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ENDOPOLYPLOIDY PATTERNS DURING DEVELOPMENT OF *CHENOPODIUM QUINOA*

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Patterns of endopolyploidy were studied in embryos and seedlings during early development. Relative nuclear DNA content was measured with DAPI staining and flow cytometry. Somatic tissue of *Chenopodium quinoa* (Chenopodiaceae) revealed extensive endopolyploidization; tissues comprised mixtures of cells with DNA content ranging from 2C to 16C in varying proportions. Endopolyploidy patterns corresponded to the developmental stage and the individual organ. Polysomaty was already present in the radicle of the embryo in the imbibited seed. During seedling development, endopolyploidization took place in many examined organs (roots, hypocotyls, cotyledons) to different extents. The C-value was highest in the differentiated root, where up to 50% of the cell underwent one or two endocycles. Endopolyploidization was not present in nuclei from leaves and the shoot apex.

Key Words: *Chenopodium quinoa*, endopolyploidy, flow cytometry, mean C-level, polysomaty.

INTRODUCTION

Endopolyploidization of nuclear DNA is a common feature in seed plants and has been estimated to occur in over 90% of all angiosperms. Variation of cell endopolyploidy levels, known as somatic polyploidy, is a developmental process involving one or several rounds of nuclear DNA synthesis without chromosomal and cellular division (D'Amato, 1989; Traas et al., 1998). Unlike true polyploidy (in the narrow sense, meaning sets of individual chromosomes) in endopolyploid nuclei the chromosomes are not separated and form bundles of sister chromatids (Sesek et al., 2005; Bauer and Birchler, 2006). Systemic somatic endopolyploidy has been reported in many species, such as *Arabidopsis thaliana* (Galbraith et al., 1991), *Brassica napus* (Chen et al., 1994), *Brasssica oleracea*, (Kudo and Kimura, 2001a), *Cucumis sativus* (Gilissen et al., 1993) and the orchid *Vanda* Miss Joaquim (Lim and Loh, 2003). Within a given species, organs differ in the degree of endopolyploidization. Generally, endopolyploidization is high in cotyledons, leaf stalks, and lower leaves; it is lower in flower organs and roots, and very low in upper leaves (Barow, 2006). Variations of DNA content have been linked with differentiation of highly specialized cell types such as vascular elements, storage cells of the endosperm, the embryo suspensor, *Arabidopsis* epidermal hair cells, and cells in the nodules of *Medicago truncatula* (Melaragno et al., 1993; Vinardell et al., 2003; Barow, 2006). There is increasing evidence that the pattern of endopolyploidy is a characteristic of tissue type and developmental stage, indicating that endopolyploidization is spatially and temporally regulated. It has also been suggested that multiplication of the genome might contribute to the differentiation of certain cell types (Galbraith et al., 1991; Lim and Loh, 2003). Functional significance has been ascribed to endopolyploidy, including a role in coordinating the gene expression required for interaction of nuclear and organelle genomes. Correlations between nuclear DNA content, cell size and nuclear volume have been found. It was suggested that during differentiation the most active cells need a certain DNA mass, which is essentially the same in all species of comparable complexity; hence endoreduplication has been proposed as a mechanism to generate a sufficient amount of DNA in anticipation of a further large increase in tissue mass. DNA endoreduplication might be an evolutionary strategy that compensates for the lack of a phylogenetic increase in nuclear DNA. The occurrence of high systemic endopolyploidy in species with small genomes such as *Arabidopsis thaliana* or *Cucumis sativus*

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seems to confirm this hypothesis (Nagl, 1976; Bryans and Smith, 1985; De Rocher et al., 1990; Galbraith et al., 1991; Gilissen et al., 1993; Sugimoto-Shirasu and Roberts, 2003).

Flow cytometry can accurately determine the DNA content of large numbers of nuclei from many tissues for study of the relationships between endopolyploidy and tissue type, developmental stage, ploidy level and environmental factors (Galbraith et al., 1991; Mishiba and Mii, 2000; Engelen-Eigles et al., 2001).

Chenopodium quinoa is a pseudograin that originated in the Andean region of South America. Its seeds are of excellent nutritional value (Risi and Galwey, 1984), so it has attracted interest in agriculture and science. There are no reports on polysomaty in *C. quinoa* although it is known that endopolyploidization occurs during the development of other species of this genus (Kolano et al., 2008). Knowledge about the endopolyploidization processes in plants is especially important for in vitro culture since differences in endopolyploidization levels in explant cells may be reflected in chromosome variation in callus culture, a possible reason for somaclonal variation in regenerated plants (Gilissen et al., 1996; Fras and Maluszynska, 2004). Here we report flow cytometric data on the occurrence of endopolyploidization and the development of polysomaty in various quinoa organs.

MATERIALS AND METHODS

MATERIAL

Seeds of *C. quinoa* were obtained from the Botanic Garden in Nancy, France (accession no. 262). Seedlings were grown in soil in a growth chamber under a 16 h photoperiod at 20±2°C. For embryo isolation the seeds were soaked in distilled water at 4°C for 18 h.

FLOW CYTOMETRY ANALYSIS

Young leaves were used for genome size estimation. The samples were chopped with a razor blade in nuclei extraction buffer (Dolezel and Gohde, 1995), filtered through 30 μm nylon mesh and stained with propidium iodide using a Partec high-resolution DNA kit according to the manufacturer's instructions. Nuclei isolated from leaves of *Lycopersicon esculentum* cv. Stupicke (2C DNA = 1.96 pg, Dolezel et al., 1994) were used as internal standard. The 2C value was taken as the average of 30 measurements (3 replicates of 10 measurements each with CV: 4.1–4.7). Ploidy patterns were determined for *C. quinoa* using plants at different developmental stages: embryos isolated from imbibited seeds (stage 0), one-day seedlings (stage 1), two-day seedlings (stage 2), three-day seedlings (stage 3), one-week plants (stage 4) and two-week plants (stage 5). For flow cytometry analysis, isolated embryos and seedlings were separated into several parts. Depending on the stage, radicles, root tips, differentiated parts of the root, hypocotyls, cotyledons, young leaves and shoot tips with immature leaves were taken for analysis. To release nuclei the tissue sample was chopped in nuclei extraction buffer (Dolezel and Gohde, 1995), filtered and stained with DAPI (4,6-diamidino-2-phenylindole). Each sample consisted of tissue from ten different plants, and for each sample 5000–8000 nuclei were analyzed. At least three replicates were analyzed. The DNA content of the isolated nuclei in the samples was analyzed with a DAKO Galaxy flow cytometer. Data processed with FloMax 2.4 (Paxtec GmbH) were plotted on a semilogarithmic scale. To show the mean degree of endopolyploidization of the analyzed organs, the mean C-value of a sample was calculated as described by Lemontey et al. (2001) according to the equation:

$$\text{Mean C value} = \sum_{i=1}^{n} \frac{C_i \times N_i}{N_{sample}}$$

where N is the number of DNA content peaks from the sample, C_i is the C-value of the nuclei of peak n_i, N_i is the number of nuclei of peak n_i, and N_{sample} is the number of nuclei of all peaks of the sample.

RESULTS

DEVELOPMENTAL STAGES

Six developmental stages were distinguished morphologically, from imbibited seed (stage 0) to seedling (stage 5) (Fig. 1). These stages were grouped in two phases: germination phase (stages 0–4) and seedling phase (stages 5 and 6). Germination of seeds started within one day (stage 1). Stage 2, a seedling with folded cotyledons, was reached after two days. Stage 3, a seedling with unfolded cotyledons, was achieved after three days. Stage 4, a seedling with lateral roots, was reached by the majority of the seedlings after seven days. The first pairs of leaves appeared when seedlings were two weeks old (stage 5). Very rapid growth of the hypocotyl-root axis occurred in the germination phase (Fig. 2).

FLOW CYTOMETRY

The genome size for *C. quinoa* was established by flow cytometry analysis of propidium iodide-stained nuclei. The 2C DNA content was determined as 3.0±0.04 pg.

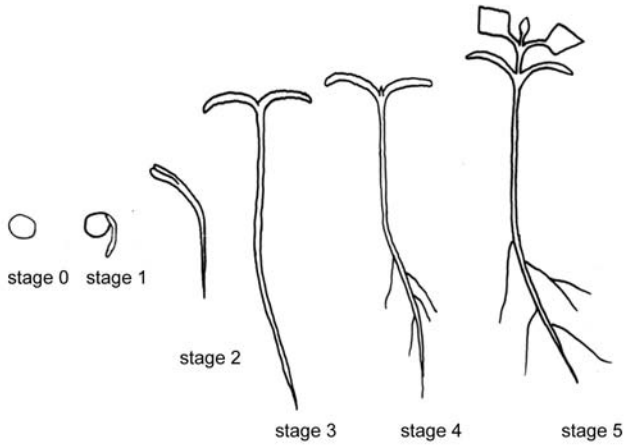

Fig. 1. Stages of *C. quinoa* seed germination and seedling development. Stage 0 – imbibited seed; Stage 1 – emergence of radicle from seed coat; Stage 2 – seedling with folded cotyledons; Stage 3 – seedling with unfolded cotyledons; Stage 4 – development of secondary roots; Stage 5 – development of first and second leaves.

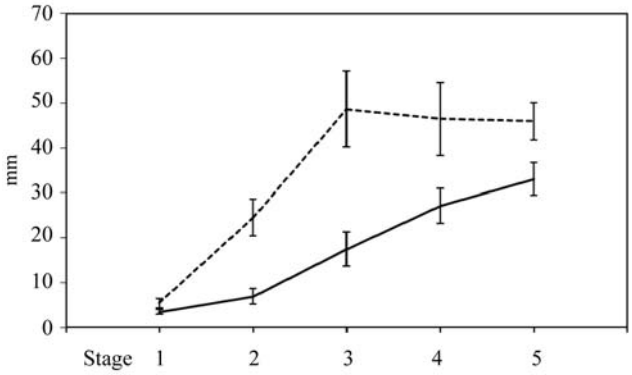

Fig. 2. Primary root (dashed line) and hypocotyl (continuous line) elongation kinetics of *C. quinoa* seedling. Whiskers indicate standard deviation.

Polysomaty of *C. quinoa* was analyzed using flow cytometry and DAPI-stained nuclei. In this work, only nuclei with DNA content higher than that in the G2 phase of the cell cycle were considered endopolyploid. Flow cytometry could not distinguish nuclei that had just entered endoreduplication from cells in the G2 phase of the cell cycle because they have the same DNA content. Endopolyploid cells appeared very early in *C. quinoa* development. In the radicle of the embryo, part of the nuclei had undergone one or two endocycles, and 8C and 16C DNA peaks were observed. However, the majority of radicle cells had 2C (51%) or 4C (39%) DNA content. Embryo cotyledons had a large peak for 2C DNA cells (86.7%) and a small one for 4C DNA cells (Tab. 1, Fig. 3).

Changes in the endopolyploidy pattern at successive stages of development between stage 1 and stage 5 were analyzed in cotyledons, hypocotyls, root tips and differentiated root parts (Tab. 1). Endopolyploidy was highest in roots. In the root tip of seedlings in stage 1, the majority of cells had 2C or 4C DNA content, but cells with 8C and 16C DNA content were also found (22%). In successive stages the number of nuclei with DNA content higher than 4C gradually decreased (Tab. 1), and in stage 5 there were only cells with 2C and 4C DNA content in root tips. In the differentiated part of the root in stage 1 the number of nuclei with diploid 2C and 4C DNA content was significantly lower than in the meristematic region, and the amount of endopolyploid cells was greater than 40% (Tab. 1). During further development the number of endopolyploid nuclei with DNA content higher than 4C initially increased to 50.1% and then decreased; however, even in stage 5 there were cells with 8C and 16C DNA content.

The patterns of polysomaty during development differed between shoot and root. In stage 1, endopolyploid cells were found only in hypocotyls (Tab. 1). At this stage the number of endopolyploid cells was relatively low (9%), but in the next stages their number increased significantly, reaching 31.5% in stage 3. The number of 2C DNA nuclei decreased from 50% in stage 1 to 29.3% in stage 3. Beginning in stage 4 the number of endopolyploid cells decreased, and by stage 5 that number was again low (10%), with no 16C DNA cells. Cotyledon tissues consisted mainly of cells with 2C and 4C DNA, but during subsequent development the number of 2C nuclei decreased from 80% in stage 1 to 41% in stage 5. In stages 4 and 5 a few cells with 8C DNA content appeared. The DNA content in shoot tips and young leaves was also analyzed for two-week-old seedlings. There were only two peaks for 2C and 4C DNA in these organs (Tab. 1). In both cases the peak for 2C DNA was significantly higher and contained 80% or more of all analyzed nuclei.

MEAN C-VALUE IN EMBRYOS AND SEEDLINGS

For embryos isolated from imbibited seeds the mean C-value was calculated at 2.3 for cotyledons and 3.5 radicles, indicating the presence of endopolyploid cells in the embryo radicle (Tab. 1). The C-value differed for every part of the seedling and changed during plant development. The mean C-level in *C. quinoa* seedlings ranged from 2.0 for organs not expressing any endopolyploidy to 6.8 for differentiated root parts at stage 2 (Tab. 1, Fig. 4). At stage 1 the mean C-value in the cotyledons, which did not express endopolyploidy, was 2.4. In all other organs it was higher than 3.0, indicating the pres-

TABLE 1. Percentage of nuclei with different DNA content in embryos and organs of developing *Chenopodium quinoa*

Stage	Organ	Percentage of nuclei with given DNA content				Mean C-value
		2C	4C	8C	16C	
0	Radicle	50.4	38.5	10.4	0.7	3.49±0.20
	Cotyledons	86.8	13.2	–	–	2.26±0.03
1	Root tip	29.9	48.0	18.5	3.6	4.58±0.32
	Differentiated root part	18.0	39.4	33.9	8.7	6.03±0.45
	Hypocotyl	50.1	40.9	9.0	–	3.35±0.09
	Cotyledons	80.3	19.7	–	–	2.39±0.03
2	Root tip	37.8	43.5	14.2	4.6	4.35±0.13
	Differentiated root part	11.9	38.0	36.8	13.3	6.80±0.15
	Hypocotyl	32.3	48.3	18.6	0.8	4.23±0.43
	Cotyledons	74.5	25.5	–	–	2.51±0.06
3	Root tip	45.9	38.0	13.5	2.6	3.95±0.36
	Differentiated root part	15.3	40.9	32.8	11.1	6.33±0.22
	Hypocotyl	29.3	39.2	28.8	2.7	4.89±0.29
	Cotyledons	62.5	37.5	–	–	2.76±0.33
4	Root tip	58.7	37.8	3.5	–	2.98±0.21
	Differentiated root part	24.3	46.3	22.7	6.8	5.21±0.32
	Hypocotyl	42.8	41.8	14.7	0.7	3.84±0.20
	Cotyledons	38.1	60.3	1.7	–	3.30±0.08
5	Root tip	60.5	39.5	–	–	2.77±0.08
	Differentiated root part	37.0	45.3	13.3	4.4	4.34±0.31
	Hypocotyl	52.0	37.1	10.9	–	3.43±0.21
	Cotyledons	40.6	57.4	2.0	–	3.23±0.65
	Leaves	91.9	8.1	–	–	2.16±0.06
	Shoot apex	80.9	19.1	–	–	2.41±0.06

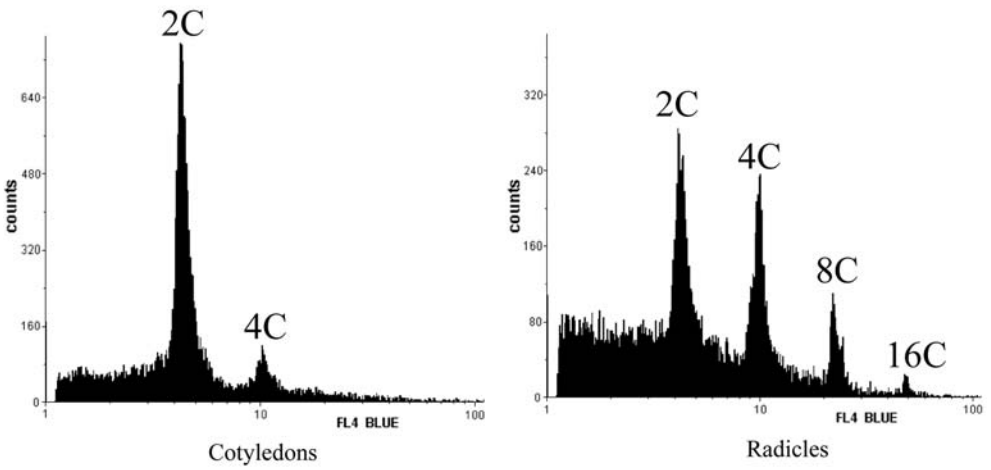

Fig. 3. Typical flow cytometric histograms (log scale) of nuclei isolated from cotyledons and radicles of *C. quinoa* embryos (stage 0).

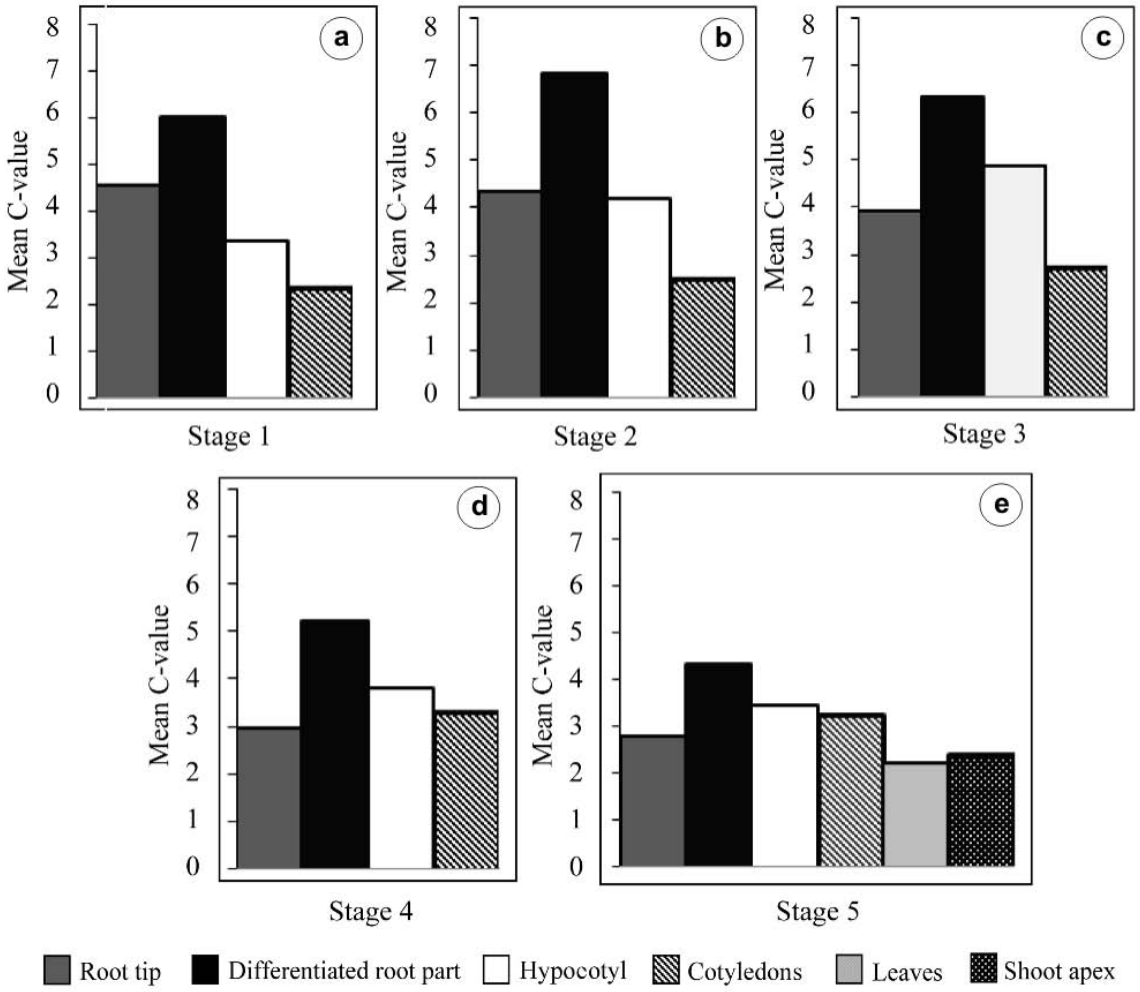

Fig. 4. Mean C-level in different organs of *C. quinoa* during seedling development. (**a**) Stage 1, (**b**) Stage 2, (**c**) Stage 3, (**d**) Stage 4, (**e**) Stage 5.

ence of cells that had undergone endocycles (Tab. 1, Fig. 4). During further development the mean C-value in root tips gradually decreased to 2.0 in two-week-old plants, while in differentiated root parts the parameter increased, reaching maximum at stage 2 when some of the cells underwent two endocycles, and then decreasing to 4.34 in stage 5. The situation was similar in hypocotyls: at germination the mean C-value increased, reached maximum at stage 3 (4.89) when about 30% of the cell underwent one or two endocycles, and then decreased to 3.46 in stage 5 (Tab. 1, Fig. 4). A continuous increase of mean C-value was observed in only cotyledons. At stage 1 the mean C-value was 2.39, similar to the value in embryo cotyledons. In subsequent stages it gradually increased, reaching 3.23 at stage 5, the highest value found for this organ. In general, during *C. quinoa* development the mean C-value was higher during germination than in the seedling phase, and varied among the analyzed organs more during the first phase than in the seedling phase (Fig. 4)

DISCUSSION

The results from flow cytometry clearly demonstrate that *C. quinoa*, like many angiosperms, is a polysomatic plant. Barow and Meister (2003) showed that endopolyploidization is related to the taxonomic position of a species. Some taxonomic groups have many species with highly endopolyploidized tissues, and others do not. Our data are in line with findings from other authors indicating that endopolyploidization is common in the Chenopodiaceae (Barow and Meister, 2003; Barow and Jovtchev, 2007; Łukaszewska and Sliwinska, 2007; Kolano et al., 2008). *C. quinoa* is an annual herb with a small

genome, so our data support the observed tendency for polysomaty to occur in species with smaller genomes and a short life cycle (de Rocher et al., 1990; Olszewska and Osiecka, 1982; Barow and Meister, 2003). However, not all groups of species show a negative correlation between genome size and endopolyploidy, especially when woody species, which often have small genomes and generally do not exhibit endopolyploidy, are included (Barow and Jovtchev, 2007). Another factor apparently correlated with endopolyploidization is life strategy. Among short-lived plants, those adapted to infertile soils seem less likely to exhibit endopolyploidy (Barow and Meister, 2003), although quinoa, which is best adapted to rocky, alkaline and saline soils, revealed endopolyploidy at least in the seedling phase.

Polysomaty was already present in the radicle of the quinoa embryo. Endopolyploidization was occurring in a number of cells of this organ in an early stage of tissue differentiation during embryo development. Such early development of polysomaty has been reported in *Cucumis sativus*, *Phaseolus vulgare* and *Spinacia oleracea* (Gilissen et al., 1993; Bino et al., 1993). In other species (*Fagus silvatica*, *Pinus nigra*, *Cichorium endivia*), embryos isolated from mature seeds contained mainly 2C DNA nuclei or, as in *Raphanus sativus*, revealed two peaks of 2C and 4C DNA (Bino et al., 1993).

Multiples of the haploid nuclear genome complement (1C) corresponding to 2C, 4C, 8C, and 16C were revealed in the quinoa root and hypocotyls, whereas in cotyledons only 2C, 4C and 8C cells were observed. Thus the maximum number of endocycles in quinoa seedlings was two. This is less than that reported for other plants such as *Beta vulgaris* (three endocycles) or *Brassica* (five endocycles) (Kudo and Kimura, 2001b; Sliwinska and Lukaszewska, 2005). Note that the results for sugar beet and *Brassica* were obtained from plants grown in vitro; we grew quinoa in soil in a growth chamber. Different conditions of plant cultivation can modify the polysomaty pattern, as endopolyploidization can be influenced by many environmental factors (Barlow, 2006).

The pattern of endopolyploidization differed between organs and developmental stages of the quinoa seedling. Such developmentally regulated polysomaty has been reported in *Arabidopsis thaliana*, *Brassica oleracea* and *Cucumis sativus*. (Galbraith et al., 1991; Gilissen et al., 1993; Kudo and Kimura, 2001a). Quinoa roots exhibit a high mean C-value, indicating the presence of endopolyploid cells. Endopolyploid cells were present even in root tips in early stages of development. The presence of endopolyploid cells in root tips might be related to early xylem cell differentiation, which is associated with endomitotic cycles (Baluska, 1990). Endopolyploidy was highest in differentiated root parts, where the mean C-value was the highest found during all of seedling development. In each analyzed stage the hypocotyls also showed a mean C-value higher than 3 (at least one endocycle), and up to 30% of the cells had DNA content higher than 4C. Similar results were obtained for *Beta vulgaris*, a species of the *Chenopodiaceae* family (Sliwinska and Lukaszewska, 2005): the number of endopolyploid cells was higher in hypocotyl and root. The finding of highest endopolyploidy in root and hypocotyl might be explained by the presence of a large proportion of vascular tissue in these organs. Large cell size is required for the functioning of this tissue, and this can be achieved by endopolyploidization (Baluska, 1990; Sliwinska and Lukaszewska, 2005). Older organs usually exhibit higher levels of endopolyploidy than younger ones (Galbraith et al., 1991; Kudo and Kitamura, 2001; Lim and Loh, 2003). Interestingly, quinoa root and hypocotyls differed in the correlation between endopolyploidy level and age. In root tips the level of endopolyploidy continuously decreased; in differentiated root parts and hypocotyls the mean C-value initially increased and then gradually decreased during seedling development. A similar development pattern for polysomaty was observed in sugar beet roots and hypocotyls (Sliwinska and Lukaszewska, 2005). The increase of this parameter seems correlated with their rapid elongation during germination, and this may be connected with vascular tissue development. The mean C-level was at maximum in stage 2 for differentiated root parts and in stage 3 for hypocotyls. In later stages the value decreased both for differentiated root parts and for hypocotyls. It is likely that most of the xylem cells undergo programmed cell death after reaching a certain level of DNA content and cannot be detected by flow cytometry.

Unlike many polysomatic plant species such as *A. thaliana* and *Cucumis sativus* (Galbraith et al., 1991; Gilissen et al., 1993), *C. quinoa* cotyledons showed only nuclei with 2C and 4C DNA content during the germination phase. Endopolyploid cells did not appear before stage 4. This is consistent with data obtained for *Beta vulgaris*, in which older cotyledons usually exhibited higher levels of endopolyploidy than younger ones (Sliwinska and Lukaszewska, 2005). Such a low level of endopolyploid cells is relatively rarely observed in the cotyledons of polysomatic species. Studies of various plant species among genera including *Vicia, Lens, Cucumsi* and *Brassica* indicated that their cotyledons consist of cells with high endopolyploidy, which can reach 64C DNA in *Lupinus consentini* for example (Bryans and Smith, 1985; Gilissen et al., 1993; Sakowicz and Olszewska, 1997; Kudo and Kitamura, 2001b; Hajdera et al., 2003).

Our analysis of the DNA content in the shoot apex and young leaves of *C. quinoa* showed a lack of

endopolyploid cells in these organs. The absence of polysomaty in the shoot meristem may be related to prevention of genetically unstable germ line cells originating from shoot apical meristem tissue; it has been observed in many plant species, for example *A. thaliana* and *Vanda* Miss Joaquim (Galbraith et al., 1991; Lim and Loh, 2003). Knowledge of the basis of endopolyploidy is important for an understanding of the regulation of gene expression in differentiated tissues, and of the nature of the tissues used for plant regeneration and transformation. It may be useful in decreasing the occurrence of somaclonal variation and polyploidization in culture in vitro (D'Amato, 1985). In endopolyploid nuclei the chromosomes are present as bundles of sister chromatids, but the degree of positional sister-chromatid alignment decreases with increasing ploidy (Schubert et al., 2006). Pre-existing endopolyploidization in explant cells is probably one of the reasons for early polyploidization in callus cells. (Fras and Maluszynska, 2004; Fras et al., 2007). When organs with endopolyploid cells serve as explants, some regenerants can be polyploid (Chen et al., 2009). Tissues expressing polysomaty should be avoided as explants, or else the regenerants should be monitored for ploidy.

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