

DEVELOPMENTAL AND CYTOCHEMICAL STUDIES OF THE ENDOSPERM CHALAZAL HAUSTORIUM OF *RHINANTHUS SEROTINUS* (SCROPHULARIACEAE)

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We examined the development of the endosperm chalazal haustorium of *Rhinanthus serotinus*, using histochemical assays and light and electron microscopy. The chalazal haustorium is a huge single cell containing two enlarged nuclei. The nuclei are located in the middle of the haustorium cell. At the chalazal end of the haustorium cell structure, ultrastructural study revealed the presence of a transfer wall forming wall ingrowths. At all examined stages of haustorium cell development we identified insoluble polysaccharides, proteins, nucleic acids and lipid droplets. Macromolecules were especially abundant in the fully differentiated haustorium cell. Our results suggest that the endosperm chalazal haustorium is a site of intense metabolic activity.

Key words: *Rhinanthus serotinus*, endosperm, chalazal haustorium, differentiation, histochemistry.

INTRODUCTION

The endosperm of numerous angiosperms produces special types of structures which function as haustoria. They most often develop at the chalazal and micropylar end or only at the chalazal end of the endosperm. It is very rare for the endosperm to produce a haustorium over its entire surface. Production of endosperm haustoria is common among angiosperms. Their presence has been reported in Cucurbitaceae (Chopra and Seth, 1977), Plantaginaceae (Mikesell, 1990), Poaceae (Mauseth et al., 1985), Scrophulariaceae (Schmid, 1906; Arekal, 1963; Tiagi, 1966; Johri and Ambegaokar, 1984), Ericaceae (Olson, 1993), Fabaceae (Dute and Peterson, 1992) and Lentibulariaceae (Płachno and Świątek, 2011; Płachno et al. 2011, 2012). The cytoplasm of endosperm haustoria usually is dense although it contains a number of small vacuoles. These cells are often deformed and filled with hydrolyzable content. It is believed that endosperm haustoria take nutrients from the mother plant tissue, transporting them to the endosperm, where sooner or later these abundant nutrients are taken up by the developing embryo (Johri and Ambegaokar, 1984). Some studies on the cytochemistry, ultra-

structure, cytoskeleton and synthetic activity of haustoria indicate that haustorial cells may be involved in the absorption, synthesis and transport of nutrients to the endosperm (Bhatnagar and Kallarackal, 1980; Torosian, 1971; Pacini et al., 1975; Nagl, 1992; Brrison and Peterson, 1975; Dute and Peterson, 1992; Olson, 1993; Świerczyńska and Bohdanowicz, 2003; Świerczyńska et al, 2005, 2006; Płachno and Świątek, 2011; Płachno et al. 2011, 2012). Here we report findings on the development and cytochemical characteristics of the endosperm chalazal haustorium of *Rhinanthus serotinus* (Scrophulariaceae).

MATERIALS AND METHODS

Seeds of *Rhinanthus serotinus* (Scrophulariaceae) were obtained from several plants growing at natural stations in the towns of Rumia and Puck in northern Poland. Flowers at various developmental stages were collected in June and July. The ovules were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) for 4 h at room temperature. Then the plant material was washed in the same buffer, dehydrated in an acetone series, and embedded in

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TABLE 1. Results of cytochemical tests at the different stages of development of the chalazal endosperm haustorium in *Rhinanthus serotinus*. + positive staining; ++ intense staining

Stain	Specificity	Haustorium cell at differentiation	Haustorium cell at full development	Haustorium cell at aging
PAS	Insoluble polysaccharides	+	++	++
Azur-B bromide	Nucleic acids	+	++	++
Aniline Blue Black	Proteins	+	++	++
Sudan Black B	Lipids	++	++	++

Spurr's resin (Spurr, 1969). Sections 1–2 μm thick were cut with glass knives using a SORVALL MT 2B ultramicrotome and placed on glass slides. For LM observations, sections were stained with 0.05% Toluidine Blue O (TBO). For TEM observations the ovules were fixed in 2.5% formaldehyde and 2.5% glutaraldehyde for 4 h, post-fixed in 1% OsO_4 overnight, treated with 1% uranyl acetate for 1 h and embedded in Spurr's resin. After contrasting with uranyl acetate and lead citrate the ultrathin sections were examined in a Philips CM 100 transmission electron microscope at 80 kV. Sections for cytochemical studies were stained with Periodic Acid-Schiff (PAS) for insoluble polysaccharides (Jensen, 1962), with Azure B bromide (Flax and Himes, 1952) for nucleic acids, with 1% Aniline Blue Black in 7% acetic acid for proteins (Jensen, 1962), and with Sudan Black B (in 70% ethanol) for lipids (Bronner, 1975). The preparations were analyzed with a Nikon Eclipse E 800 microscope fitted with a Nikon CCD camera.

RESULTS

The endosperm of *Rhinanthus serotinus* develops after double fertilization from a central cell of the embryo sac according to the cellular type. The endosperm mother cell is divided into two unequal cells: the smaller micropylar and the larger chalazal one. The micropylar cell enlarges and divides longitudinally and then transversely, forming the endosperm micropylar haustorium along with initial cells of the endosperm proper. The large chalazal cell increases as the embryo grows, and forms a unicellular endosperm chalazal haustorium. During development, the nucleus of the chalazal haustorium cell undergoes only one division. The binuclear chalazal haustorium enlarges and damages adjacent tissues (Fig. 1).

In *R. serotinus* the following stages can be distinguished during the development of the endosperm chalazal haustorium cell: (i) differentiation of the chalazal haustorium (at the several-cell stage of the endosperm proper); (ii) full development of the chalazal haustorium (when the endosperm

proper has a few dozen cells); and (iii) degeneration of the chalazal haustorium (when the endosperm proper has several hundred cells) (Fig. 2).

CHALAZAL HAUSTORIUM CELL DURING DIFFERENTIATION

This is the stage in which the chalazal haustorium cell of *R. serotinus* gradually differentiates. The haustorium cell greatly elongates to 600 μm and its diameter reaches 150 μm . The haustorium has two enlarged nuclei. Its micropylar end adheres to cells of the endosperm proper (Fig. 3). Its chalazal end adheres to the several-cell pedestal residues and the cell wall is distinctly thickened (Figs. 3, 4). Table 1 gives the cytochemistry results for the different developmental stages of the chalazal endosperm haustorium. The haustorium walls are PAS-positive. A PAS-positive thickened wall is present at the chalazal end of the haustorium cell (Fig. 5). Protein staining in the cytoplasm of the haustorium cell and in nucleolus proteins is more intense than in the cells of the endosperm proper (Fig. 6). Similarly, staining with Azure B bromide (Fig. 7) shows the presence of RNA and DNA in the enlarged chalazal haustorium cell nucleoli. There are many evenly distributed lipid droplets in the haustorium cell cytoplasm (Fig. 8); they also occur in the cells of the endosperm proper (data not shown).

CHALAZAL HAUSTORIUM CELL DURING FULL DEVELOPMENT

A completely developed chalazal haustorium cell is kidney-shaped. Haustorium length reaches 800 μm and its diameter 250 μm . In the middle of its length the cell has two enormous polytene nuclei. At the micropylar end the haustorium adheres to cells of the endosperm proper. The chalazal wall of the haustorium grows into the ovule tissues in the direction of the vascular bundle (Fig. 9). It is equipped with a labyrinth of wall ingrowths reaching deep inside the cytoplasm (Fig. 10). PAS-positive wall ingrowths are formed in the chalazal part of the haustorium wall, which are longer and wider than in the previous stage (Fig. 11). The concentration of

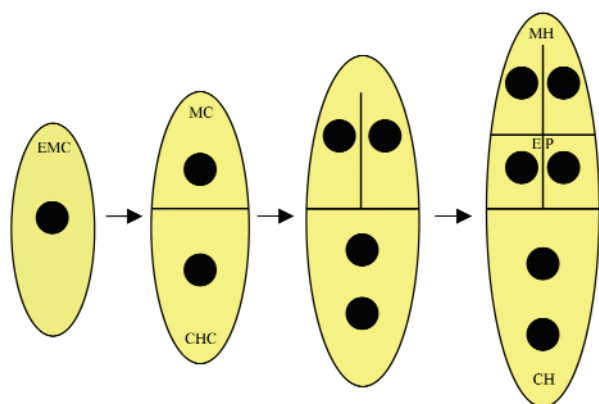


Fig. 1. Scheme of development of *Rhinanthus serotinus* endosperm. Endosperm mother cell (EMC), micropylar cell (MC), chalazal cell (CHC), micropylar haustorium (MH), endosperm proper (EP), chalazal haustorium (CH).

proteins in the haustorium cytoplasm is higher than in the preceding stage (Fig. 12). Azure B bromide staining reveals enlarged nucleoli with high RNA and DNA content in the nuclei of the chalazal haustorium cell (Fig. 13). The cytoplasm of the chalazal haustorium contains numerous lipid droplets uniformly distributed throughout the cell (Fig. 14).

CHALAZAL HAUSTORIUM CELL DURING AGING

During aging the chalazal haustorium cell does not enlarge, and the endosperm proper contains several hundred cells. Some symptoms of degeneration can be observed within the chalazal haustorium cell, manifested primarily as increased cytoplasmic vacuolization. The haustorium cell cytoplasm is noticeably thinner than in the previous stage (Fig. 15). The chalazal end of the haustorium cell wall adheres to crushed ovule tissues and the wall ingrowths continue to expand (Fig. 16), while the micropylar end is adjacent to the cellular endosperm proper (data

not shown). The PAS-positive chalazal haustorium wall ingrowths enlarge further, are strongly branched, and grow deeply into the cytoplasm (Fig. 17). At this stage the levels of Aniline Blue-Black-stained proteins (Fig. 18) and of the more intensely Azure-B-bromide-stained nucleic acids (RNA and DNA) are higher (Fig. 19). A larger number of lipid droplets, which are slightly bigger than in cells of the endosperm proper, are visible in the chalazal haustorium cell (Fig. 20).

DISCUSSION

Three types of endosperm development are described in angiosperms: nuclear, ab initio cellular, and helobial (Vijayaraghavan and Prabhakar, 1984). In many members of Scrophulariaceae the endosperm produces micropylar and/or chalazal haustoria, which penetrate ovular tissues. Usually the endosperm haustoria are fast-developing, highly specialized and synthetically active structures which absorb food materials from the maternal tissues and transfer them to the endosperm (Johri and Ambegaokar, 1984). In the genus *Rhinanthus* the endosperm conforms to the cellular type of development and several members of this genus develop both micropylar and chalazal endosperm haustoria (Schmid, 1906). The endosperm chalazal haustorium of *Rhinanthus serotinus* is a huge, long cell containing two enlarged nuclei with polytene chromosomes. The large size of the haustorium cell and its nuclei is associated with multiplication of its basic nuclear DNA content (Świerczyńska et al., 2005). Only a few reports have described the formation, ultrastructure (Nagl, 1992; Bohdanowicz et al., 1993) and cytoskeleton (Świerczyńska and Bohdanowicz, 2003; Świerczyńska, 2004; Świerczyńska et al., 2006) of such intriguing polytene structures as are found in endosperm chalazal haustoria in the genus *Rhinanthus*.

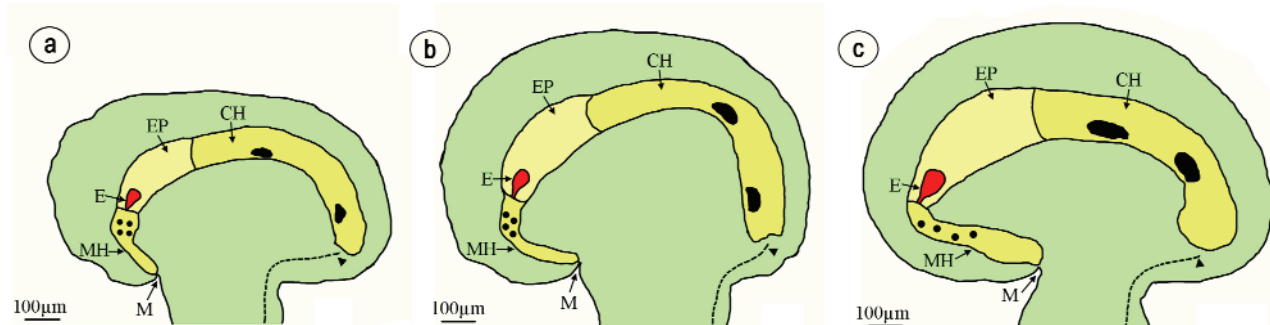
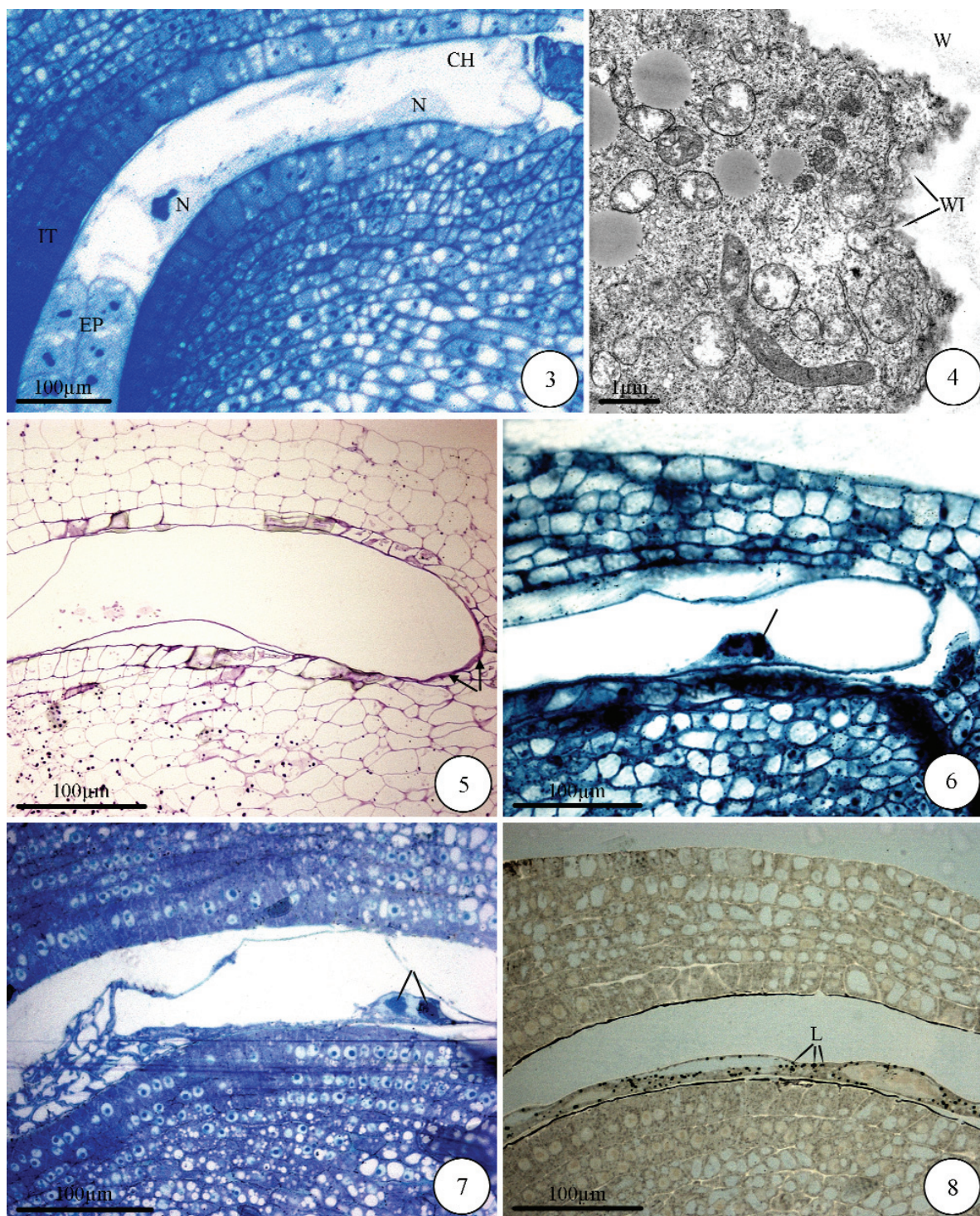
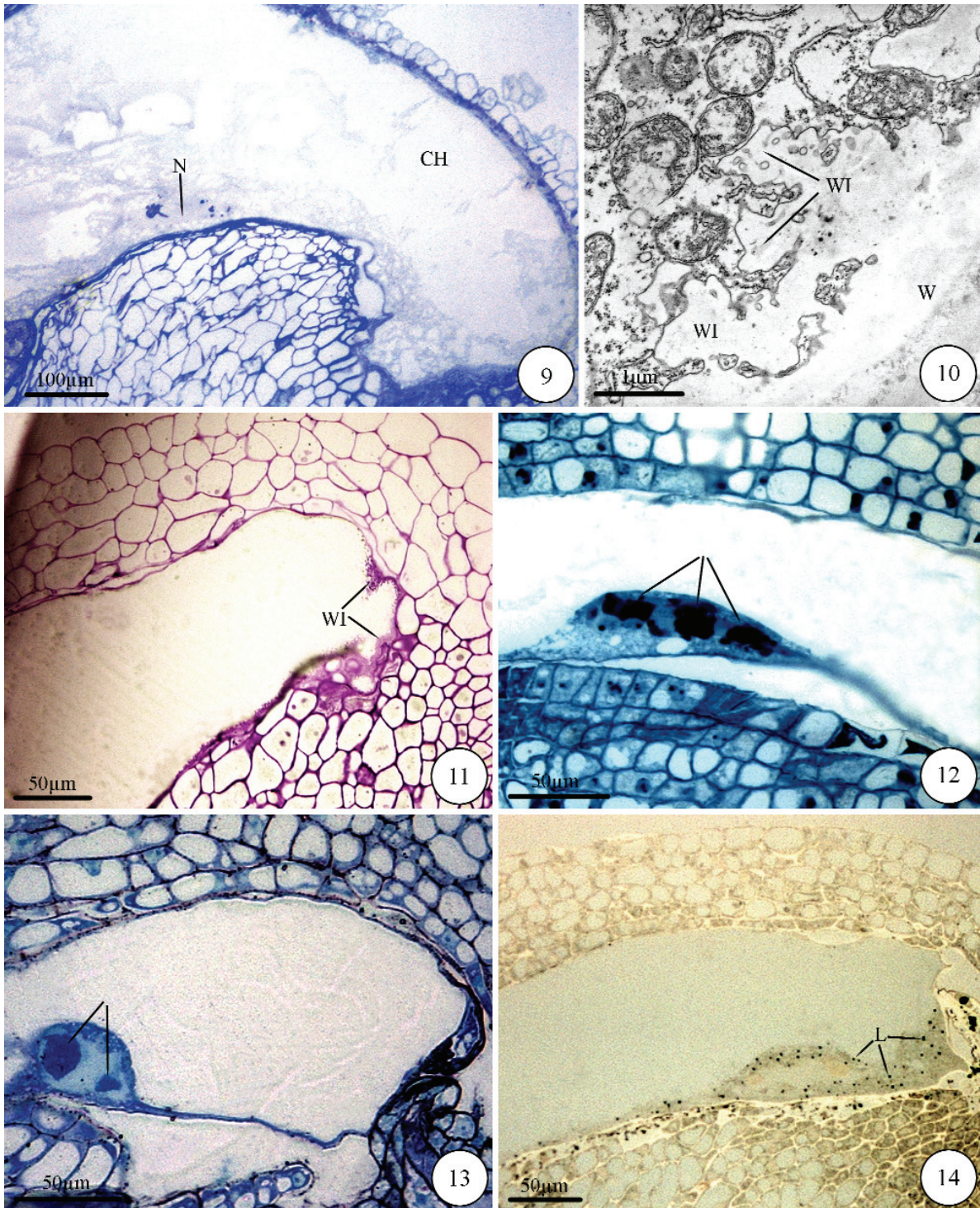


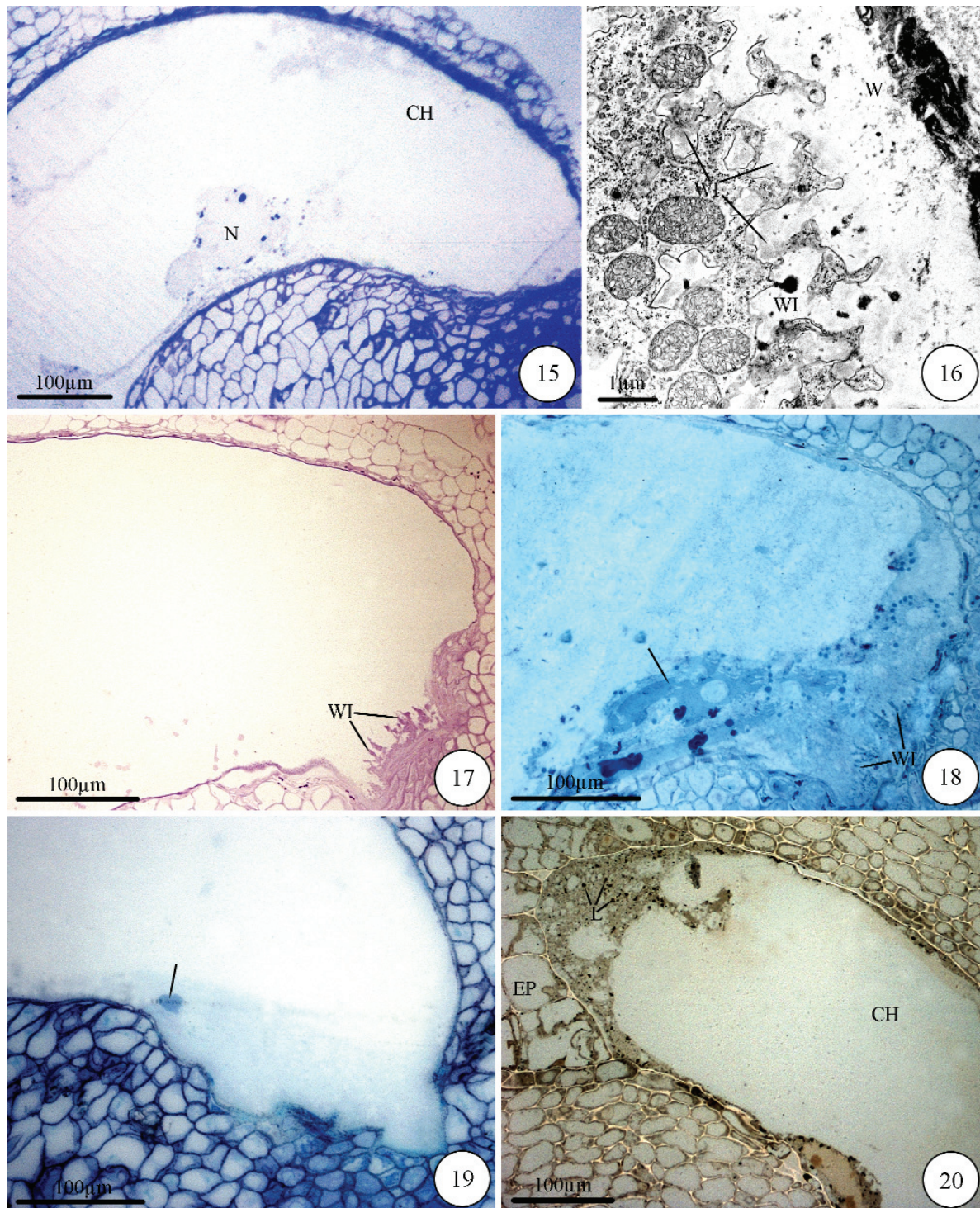
Fig. 2. Schematic longitudinal sections showing stages of development of *Rhinanthus serotinus* endosperm chalazal haustorium cell. (a) Differentiation of chalazal haustorium cell, (b) Full development of chalazal haustorium cell, (c) Degeneration of chalazal haustorium cell. Micropyle (M), micropylar haustorium (MH); embryo (E), endosperm proper (EP), chalazal haustorium (CH), vascular bundle (arrowhead).



Figs. 3–8. *Rhinanthus serotinus* endosperm chalazal haustorium. Stages of differentiation of chalazal haustorium cell after staining and cytochemical reactions. **Fig. 3.** Semithin section showing young chalazal haustorium cell with two nuclei (N) and nucleoli, several cells of endosperm proper (EP), and integumentary tapetum cells (IT). **Fig. 4.** Magnified fragment of haustorium cell; visible are thickened chalazal wall (W) and wall ingrowths (WI). **Fig. 5.** Polysaccharides stained by PAS reaction are visible in walls, especially on the chalazal end of the haustorium cell (arrows). **Fig. 6.** Protein staining with Aniline Blue Black shows intense staining in nucleoli (line). **Fig. 7.** DNA and RNA staining with Azure B bromide reveals their presence in nucleoli (lines). **Fig. 8.** Lipid (L) staining with Sudan Black B shows lipid droplets in haustorium cell cytoplasm (lines).



igs. 9–14. *Rhinanthus serotinus* endosperm chalazal haustorium. Stage of complete development of chalazal haustorium cell; staining and cytochemical reactions. **Fig. 9.** Semithin control slice stained with Toluidine Blue O shows strongly vacuolized cell of chalazal haustorium (CH) and one of the nuclei (N). **Fig. 10.** Highly magnified fragment of chalazal haustorium wall (W), showing wall ingrowths (WI). **Fig. 11.** Polysaccharide staining by PAS reaction; lines point to PAS-positive wall ingrowths (WI) of chalazal wall of haustorium cell. **Fig. 12.** Protein staining with Aniline Blue Black; one of the nuclei with strongly stained nucleoli (lines) visible in haustorium fragment. **Fig. 13.** DNA and RNA staining with Azure B bromide; fragment of haustorium with strongly stained nucleoli (lines). **Fig. 14.** Lipid (L) staining with Sudan Black B; spherical lipid droplets present in haustorium cytoplasm.



Figs. 15–20. *Rhinanthus serotinus* endosperm chalazal haustorium. Degeneration/aging of chalazal haustorium cell; results of staining and cytochemical reaction. **Fig. 15.** Semithin control slice of chalazal haustorium (CH) stained with Toluidine Blue O, showing one of the nuclei (N). **Fig. 16.** Fragment of chalazal haustorium wall (W), showing expanding wall ingrowths (WI). **Fig. 17.** Polysaccharide staining by PAS method; PAS-positive wall ingrowths (WI) of chalazal wall of haustorium cell. **Fig. 18.** Protein staining with Aniline Blue Black, showing one of the nuclei (line) with highly enlarged nucleoli in the haustorium fragment. Lines indicate wall ingrowths (WI). **Fig. 19.** DNA and RNA staining with Azure B bromide; fragment of haustorium cell with strongly stained nucleoli (line). **Fig. 20.** Lipid (L) staining with Sudan Black B; part of endosperm chalazal haustorium (CH) and endosperm proper (EP).

Our study tracked a number of changes in the structure of the endosperm chalazal haustorium of *Rhinanthus serotinus* through its development stages. These changes are associated with increased activity of the haustorium cell in metabolite uptake, probably related to meeting the needs of the growing endosperm proper. Observations of the structure of the haustorium cell, especially that of the chalazal wall, support that suggestion. The chalazal end of the haustorium cell of *R. serotinus* produces a PAS-positive wall during early development, which becomes most extensive as the haustorium cell reaches early senescence. The wall ingrowths enlarge and branch further during differentiation of the haustorium cell. The presence of characteristic wall ingrowths is an attribute of so-called transfer cells (Gunning and Pate, 1969, 1974; Pate and Guning, 1972). In *R. serotinus* such a transfer wall structure occurs in the chalazal haustorium but also in cells of the endosperm proper. Transfer cells are specialized for short-distance active transport of nutrients, typically involving an increase in the contact area between the plasmalemma and the external environment by means of the formation of wall ingrowths. Ultimately this provides easier transport of metabolites. Transfer ingrowths have been found in the endosperm haustorium cells of several species: *Lobelia dunnii* (Torosian, 1971), *Vaccinium macrocarpum* (Brisson and Peterson, 1975), *Glycine max* (Dute and Peterson, 1992), *Rhinanthus minor* (Nagl, 1992), *Rhinanthus serotinus* (Bohdanowicz et al., 1993) and *Utricularia intermedia* (Płachno and Świątek, 2011). The wall ingrowths of those show structure typical for transfer cells. Active transport of metabolites by the plasmalemma requires an energy supply, which is likely to be generated by the mitochondria present in large quantities near the transfer walls of *Rhinanthus* and several other plant species (Gunning and Pate, 1969, 1974; Wooding, 1969; Pate and Gunning, 1972; Nagl, 1992; Bohdanowicz et al., 1993).

Cytochemical reactions revealed the presence and distribution of polysaccharides, lipid droplets, proteins and nucleic acids in the endosperm chalazal haustorium cell of *R. serotinus* at different developmental stages. The presence of a pool of proteins and fairly numerous lipid droplets in the haustorium cytoplasm presumably is associated with the extensive endoplasmic reticulum (ER) membrane system present in the haustorium cell (Świerczyńska, 2004). Aniline Blue Black staining of protein revealed an increase of protein at maturity and during early degeneration, when ER profiles expand. Protein substances synthesized in the haustorium can be used to expand ER membranes or can accumulate inside the ER profiles. The ER is equipped with the whole system of enzymes involved in synthesis and secretion of lipids, which partici-

pate in building ER membranes and also are stored in oil bodies (Woźny, 2001). During development of the haustorium, DNA and RNA content increases markedly. Nucleic acid content reaches maximum during early degeneration of the haustorium cell. Undoubtedly this is connected with endopolyploidization of the nuclei (Świerczyńska et al., 2005). Multiplication of genome number in the nucleus of an endopolyploid cell usually leads to a proportionate increase in its physiological activity (D'Amato, 1989). Many observations of endosperm haustorium structure confirm its role in synthesis and/or specialization in active transport (Bhatnagar and Kallarackal, 1980; Torosian, 1971; Pacini et al., 1975; Nagl, 1992; Dute and Peterson, 1992; Bohdanowicz et al., 1993; Świerczyńska, 2004).

Our analyses of the development and cytochemistry of the endosperm chalazal haustorium of *Rhinanthus serotinus* confirm that the chalazal haustorium is an active transfer cell absorbing nutrients from the mother plant tissue, metabolizing and translocating them to the endosperm.

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