.1	13.3±3.0	18.8±4.4	11.0±3.5
Cabbage	0	Embryo	100.0±0				
	1	Embryo	80.0±4.3	20.0±4.3			
	2	Shoot tip	76.8±3.1	23.2±3.1			
		Cotyledons	19.8±2.1	54.8±5.1	25.3±6.0		
		Hypocotyl	29.0±3.3	39.3±2.5	19.6±1.9	12.0±3.1	
	3	Shoot tip	78.1±3.1	21.3±3.1			
		Leaf	37.8±3.7	51.0±3.2	11.2±1.8		
		Cotyledons	18.7±2.0	42.5±1.5	28.2±2.8	10.7±3.0	
		Hypocotyl	27.5±2.6	33.0±2.4	16.7±2.7	14.5±2.2	9.8±3.4
Broccoli	0	Embryo	100.0±0				
	1	Embryo	81.2±3.1	18.8±3.1			
	2	Shoot tip	83.5±2.3	19.8±2.3			
		Cotyledons	21.2±2.0	55.0±4.0	23.8±4.8		
		Hypocotyl	25.7±3.3	34.7±3.4	20.5±1.8	19.2±2.7	
	3	Shoot tip	78.0±3.8	22.0±3.8			
		Leaf	49.8±8.0	41.8±7.6	8.3±2.7		
		Cotyledons	20.3±2.6	42.8±2.0	28.7±0.8	8.2±2.8	
		Hypocotyl	22.5±2.0	31.8±0.6	13.1±2.1	23.3±2.6	9.2±3.1
Cauliflower	0	Embryo	100.0±0				
	1	Embryo	81.8±4.0	18.2±4.0			
	2	Shoottip	82.3±3.1	17.7±3.1			
		Cotyledons	22.6±3.2	54.7±5.2	22.7±7.3		
		Hypocotyl	24.8±0.8	34.8±1.6	22.8±2.9	17.5±2.2	
	3	Shoottip	78.8±3.9	21.2±3.9			
		Leaf	48.5±7.6	42.2±7.6	9.3±1.3		
		Cotyledons	19.3±1.5	29.7±1.9	42.7±1.4	8.5±1.9	
		Hypocotyl	22.7±2.1	34.3±1.7	13.5±2.2	13.8±2.6	15.7±2.8

[a] For each value: means±standard deviation

ples of the haploid nuclear genome complement (1C) corresponding to 2C, 4C, 8C, 16C, 32C and 64C were observed, while *B. oleracea* exhibited a mixture of cells with five ploidy levels, 2C, 4C, 8 C, 16C and 32C. Multiploidy may be a common property of *Brassica* species.

Multiploidy was progressive; in general, older tissue showed higher levels of multiploidy than younger tissues within the same seedlings. The amount of DNA in each cells of the *Brassica* seedling may be systemically controlled. The seedlings appeared to become multipolyploid by repeated rounds of replication of its entire genome in the absence of mitosis. Similar systemic endopolyploidy has been reported in *A. thaliana* (Galbraith et al. 1991), tomato (Smulders et al. 1994) and cucumber (Gilissen et al. 1993). In these species, a mixture of diploid and polyploid cells in a given organ is the rule rather than exception. Galbraith et al. (1991) reported that endopolyploidy up to the 16C level occurs in the vegetative tissues but not in the floral structures of *A. thalaina*. Since the absolute DNA values of *B. oleracea* and *B. rapa* are much larger than that of *A. thalaina* (Croy et al. 1993, Prakash et al. 1999), the suggestion that endopolyploidy is most prevalent in plants with small genomes (Nagl 1978) does not seem to be a universal generalization. De Rocher et al. (1990) demonstrated the occurrence of endopoyploidy in nine species of succulents with relatively small genomes (<3.5 pg of DNA) and the lack of multiple ploidy levels in two other succulent species with large genomes (>32.0 pg of DNA). Thus, for some groups of plants, the occurrence of multiploidy may be related to the absolute size of the genome.

The embryo at stage 0 produced only a 2C peak, irrespective of the species tested. This indicates that the quiescent embryos had arrested the cell cycle at the presynthetic G_1 phase of nuclear division. As previously described in other species (Bino et al. 1992), this might reflect a stringent control over the nuclear division cycle.

During the development of the seedlings, shoot tips remained at a normal diploid (2C) level. For these tissues, the distribution of nuclei was skewed toward 2C and 4C, demonstrating that only mitotic divisions occurred. Because the germ cells originated from the shoot apical meristem, repression of endoreduplication in the meristem could be a mechanism to ensure the genetic stability of the germ line.

Endoreduplication has often been observed in association with cell growth, and in plants a strong correlation between cell size and endoreplication was found (Melaragno et al. 1993). These observations have led to the widely accepted view that endoreduplication favors sustained cell elongation in the absence of mitosis. However, it could also be important for other reasons. Endoreduplication could present a means for organisms to increase the numbers of functional genes copies within each cell, thereby acting to mitigate any adverse effects of environmental influences – for example ultraviolet irradiation – on transcription of the genome.

The tissue-specific pattern of endopolyploidy suggests that endoreduplication cycles in plants constitute an essential part of the developmental program that are necessary for differentiation and for the specialized function of given cells and tissues. Regulation of endopolyploidy may operate at multiple cellular levels. Transformation of the mitotic cycle to the endoreduplication cycle by inhibition of the G2/M transiton is required, and the number of endoreduplication cycles is probably controlled by given cells and tissues. Therefore, the formation of differentiating endopolyploid tissues is likely to be controlled by components of the cell cycle machinery. Indeed, Grafi and Larkins (1995) demonstrated that endoreduplication in the development of maize endosperm proceeds as a result of both the inactivation of M-phase-related cyclin-dependent kinases (CDKs) with a inhibitor and the induction of S-phase-related CDKs. Drugs known to inhibit protein kinases have been shown to induce endoreduplication in plant cells (Nagl 1993). Recently, Cebolla et al. (1999) identified a plant mitotic inhibitor that links cell proliferation to cell differentiation and promotes endoreduplication. Jacqmard et al. (1999) described a cell cycle gene, *CKS1At*, that was associated with the endoreduplication cycle. Expression of *CKS1At* was present in endoreduplicating tissues in *A. thaliana*. Moreover, endoreduplication seems to be under the control of growth regulators. In tobacco protoplasts, an auxin-only signal induces endoreduplicatoin and cell expansion (Valente et al. 1998). Treatment of apricot fruits with auxin resulted in an increase in mesocarp volume due to cell enlargement and endopolyploidy (Bradley and Crane 1955).

Explants with multiple ploidy levels may have an effect on the ploidy level of the regenerants. Cells in the tetraploid cell cycle will produce tetraploid regenerants if their competence of regeneration has been maintained. In transformation work with *Brassica*, explants are usually pieces of young seedlings grown in vitro, especially hypocotyls and cotyledons (Moloney et al. 1989; Radke et al. 1989; Metz et al. 1995). Metz et al. (1995) reported that hypocotyl explants from in vitro grown seedlings of cabbage regenerated a high percentage (33%) of tetraploid transformed plants. In protoplast culture of rapid-cycling *B. oleracea* (Hansen and Earle 1994) and *B. campestris*, syn. *B. rapa*, (Olin-Faith 1996), a majority of the regenerated plants were tetraploid.

An understanding of the basis of endopolyploidy is important for understanding both the regulation of gene expression in differentiated tissues and the nature of tissues used for plant regeneration and transformation. Parallels can also be drawn to the regulation of epigenetic silencing. In *Nicotiana sylvestris* plants transgenic for a chitinase gene, epigenetic transgene silencing was developmentally regulated (Hart et al. 1992). Ploidy changes also influence gene expression. Mittelsten Scheid et al. (1996) observed the reduced gene expression of a transgene in triploid versus diploid *A. thaliana*. Galitski et al. (1999) identified genes showing ploidy-dependent expression in isogenic yeast (*Saccharomyces cerevisiae*) strains that varied in ploidy from haploid to tetraploid. These genes were induced or repressed in proportion to the number of chromosome sets. It is possible that differential gene expression might be involved in cells of different ploidy levels.

In conclusion, our flow cytometric data indicated that most of the somatic tissues of *Brassica* seedlings are multiploid and that this phenomenon is developmentally regulated. The availability of adequate molecular probes will prove beneficial in gaining to an understanding of this important aspect of differentiation in plants.

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