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CELLULAR ACCUMULATION OF PROTEIN BODIES AND CHANGES IN DNA PLOIDY LEVEL DURING SEED DEVELOPMENT OF *LATHYRUS TUBEROSUS* L.

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Mitotic activity, changes in nuclear DNA content and accumulation of protein bodies during endosperm and embryo development of *Lathyrus tuberosus* L. (2C DNA 15.1 ± 0.08 pg) were analyzed. A gradual decrease in mitotic activity of embryo cells was observed during seed development. At the final stages of embryogenesis, mitotic activity was noted only in tip meristems and provascular tissue. The decrease in mitotic activity in endosperm was accompanied by an increase in amitosis frequency. Cytophotometry of nuclear DNA revealed that in endosperm the nuclei underwent six endoreplication rounds, reaching 192C. The C DNA level was much higher at the chalazal pole than at the micropylar pole, where it was only 12C. In the cotyledon the nuclear DNA level reached 64C (five endoreplication rounds), while in the hypocotyl-root axis it increased from 2C to 4C. Cells of all *Lathyrus tuberosus* embryo organs are involved in the synthesis of storage proteins and their accumulation in the form of protein bodies. This process starts ~21 days after fertilization (DAF). Cells containing protein bodies are capable of division. The authors characterized the central and basal zones of cotyledons according to the amount of protein bodies per 400 μm^2 of cytoplasm area. Double staining (Feulgen/Coomassie Brilliant Blue R250) was used for measurement of nuclear DNA and storage protein content in the same cells. The ploidy of nuclei correlated with total protein body volume (r = 0.97) in *Lathyrus tuberosus* cotyledon cells.

Key words: *Lathyrus tuberosus*, seed development, embryo, cotyledon, endosperm, endoreplication, protein bodies.

INTRODUCTION

The seed is where zygotic embryo development occurs; thus it plays an important role in maintaining the succession of generations in angiosperms (Esau, 1977). Seed development is a sequence of specific spatial and temporal events. The primary phase of cell divisions is followed by elongation, differentiation, and accumulation of storage substances (protein, carbohydrates, lipids). The latter phase is one of the primary functions of seeds (Esau, 1977).

Seeds of leguminous plants (Fabaceae) are an important source of plant protein, second only to cereals. Fabaceae seeds, unlike cereal, are relatively poor in tryptophan and sulfur-containing amino acids such as cysteine and methionine but contain much lysine, so from a nutritional point of view these plants are complementary (Duranti and Gius, 1997). The major storage proteins of Fabaceae seeds are globulins, that is, legumin and vicilin. They belong to a large group of proteins termed secretory proteins. They are transported through a system of membranes from the endoplasmic reticulum (ER) via the Golgi apparatus (Gapp) to vacuoles. Finally, storage proteins are accumulated in the form of protein bodies which in dicotyledonous plants are formed mainly from protein storage vacuole (PSV) fragmentation (Vitale and Raikhel, 1999).

The expression of storage protein coding genes as well as the localization of their products in developing seeds are precisely regulated. Moreover, there are tissue differences in storage protein coding gene expression between mono- and dicotyledonous species; it takes place mainly in endosperm and embryo tissues, respectively; storage protein gene expression occurs during the cell expansion stage when the fresh weight of seeds increases (Corke et al., 1987). In *Vicia faba*, however, apart from the well documented main storage phase of protein deposition starting at cotyledon stage, a short period of formation of a small number of little protein bodies in the embryo proper, suspensor and endosperm was observed at early globular stage (Panitz et al., 1995). Moreover, the legumin B gene from

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Vicia faba transferred to *Nicotiana tabacum* exhibited the same double-phase expression pattern (Panitz et al., 1997, 1999). The main phase of storage protein accumulation at the final stages of embryogenesis provides a large amount of nutritional substances used by the young seedling after germination (Tiedemann et al., 2000).

Development of plant organs is mainly the result of the mitotic activity of existing or newly formed meristems. After the arrest of cell divisions, further differentiation of organs can be accompanied by DNA endoreplication and increase in cell volume. DNA endoreplication, then, is observed already during plant embryogenesis, and is closely connected with differentiation (Nagl, 1978). Endoreplication is precisely controlled genetically (Joubs and Chevalier, 2000). It appears at certain differentiation stages in specific cells and tissues with high metabolic activity (Grafi, 2002), and it results in the formation of endopolyploid nuclei (Nagl, 1978). The dynamics of endoreplication depends on 2C DNA content, the species, and the type of life cycle of the plant (Olszewska and Osiecka, 1983). It has been suggested that endoreplication allows higher gene expression than at normal DNA content levels, depending on the cell's needs (Larkins et al., 2001).

In the present research, changes in mitotic activity and nuclear DNA content during the development of *Lathyrus tuberosus* endosperm and embryo were analyzed. The results were used to specify the time of appearance and distribution of protein bodies in *Lathyrus tuberosus* endosperm and embryo. The aim of the study was to determine how mitotic activity influences storage protein synthesis and whether the ploidy level of cotyledon parenchyma cells affects the amount of protein bodies amassed during the main phase of protein accumulation.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds from wild-growing plants of *Lathyrus tuberosus* L. (Fabaceae) were used in this study. Embryos at different developmental stages were analyzed, estimated on the basis of size (Marciniak, 1991b) and the number of days after fertilization (DAF). The relationship between embryo length and successive developmental stages of endosperm was also determined.

METHODS

Seeds were fixed in MAF (96% methanol: 40% formaldehyde:glacial acetic acid, v:v:v, 80:15:5) for 24 h at RT (room temperature 22˚C) or in FAA (70% ethanol:40% formaldehyd:glacial acetic acid, v:v:v, 90:5:5) for 24 h at RT, or in 2,5% glutaraldehyde: 50 mM sodium cacodylate, 15% sucrose (pH 7.2) for 12 h at 4˚C. It is well known that fixatives containing formaldehyde are recommended for analyses where good preservation of DNA and proteins is required.

Before the Feulgen procedure the seeds were cut longitudinally for better penetration of reagents. Seeds fixed in MAF were hydrolyzed for 1 h with 4 N HCl at RT, and nuclear DNA was stained by the Feulgen method (Schiff's reagent was made of pararosanilin, Sigma) for 1 h in dark, at RT. After washing in SO_2 water, embryos proper and endosperms were isolated from the ovules. Squashed preparations were made by the dry ice method. The preparations were mounted in Canada balsam. The mitotic index was determined by counting the number of cells in mitosis per 1,000 randomized cells from 4 embryos for each stage.

CYTOPHOTOMETRY

Cytophotometric measurements of DNA content were performed at 550 nm with a CCD camera-based image processing system (IMAL-1024). Twenty telophase and metaphase plates from primary root meristems of young seeds were used to determine 2C and 4C DNA content values. In each experimental series, DNA content in 100 nuclei of embryo cells was measured. The ploidy level of nuclei of embryo cells was determined based on the 2C and 4C values. The DNA content values were calculated based on cytophotometric measurements and expressed in arbitrary units (AU). The absolute DNA amounts measured by Feulgen cytophotometry were calibrated by using the 2C value for *Allium cepa* (33.5 pg) as a standard (Bennett and Smith, 1976). The material was fixed for 2 h in freshly made ethanol-acetic acid (3:1, v/v) and stored in 70% ethanol at 0–4˚C. Six roots from *Lathyrus tuberosus* embryos were hydrolyzed, stained and washed in the same baths together with *Allium cepa* roots. Feulgen absorbance measurement values were converted to weight units (picograms) using *Allium cepa* cytophotometric measurements as a standard (2C = 33.5 pg) (Bennett and Smith, 1976).

ANALYSIS OF PROTEIN BODIES

After FAA or 2.5% glutaraldehyde fixation, seeds were dehydrated in an ethanol series and embedded in Paraplast Plus (Sherwood Medical, St. Louis, U.S.A.). Sections were cut 8 µm thick with a steel blade on a rotary microtome, mounted on poly-L-lysine-treated slides (Polysine, Menzel-Glaser), and dried for 24 h at 37˚C (Borisjuk et al., 1995).

Deparaffinized and hydrated sections were hydrolyzed for 1 h with 4 N HCl at RT, and stained by the Feulgen method. Following Feulgen staining, the sections and squashed preparations were stained with 0.02% Coomassie Brilliant Blue R 250 in Clarke's solution (pH 2) for 20 h. Negative controls for protein

TABLE 1. Mean mitotic activity during seed development, % ± SE

Component	Embryo length [mm]									
	0.1	0.5	1.0	2.0	3.0		4.5		5.0	
Embryo	Whole embryo				A	C	A	C	A	C
	11.9±1.41	9.2±0.44	8.5±1.94	7.6±0.24	6.3±0.52	3.3±0.49	5.2±0.11	0.9±0.27	0.0	0.0
Endosperm	49.1±3.94	18.2±8.80	4.8±2.77	2.8±1.37	0.0		0.0	0.0		

A – axis; C – cotyledons

analysis (Irwing, 1984) were performed by treating sections with 0.2% trypsin (lyophilized; Sigma Chemical) in 0.05 M Tris buffer (pH 8) at 35˚C for 20 h or with 0.05 M Tris buffer and then stained in Coomassie Brilliant Blue R 250.

Tissue preparations were analyzed with an OLYMPUS BX 60 microscope equipped with a SONY DXC–950 P video camera, using an image analysis system (software for SIS, Analysis ver. 3.0). In order to standardize the results, the diameter and total volume (μm^3) occupied by protein bodies were measured for cytoplasm area units of 400 μm^2 for embryo and 2500 μm^2 for endosperm. Total volumes of protein bodies per cell/endosperm were calculated on the basis of their sizes and number. Cytophotometric measurements of endosperm protein body absorbance were carried out at 560 nm and protein content was expressed in arbitrary units (AU).

Protein body content during the accumulation phase in different *Lathyrus tuberosus* embryo tissues was analyzed in semithin sections. Based on preliminary histological and histochemical studies, three embryo developmental stages were chosen for further analyses: (I) stage of preliminary accumulation of protein bodies, embryos 2.9–3.3 mm long; (II) stage of advanced accumulation, embryos 3.5–4.0 mm long; and (III) final stage of storage protein accumulation, embryos 4.7–5.5 mm long. Four embryos were analyzed for each stage. The protein body size and number were determined based on 60 measurements for each examined tissue (720 measurements for each stage).

Developing cotyledons of *Lathyrus tuberosus* were used for analysis of protein body accumulation in cells with different ploidy levels. Based on embryo length and organization, three cotyledon developmental stages were chosen for analyses: (I) embryo 4.0 mm long, (II) embryo 4.5 mm long, and (III) embryo 5.0 mm long. Squashed preparations of three cotyledons of embryos from different plants were made for each stage. The preparations were stained with Feulgen/Coomassie Brilliant Blue R 250. The relationship between the ploidy level and the rate of protein body accumulation was established based on analysis of the diameter and total volume of protein bodies in cell populations representing 4C, 8C, 16C and 32C nuclear DNA levels. Measurements were performed for cells representing 5 cotyledon zones: basal, apical, abaxial, adaxial and central. In each cotyledon zone, the ploidy level of 120 cells was determined and plotted against the number and sizes of protein bodies per cytoplasm area unit (400 μm^2).

For analysis of protein body accumulation in endosperm, nine developmental stages were chosen based on the length of the embryo proper in ovules from which the examined tissue was taken: (I) 0.12 mm, (II) 0.32 mm, (III) 0.56 mm, (IV) 0.88 mm, (V) 0.95 mm, (VI) 1.65 mm, (VII) 2.00 mm, (VIII) 2.20 mm and (IX) 3.00 mm. For each stage, the protein body accumulation was analyzed at both endosperm poles: micropylar and chalazal. Three endosperm preparations were used for each developmental phase.

STATISTICAL ANALYSIS

Data were analyzed with Statistica for Windows 98, (StatSoft Tulsa, U.S.A.) and Excel'98. Data are presented as means (%) and means ± standard error. One-way ANOVA was performed with the Tukey test, and coefficients of correlation were determined. Significance levels are given only if lower than $p = 0.05$.

RESULTS

MITOTIC ACTIVITY IN EMBRYO AND ENDOSPERM

Mitotic divisions were observed at all developmental stages except for embryos 5.0 mm long. In embryos over 2.0 mm in length, mitotic activity was determined separately for the hypocotyl-root axis and for cotyledons. In the globular-stage embryos (0.1 mm in diameter), the mitotic index (MI) was 11.9% (Tab. 1), and decreased at successive developmental stages to 0.9% in cotyledons of embryos 4.5 mm long. In 3.0 mm and 4.5 mm long embryos, the mitotic activity of the hypocotyl-root axis was higher than that of cotyledons. Moreover, cotyledon parenchyma cells of these embryos did not show any mitotic activity. However, mitotic divisions occurred in provascular and epidermal cells (Tab. 1).

Mitotic activity at successive stages of endosperm development showed wave-like fluctuations. The highest mitotic activity was found in endosperm isolated from a seed whose embryos were 0.1 mm long (49.1%).

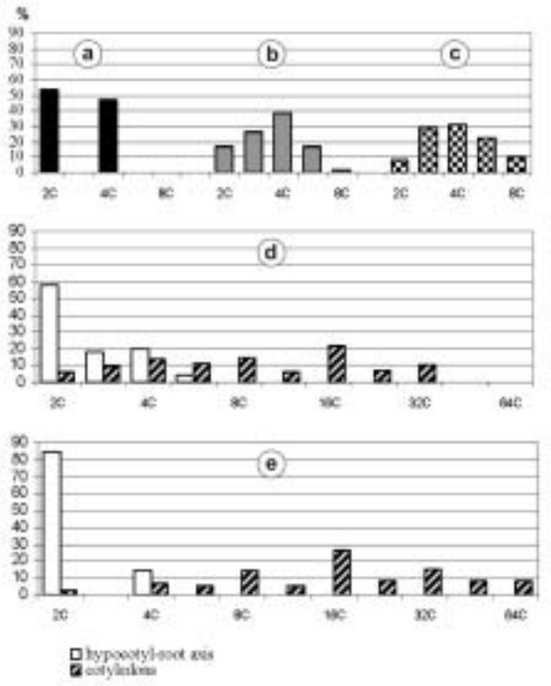

Fig. 1. Changes in nuclear DNA content in cells during successive stages of embryo development. Developmental stages were correlated with embryo length: (**a**) 0.12 mm, (**b**) 0.5 mm, (**c**) 2.5 mm, (**d**) 3.5 mm, (**e**) 4.6 mm.

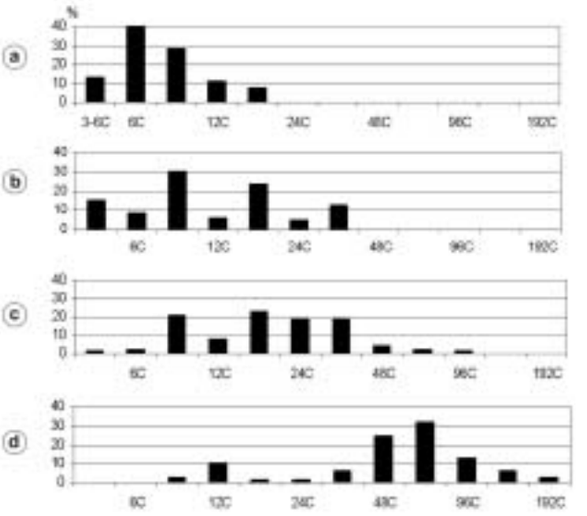

Fig. 2. Changes in nuclear DNA content in nuclear endosperm from seeds at different developmental stages of embryos (correlated with embryo length): (**a**) 0.1 mm, (**b**) 0.5 mm, (**c**) 0.7 mm, (**d**) 2.5 mm.

At succeeding developmental stages it decreased significantly. MI was 2.8% in endosperm from seeds containing embryos 2.0 mm in length (Tab. 1). Mitotic activity was highly variable at all endosperm developmental stages. In endosperm surrounding embryos 3.0 mm long, no mitotic divisions were found (Tab. 1). On the contrary, endosperm degradation was observed.

CHANGES IN DNA CONTENT DURING THE EMBRYO AND ENDOSPERM DEVELOPMENT

For *Lathyrus tuberosus*, 2C DNA content was determined to be 15.1 ± 0.08 pg. In embryos 0.12 mm long, nuclear DNA content was 2C (53.1%) and 4C (46.9%) (Fig. 1a). In embryos 0.5 mm long, 4–8C (16.7%), and 8C DNA (1.8%) nuclei occurred, in addition to nuclei with DNA content levels characteristic of proliferation (2C, 2–4C and 4C DNA). Nuclei with 4C DNA (38.9%) dominated in the analyzed population (Fig. 1b). Similar content of nuclear DNA was also observed in embryos 2.5 mm long (Fig. 1c); the number of 2C DNA nuclei decreased (8.2%) in comparison with the previous stage, and those with 8C DNA increased (10.2%) (Fig. 1c). DNA content in embryos more than 2.5 mm long was analyzed separately for the hypocotyl-root axis and cotyledons. In the hypocotyl-root axis of embryos 3.5 mm long, nuclear DNA content ranged between 2C and 4–8C, with 2C DNA nuclei dominating (48.6%), and only 4% of the nuclei had 4–8C DNA (Fig. 1d). In the hypocotyl-root axis of embryos 4.6 mm long from completely dehydrated seeds, only 2C (85.2%) and 4C DNA (14.8%) nuclei were observed (Fig. 1e). In cotyledons of embryos 3.5 mm long, nuclear DNA content ranged from 2C to 32C. In cotyledons of embryos 4.6 mm long, 16C DNA nuclei dominated (26.2%); the highest DNA content found was 64C (8.3%) (Fig. 1e).

In the endosperm of seeds in which the embryos were 0.12 mm long, nuclear DNA content ranged from 3–6C (13.4%) to 12–24C (7 .3%) (Fig. 2a), with 6C DNA nuclei dominating (40.2%). Endosperm development was accompanied by an increase in the number of nuclei with higher DNA content (Fig. 2b,c). In the endosperm from seeds in which the embryos were 2.5 mm long, nuclear DNA content reached 192C. Classes of nuclei with DNA content below 6–12C level were not found, and the dominating levels were 48C (24.6%), 48–96C (31.9%) and 96C (13.0%) (Fig. 2d).

Cytophotometric measurements of nuclear DNA content at the micropylar and chalazal poles revealed significant differences in ploidy levels between the respective nuclei. At the micropylar pole, regardless of endosperm developmental stages, nuclear DNA content did not exceed 12 C. At the chalazal pole, however,

TABLE 2. DNA content (C) in micropylar (M) and chalazal (CH) regions of endosperm during successive stages of seed development

Pole of endosperm	Stage						
	I	II	III	IV	V	VI	VII
M	3–6 C 6 C 6–12 C	3–6 C 6 C	3–6 C 6 C	6 C 12 C	6 C 12 C	6 C 12 C	12 C
CH	6 C 12 C 12–24 C	12 C 12–24 C	24 C 24–48 C	48 C 96 C	24 C 48 C 48–96 C	24 C 48 C 48–96 C	48 C 96 C 192 C

DNA content increased with consecutive developmental stages, reaching 192C (Tab. 2).

In endosperm, cytophotometric measurements showed that decreased mitotic activity was accompanied by an increase in the frequency of amitotic divisions. During the first developmental stages only a few nuclei divided amitotically, but as endosperm developed their number increased; in endosperm surrounding embryos 3.0 mm long such nuclei were common. Individual fragments of amitotically dividing nuclei contained from 2C to 48C DNA, depending on the number of fragments. A few of them had average values, that is, 6–12C or 12–24C DNA.

PROTEIN BODY ACCUMULATION IN THE EMBRYO

Synthesis and accumulation of storage proteins began in embryos ~3.0 mm long, 21 days after fertilization (DAF).

The first protein bodies detectable by light microscopy appeared at the first stage of accumulation (I) in cotyledon parenchyma cells. Protein bodies were observed in the central, abaxial, adaxial and basal zones of cotyledons. In all these zones the dominating protein bodies were smaller than 0.5 μm in diameter (Tab. 3) and their total volume was less than 60 μm^3 (Fig. 3).

During further embryo development, protein bodies appeared first in parenchyma cells of the cotyledon apical zone, then in parenchyma cells of the hypocotyl-root axis and in epidermis. Shoot meristematic cells as well as provascular cells of the cotyledon and hypocotyl-root axis accumulated protein bodies last.

At stage II of accumulation, protein bodies were visible in all examined parts of the embryo, but the intensity of their accumulation varied in different types of cells. It was higher in parenchymatic cells of cotyledons, the hypocotyl-root axis and the adaxial epidermis, than in other cell types of the developing embryo (Tab. 3). In all cells of embryos during stage II of accumulation, protein bodies up to 1 μm in diameter dominated. In parenchymatic cells of cotyledons, the hypocotyl-root axis and the adaxial epidermis of cotyledons, the largest protein bodies were 6.0 μm in diameter. In plumula and in the epidermis of the embryo axis, small protein bodies were observed in mitotically active cells.

TABLE 3. Localization of protein bodies during successive stages of embryo development

Tissue		Stage		
		I	II	III
Cotyledon parenchyma (zone)	apical	-	++	+++
	central	+	++	+++
	abaxial	+	++	+++
	adaxial	+	++	+++
	bazal	+	++	+++
Plumula		-	+	+++
Cotyledon provascular system		-	+	+++
Axis provascular system		-	+	+++
Axis epidermis		-	+	+++
Cotyledon abaxial epidermis		-	+	+++
Cotyledon adaxial epidermis		-	+	+++
Hypocotyl-root axis parenchyma		-	+	+++

Occurrence of protein bodies:
+ – beginning stage of protein body accumulation
++ – advanced stage of protein body accumulation
+++ – final stage of protein body accumulation

The greatest protein body volumes at accumulation stage II were observed in cotyledon basal (835.8 μm^3), central (666.8 μm^3) and adaxial zones (660.9 μm^3) (Fig. 3b). The lowest mean volumes were observed in plumula cells (33.6 μm^3) and in axis provascular cells (33.2 μm^3) (Fig. 3a).

Embryos at the final (III) stage of protein body accumulation were mature (~34 DAF). In all examined cell types, the number of the smallest protein bodies (<0.5 μm in diameter) decreased as the largest ones (>4.5 μm in diameter) disappeared. At stage III of protein body accumulation, storage protein volume was double that of the previous stage in all cell types except axis parenchyma, where the increase was slight (from 447.1 to 482.0 μm^3) (Fig. 3a,b). In parenchyma and adaxial cotyledon epidermis, protein bodies occupied the greatest

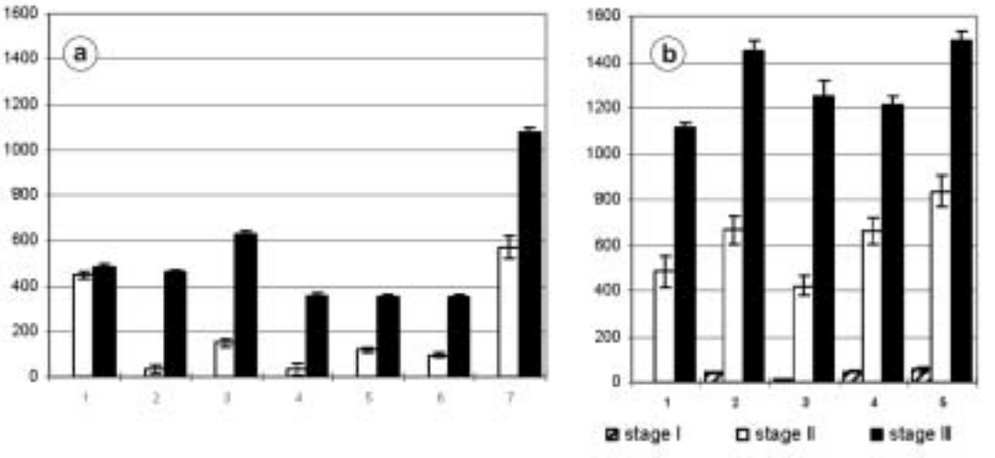

Fig. 3. Total protein body volume ± SE measured per 400 μm^2 area. (**a**) Different tissues. 1 – axis; 2 – meristem; 3 – cotyledon provascular system; 4 – axis provascular system; 5 – axis epidermis; 6 – cotyledon adaxial epidermis; 7 – cotyledon abaxial epidermis, (**b**) Cotyledon zones of three stages of protein body accumulation. 1 – apical; 2 – central; 3 – adaxial; 4 – abaxial; 5 – basal. Bars at top indicate SD.

total volume (from 1076.6 μm^3 to 1493.1 μm^3). In the other tissues the volume ranged from only 349.4 μm^3 in axis epidermis to 625.2 μm^3 in the cotyledon provascular system f (Fig. 3a,b).

In order to determine any differences between cotyledon zones and cotyledon adaxial epidermis (variant I) and the other tissues (variant II) at stage III, one-way ANOVA was performed with the Tukey test (Figs. 4, 5). Analysis at $p = 0.0001$ revealed that the central and basal zones were characterized by the highest total volume of protein bodies, significantly higher than the other zones. The differences in volumes between the apical, abaxial and adaxial zones and cotyledon adaxial epidermis were not statistically significant (Fig. 4). The lowest values of total volume of protein bodies were in axis epidermis, cotyledon abaxial epidermis and provascular system (Fig. 5).

RELATIONSHIP BETWEEN DNA AND TOTAL VOLUME OF PROTEIN BODIES

The total volume of protein bodies was estimated per 400 μm^2 of cytoplasm. Cotyledon zones of embryos 4.0 mm long (stage I) exhibited differences in total protein body volume (μm^3) in cells with the same level of ploidy (Fig. 6a). Protein body volume in 4C DNA cells ranged from 20.7 μm^3 in the apical zone to 489.3 μm^3 in the central zone. The situation was similar in 8C, 16C and 32C DNA cells. In the apical, abaxial and adaxial zones, increased ploidy level was accompanied by a slight increase in storage protein volume. No such correlation was observed in the central and basal zones, where the differences in total volume of protein bodies in cells containing 4C, 8C, 16C and 32C DNA were negligible (490–627 μm^3 in central zones and 194–250 μm^3 in basal zones) (Fig. 6a). Statistical analysis did not reveal any correlation between nuclear DNA content and total volume of protein bodies in cells at this stage (Pearson's correlation coefficient $r = 0.39$).

In cotyledons of embryos 4.5 mm long (stage II), protein body volume was increased in all cotyledon zones (Fig. 6b). In the central zone the increase was slight. Moreover, protein body volume increase along with DNA content increase reached a higher level in 32C cells (as much as 5-fold in the apical zone) than in 4C DNA cells. Statistical analysis showed a high correlation between DNA content and protein body volume in cotyledon cells at stage II (Pearson's correlation coefficient $r = 0.79$).

In all examined zones of mature cotyledons (stage III), increased nuclear ploidy was accompanied by increased protein body volume (Fig. 6c), which grew gradually but did not double in cells with higher ploidy level. Two-fold increases were observed only between 4C, 8C and 16C DNA cells. Pearson's correlation coefficient indicated a high correlation between DNA content and total volume of protein bodies. It was statistically significant even for significance level $p = 0.000001$.

PROTEIN BODY ACCUMULATION IN ENDOSPERM

In *Lathyrus tuberosus*, endosperm synthesis of storage proteins and their accumulation as protein bodies occurred already at the globular stage of embryogenesis. In the initial stages of endosperm development (phases I-V), mean storage protein content at both poles, which

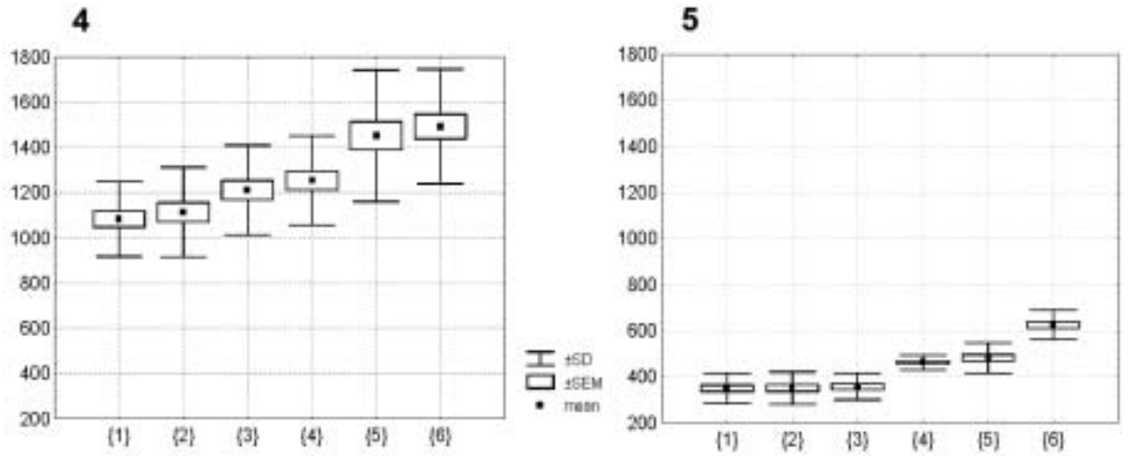

Fig. 4–5. Graphic presentation of one-way ANOVA. **Fig. 4.** First variant. 1 – adaxial epidermis of cotyledons; 2 – apical; 3 – adaxial; 4 – abaxial; 5 – central; and 6 – basal zone of cotyledons. **Fig. 5.** Second variant. 1 – axis epidermis; 2 – abaxial epidermis of cotyledons; 3 – axis provascular system; 4 – plumula; 5 – hypocotyl-root axis parenchyma; 6 – cotyledon provascular system.

varied in ploidy, reached similar levels (5000 to 6700 AU). When the embryo became 1.6 mm long, transient disappearance of visible protein bodies occurred (phase VI). Reappearance of storage protein synthesis in endosperm (which takes place starting at 15 DAF) and storage protein accumulation was observed until this trophic tissue disintegrated. Protein content at the micropylar pole then increased to the level recorded at the beginning of endosperm development (5061 AU). No such phenomenon was observed at the chalazal pole, and maximum protein content was lower than 2000 AU (Fig. 7).

Protein bodies were also observed in endosperm zones characterized by high mitotic activity. In the regions of mitotic activity, the examined structures were absent only from phragmoplasts.

DISCUSSION

Among the species of the genus *Lathyrus*, 2C DNA content ranges from 6.9 to 29.2 pg (Bennett and Smith, 1991). In our study, the 2C DNA content in *Lathyrus tuberosus* was estimated at 15.1 ± 0.08 pg. This value is lower than the 18.6 and 19.5 pg 2C DNA content reported by Bennett and Leitch (2005) for the same species. These differences can be attributed to differences in the methodology of DNA content measurement. Bennett and Smith (1976) thoroughly discussed the possible sources of these kinds of differences.

It is well documented that the highest mitotic activity occurs in early stages of embryogenesis in globular embryos (Rodkiewicz at al., 1996). In endosperm, mitotic divisions start soon after fertilization. In *Triticum aestivum* the first mitotic division of endosperm nuclei is usually observed 6 h after pollination. The first division of the zygote takes place several hours after fertilization (Raghavan, 1997). Starting from the heart-shaped stage, mitoses gradually decrease in frequency and then stop altogether (Rodkiewicz et al., 1996). In *Vicia faba*, mitoses in the hypocotyl-root axis, plumula and provascular tissues persist until late cotyledon stage, while in cotyledon parenchyma cells they persist until middle cotyledon stage (Borisjuk et al., 1995).

The results reported here also indicate a gradual decrease in mitotic activity in the developing *Lathyrus tuberosus* embryo. In cotyledon parenchyma cells, mitotic activity declined in embryos over 1.8 mm in length. In the remaining tissues, mitotic activity persisted until nearly the end of seed maturation, when they reached 4.5 mm in length. Hypocotyl-root axis cells exhibited higher mitotic activity than cotyledon cells, while in endosperm the mitotic activity stopped when the embryo reached 3.0 mm in length. At subsequent developmental stages, endosperm degradation by programmed cell death (PCD) (Wojciechowska and Olszewska, 2002) was observed, leading to the appearance of nonendospermic seeds. Decreased mitotic activity in *Lathyrus tuberosus* endosperm was accompanied by an increase in the number of contracting nuclei dividing amitotically. Suggested amitosis or fusion of nuclei during endosperm development has been described in *Pisum sativum* and *Vicia faba* ssp. *minor* (Marciniak, 1991a).

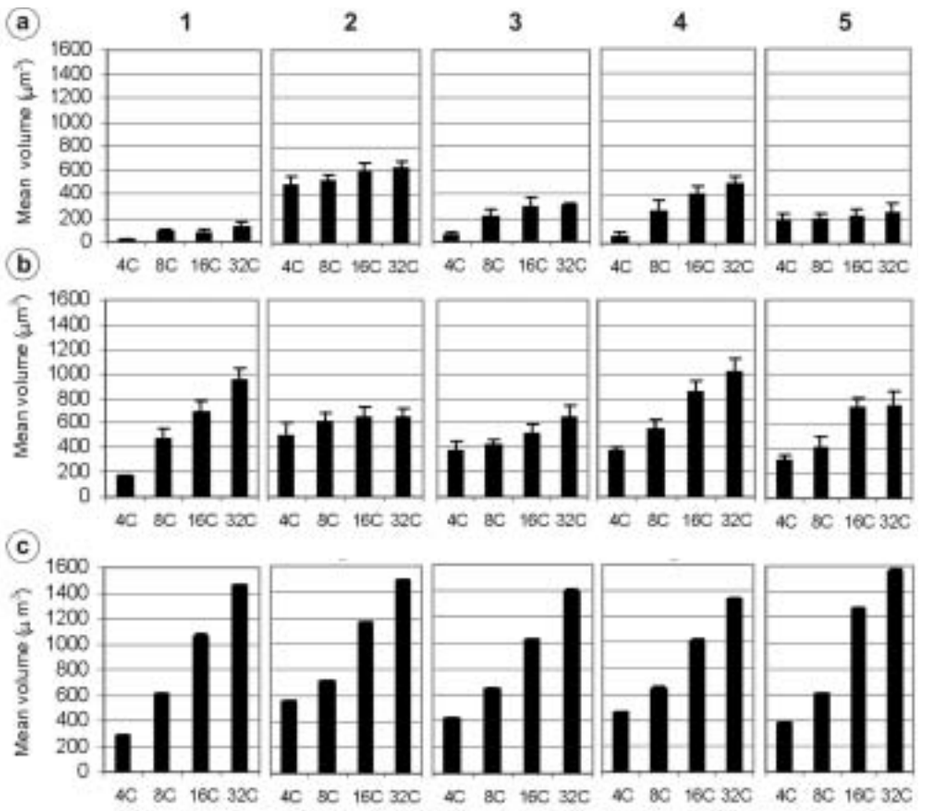

Fig. 6. Total protein body volume ± SE measured per 400 µm^2 of cytoplasm in cells with different ploidy levels in the examined cotyledon zones at three developmental stages. (**a**) Cotyledon stage I, (**b**) Cotyledon stage II, (**c**) Cotyledon stage III. 1 – apical zone; 2 – central zone; 3 – abaxial zone; 4 – adaxial zone; 5 – basal zone. Bars at top indicate SD.

After the period of endosperm development characterized by intensive mitotic divisions, nuclear DNA content increased by endoreplication. Endoreplication is thought to be a common, basic process accompanying endosperm development in mono- and dicotyledonous species (Marciniak, 1991b). In *Zea mays* endosperm, a decrease in mitotic activity is followed by an increase in nuclear DNA content to 384C (Kuran and Marciniak, 2002). A similar phenomenon was observed in *Pisum sativum* ssp. *arvense* cotyledon (Smith, 1973) as well as in endosperm and embryo of *Lathyrus tuberosus*. Generally, storage tissues (endosperm and cotyledon parenchyma) are characterized by a significantly higher DNA endoreplication level (at least several replication rounds) than, for example, root or leaf parenchyma (Marciniak, 1991b).

In *Lathyrus tuberosus*, 2C–4C DNA content was observed in the hypocotyl-root axis; thus this part of the embryo is diploid. Endoreplication takes place in the cotyledon, reaching 64C at the end of seed development. Similarly, when DNA content in cotyledons reaches 96C in *Vicia faba*, nuclear DNA content in root cells is only 2C and 4C (Borisjuk at al., 1995). In *Lathyrus tuberosus* endosperm, the endoreplication level reaches 192C at maximum at the chalazal pole, while at the micropylar pole it is 12C. Spatial differences in the ploidy of nuclei were described in *Vicia faba* endosperm, which, like *Lathyrus tuberosus*, is of the nuclear type. In this endosperm type, nuclear size gradually increases from the region surrounding the embryo to the chalazal region of the endosperm, where the nuclei are largest (Panitz et al., 1995).

Continuation of DNA synthesis in non-dividing cells suggests that polyploidization results in extra copies of storage protein genes. However, neither in monocotyledonous nor in dicotyledonous species has distinct selective amplification of the genes that code storage proteins during seed development been observed. Most genes for these proteins had one or three but no more than five copies per haploid genome (Bewley and Black, 1994).

Different embryo tissues are engaged in storage protein accumulation. In mature *Prosopis velutina* (Fabaceae) embryos, all tissues except the embryo root and

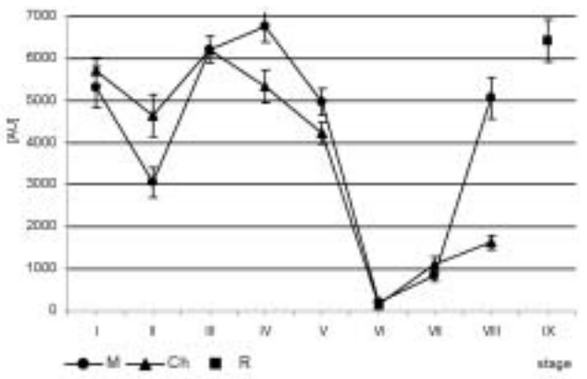

Fig. 7. Relative content of protein body storage proteins at successive stages of endosperm development (I–IX). M – micropylar; Ch – chalazal; R – residual. Bars indicate SD.

cotyledon epidermis accumulate protein bodies (Irwing, 1984). In the *Vicia faba* embryo, no protein bodies are observed in meristematic cells of the axis and shoot or in provascular tissues (Borisjuk et al., 1995). Similarly, in mature embryos of transgenic *Nicotiana tabacum*, neither provascular tissue nor shoot and root tip meristems are engaged in legumin accumulation (Panitz et al., 1997). However, in the examined *Lathyrus tuberosus*, all organs and tissues of the embryo were found to participate in synthesis and accumulation of storage proteins. Similarly, all tissues of the mature *Capsicum annuum* embryo contain protein bodies (Chen and Lott, 1992). Thus, one cannot characterize one spatial system of protein body accumulation in embryos.

The main storage organ in Fabaceae plants is the cotyledon. ELISA analysis has revealed that the cotyledons of a mature *Vicia faba* embryo accumulate 100 times more storage proteins per mg of soluble protein extract than other tissues (Borisjuk et al., 1995). The present study showed the significance of differences in total volume of protein bodies between cotyledon and hypocotyl-root axis tissues of the embryo. As the embryo developed, the total volume of protein bodies increased in all tissues. In the mature embryo, the highest protein body volume was observed in cotyledon parenchyma and adaxial epidermis cells. In the other types of cells it was 50% lower. In the cotyledon, the greatest total protein body volume measured per 400 μm^2 appeared in the central and basal zones. Moreover, a gradient of intensity of protein body accumulation was observed from the basal and adaxial zones to the abaxial and apical zones of the cotyledon. In *Pisum sativum* cotyledons, vicilin expression patterns are similar; it starts in the adaxial zone and proceeds to the abaxial zone (Hauxwell et al., 1990). Similarly, vicilin and legumin gene expression in *Vicia faba* cotyledons starts in the older adaxial region and spreads towards other regions except for epidermis and provascular tissue (Borisjuk et al., 1995). During *Brassica napus* embryogenesis, storage protein (napin and cruciferin) synthesis starts in the axis (including provascular tissue and meristematic cells), then appears in other parts of the cotyledon, later in its basal and finally in its central zone (Höglund et al., 1992). In *Pisum sativum* (var. *amino* and var. *cameor*) cotyledons in which three zones were identified, – peripheral, middle, and central – protein content per cytoplasm unit was highest in the central and peripheral zones (var. *amino*) and in peripheral and middle zones (var. *cameor*) (Le Gal et al., 1984a).

The correlation between endopolyploidy level and storage protein amount in cotyledons (dicotyledonous) and endosperm (monocotyledonous) has been investigated in several experiments in which DNA content was determined cytophotometrically and protein content either by the content of protein nitrogen per mg endosperm (Cavallini et al., 1995) or by means of dense protein material (DPM) content (Le Gal et al., 1986). The results in those studies indicate such a correlation; that is, the increase in mean DNA content per cell was accompanied by increased DPM (Le Gal et al., 1986b). Panitz et al. (1999) suggest, however, that storage protein accumulation is not correlated with endoreplication because it takes place in cells with normal diploid DNA content, and blockage of endoreplication by inhibition of DNA synthesis does not impair storage protein accumulation. Nor is it limited to morphologically or functionally specific cells such as parenchyma cells.

The nuclear DNA analysis performed in the present research showed that intensification of endoreplication in *Lathyrus tuberosus* cotyledons correlated with the onset of the storage protein accumulation phase. Statistical analysis revealed a close correlation between DNA content and the total volume occupied by protein bodies in individual cotyledon cells of the mature *Lathyrus tuberosus* embryo (Pearson's correlation coefficient r = 0.97). This correlation appears at the second stage of cotyledon development, indicating the biological effects of DNA content multiplication. Cells of the same ploidy level accumulate similar amounts of protein bodies, regardless of the cotyledon zone. Differences in the amount of accumulated protein bodies may result from a combination of cells of different ploidy level. Significant differences in ploidy level between cotyledon zones were observed in *Pisum sativum*, *Vicia faba* and *Lupinus albus* var. *lucky* (Le Gal et al., 1986). In cotyledons of those species, the increase in DNA content proceeded from the peripheral to the central parts of the cotyledon. However, other factors, such as the size of cells in a given cotyledon zone, should not be excluded, as a correlation between cell size and

storage protein deposition has been reported (Corke et al., 1990). Cell volume may be a factor determining which cell starts to accumulate storage proteins (Panitz et al., 1999). On the other hand, no influence of cell nucleus ploidy level on the amount and size of accumulated protein bodies was observed in *Lathyrus tuberosus* endosperm. At the initial stages of endosperm development, mean content of storage proteins at both poles, differing with regard to the ploidy level of nuclei, remained at similar levels. Contrary results were obtained from *Vicia faba* endosperm, where protein bodies were localized in regions with higher ploidy levels of nuclei (Panitz et al., 1995). When protein body accumulation in endosperm reaches maximum, the process starts in the embryo proper. Endosperm degenerates at the middle cotyledon stage, and protein accumulation then takes place in the embryo (Borisjuk et al., 1995).

Storage protein accumulation appears at different developmental stages in both the endosperm and embryo. Storage protein gene expression in seeds is species-specific; it can be seen in different seed tissues regardless of their development and origin, that is, both in differentiated and in meristematic cells. Thus this process must be considered as part of the general ontogenetic program controlling the production of transient and stable reserves of nutrients for the embryo and developing seedling according to demand (Panitz et al., 1999).

It is generally assumed that protein bodies are accumulated only in cells of nonproliferating tissue at the final stage of differentiation (Corke et al., 1990). The hypothesis that storage protein gene expression is limited only to the cell growth phase in embryogenesis is based mainly on studies on the main phase of accumulation of these proteins. Research on *Nicotiana tabacum* contradicts it, providing evidence that cells containing protein bodies are able to divide; protein bodies were observed not only in interphase cells but also in mitotically active ones (Panitz et al., 1999). The results of our research also argue against this hypothesis, as *Lathyrus tuberosus* cells containing protein bodies are also able to divide. In parenchyma cells of *Lathyrus tuberosus*, protein bodies accumulated in cotyledon during cell expansion. In epidermis, provascular tissue and parenchyma tissue of the embryo axis, protein bodies were present in cells exhibiting mitotic activity. Also, in *Lathyrus tuberosus* endosperm there were protein bodies in areas with mitotic activity.

Double staining with Feulgen/Coomassie Brilliant Blue R250 allowed simultaneous estimation of nuclear DNA and storage protein content in single cotyledon cells. The obtained results indicated a distinct correlation between nuclear ploidy level and total protein body volume in *Lathyrus tuberosus* cotyledons.

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