ACTA BIOLOGICA CRACOVIENSIA Series Botanica 48/2: 59–65, 2006

SUCCINATE DEHYDROGENASE AND ACID PHOSPHATASE ACTIVITY IN *PHASEOLUS LUNATUS* TESTA

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Received April 20, 2006; revision accepted August 10, 2006

Succinate dehydrogenase (SDH) and acid phosphatase (AcP) activity in *Phaseolus lunatus* seed testa are demonstrated in enzyme tests, and uptake and transport of vital and indicator dyes such as methylene blue, Congo red and tetrazolium in the seed testa are examined by light and transmission electron microscopy. SDH activity was observed in the vascular bundles (endotesta) and in some cells in endo- and mesotesta. AcP activity was located near cell walls in both meso- and endotesta. In the vascular bundles there was very little AcP activity. Vital and indicator dyes were conducted from the exotesta (hilum) to endotesta. Vesicle mobilization was observed in the mesotesta. Strong enzyme activity in the meso- and endotesta and vesicle mobilization in the mesotesta suggest the potential active role of testa strata in imbibition and the initial nutritional stage of germination.

Key words: *Phaseolus lunatus*, Congo red, succinate dehydrogenase, acid phosphatase, methylene blue, testa, tetrazolium.

INTRODUCTION

The seed testa is formed from the integuments of the ovule, the progenitor of the seed (Campbell et al., 1999). As the cover of a seed, the testa employs different strategies in higher plant life and in its adaptation to different environments (Windsor et al., 2000). The seed testa is very diverse in form, structure and function (Fahn, 1974; Nevell and Hymowitz, 1978; Lersten, 1979; Werker, 1997; Karcz et al., 2005; Aniszewski et al., 2006). Like many other plant species, *Phaseolus lunatus* exhibits variation in testa patterns in both shape and color (Lioi and Galasso, 2002). The testa of this species can be full-colored, speckled, mottled or locally pigmented, and can vary in color from white, green, grey, yellow, brown, red and purple to black (Bailey, 1963; Baudoin, 1988). Vargas et al. (2003) reported that the seeds of 38 wild populations of *Phaseolus lunatus* showed significant testa polymorphism between populations. An analysis of 29 local ecotypes of this species showed that the wild lima bean preserves most of its isozyme variation (Bi et al., 2003). In our previous study a precise regionalization system for the *Phaseolus lunatus* testa was presented. We postulated the use of the central point of the testa as an important basic topographic marker for comparability of testa anatomy data (Aniszewski et al., 2006). We also found that the white fully matured testa has three parts at this topographic marker: the exo-, meso- and endotesta. The exotesta is divided into cuticle, palisade and crushed cell layers. The mesotesta is composed of stapes cells and a layer of crushed cells. The endotesta is composed of support tissue, three crushed cell layers, phloem, xylem and the testa base (Aniszewski et al., 2006). Recently, SDH and AcP were found as active enzymes in both the endo- and mesotesta of *Phaseolus lunatus*, which strongly suggests that there are some living cells (Aniszewski, 2005). In light of this, the role of the fully matured testa in the seed life strategy seems very important. It is known that the water permeability strategy of seeds is connected with the testa and its diversity (Rudrapal et al., 1992; Trillo and Carro, 1993). Smaller seeds, along with hardseeded and rough-seeded lines, exhibited thicker testa than softseeded and smoothseeded lines (Miao et al., 2001). Although many studies have addressed the protective function of the testa in legumes in relation to water uptake and imbibition (Duke and Kakefuda, 1981; Tully et al., 1981; Duke et al., 1983; Powell

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PL ISSN 0001-5296
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and Matthews, 1978; Powell et al., 1986: Powell, 1990; Legesse and Powell, 1992; Hahalis et al., 1996), there is insufficient knowledge of the participation of the cover in other seed strategies. The participation of different testa strata in these strategies is a particularly challenging subject for study. The internal means of water transportation and enzyme activity in the different parts of the testa are very new study subjects (Aniszewski, 2005; Aniszewski et al., 2006). Here I study seed testa, using vital and indicator dyes such as methylene blue, Congo red and tetrazolium to examine the strata of white *Phaseolus lunatus* testa in relation to enzymatic activity and vital and indicator dye uptake and their transport. I address basic questions about the fully matured seed testa: how are the vital dyes driven, and what activities of succinic dehydrogenase and acidic phosphatase take place in different testa strata?

MATERIALS AND METHODS

PLANT MATERIAL AND TESTA TOPOGRAPHY

White seeds of *Phaseolus lunatus* genotype AC 2441 (Fig. 1) were obtained from the Institute of Applied Botany of the University of Hamburg (Germany) and stored at room temperature at the Laboratory of Applied Botany of the Department of Biology of the University of Joensuu. The seeds were checked for normality. No pathological or species-deviant seeds were observed in the samples. The testae were also checked for internal normality using a scalpel, tweezers and glass base and stereo microscope.

The testa investigation was focused on the central point (CP) on the vertical side. The CP location was determined as described in a previous study (Aniszewski et al., 2006). Pieces of the testa were cut from this CP under a stereo microscope using a scalpel, tweezers and glass base.

TESTA PIECES AND MICROSCOPY STUDIES

Pieces of the testa were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer rinsed after fixation, postfixed for 3 h in 1% OsO_4 in 0.1 M sodium cacodylate buffer, and rinsed in sodium cacodylate buffer. After dehydration, pieces of the seed testa were embedded in Epon prepared according to the standard method. For light microscopy, semithin (2 μm) sections were cut with an LKB 2188 ultramicrotome (LKB Ultrascan XXL, Bromma, Sweden) and stained with 1% toluidine blue. For TEM, ultrathin sections were cut with a diamond knife and stained with 2% uranyl acetate and 1% lead citrate (pH 12.0).

Testa samples were examined with light (Dialux 20 EB Leitz) or electron microscopes (TEM EM 900 with Mega Vision 2003). The light microscope was connected to a camera (Olympus dp 10) and a computer system (Olympus C21 W95E).

HISTOCHEMICAL TECHNIQUES

Methylene blue (MB, Merck, Darmstadt, art. 1283) and Congo red (CR, Merck, Darmstadt, art. 1340) were used to check dye flow and to stain tissues and substances in the testa strata. These dyes are commonly used in histo- and clinical chemistry. MB is a cationic dye, which attracts acidic groups such as pectins (Nari et al., 1991) and lignins (Fineran, 1997) and indicates proline synthesis and increased oxidative pentose phosphate pathway (OPPP) activity (Hare et al., 2003). CR is well known for its attraction to some cell wall polysaccharides such as cellulose (Verbelen and Stickens, 1995) and some proteins (Gueneron et al., 2000). MB and CR were prepared and used as 0.1% aqueous (MB) and 0.1% NH_2OH solution (CR). Sections of dyed testae were obtained and examined with a Dialux 20 EB Leitz light microscope connected to an Olympus dp 10 camera system.

The seeds were treated with MB and CR in Petri dishes with absorbant paper for 24 h. An additional water imbibition control test was also set up.

The tetrazolium (TZ, 2,3,5-Triphenyltetrazoliumchloride test, Merck, Darmstadt, art. 8380) was used to indicate the presence of living cells in the testa. This vital dye is commonly used in histo- and clinical chemistry. TZ is a chemical which, upon reduction, forms deeply colored pigments, known as monoformazans, and is used for histochemical detection of dehydrogenases in enzyme diagnostic and clinical chemistry (Serva, 2004; Oritani et al., 2004).

Seeds were treated with 0.5% tetrazolium in Petri dishes with absorbant paper for 24 h. After treatment the seeds were photographed using a Wild Makroskop (M420, Heerbrugg, Switzerland) with L100 macroscope light filter (Fiberoptic – Heim AG, Switzerland), two sample light sources (KL 150B, Schott, Mainz, Germany) and the Olympus dp 10 camera system. Sections of dyed testa were examined with a Dialux 20 EB Leitz light microscope connected to an Olympus dp 10 camera system.

ENZYME TEST

Succinic dehydrogenase (SDH) and acid phosphatase (AcP) in testa strata were assayed by histochemical testing. For SDH activity, soft white paper was watered with succinate and nitroblue tetrazolium containing a substrate solution (Pearse, 1972). The seed was stained for 22 h in aluminum foil at room temperature and the testa pieces were put into

4% formaldehyde and embedded after ethanol dehydration in Euparal. For AcP activity, the seeds were incubated in wet paper for 22 h in aluminum foil, then pieces of the testa were fixed in cold 4% formaldehyde for 4 h, dehydrated in ethanol and embedded in ester wax. Next, the ester wax-embedded pieces of testa were cut with a microtome into 10 μm sections. After removal of the ester wax with ethanol, the sections were incubated in a substrate solution (pH 5.2) with Naphtol AS-MX phosphate as the substrate and Fast Blue BBN as the tetrazolium salt (Burstone, 1961).

RESULTS

The general scheme of testa structure is presented in Figure 2. There are exo-, meso- and endotesta. The structure of the exotesta constructed of palisade and crushed cell layers, and of the mesotesta constructed of stape cells, is shown in the inset of Figure 2. The palisade cells are relatively large sclereids; some are sharp-pointed and some not. These sclereids are connected to a very thin layer of crushed cells, an adhering layer between the exo- and mesotesta. The stape cells are parenchymatous cells, and together with crushed cells they form the mesotesta. The endotesta is a combination of support, vascular and testa bottom cells.

The initial uptake of the dyes (CR, MB) occurs across the hilum, and they flow to the vascular bundles. The dyes are good indicators, visible on the surface of the testa. At the beginning of imbibition the bundles are swelled (Figs. 3, 4). Both dyes were conducted from the hilum situated in the exotesta to the endotesta (Figs. 3–5). Light microscope images of a cross section of the endostratum demonstrate that, in the first step of imbibition, during the first 24 h of contact with the dye, MB accessed all the parts of the endotesta, a part of the mesotesta but still not the cotyledon (Fig. 5). Between the cotyledon and testa base is the crushed cell layer (CC) seen in Figure 5.

The vascular bundles in the endotesta are visible as dark coloring in the stratum (Fig. 6).

The TZ test results show enzymatic activity in the testa (Fig. 7). Dehydrogenase activity was found in the testa compartments in the endo- and mesotesta between the vascular bundles (Fig. 7). Enzymatic activity is evident in the meso- and endotesta. Vascular bundles start from the hilum situated in the exotesta. There are no indications (red color) of enzymatic activity. The vascular bundles lead from the hilum (exotesta) across the mesotesta to the endotesta, where there are clear signs of enzymatic activity. At the beginning of water imbibition (first 24 h) the indication is clear (Fig. 7a) but becomes stronger after 24 h (Fig. 7b).

Nutrient mobilization (in vesicles) occurs in the mesotesta during early imbibition (Fig. 9). Identification of these nutrients is a task for future cytochemical studies.

The highest activity of succinic dehydrogenase (SDH) was localized in the vascular bundles (Fig. 8a). There was also some SDH activity in the endo- and mesotesta structures between and near the vascular bundles, indicated by brown points outside the vascular system (Fig. 8a).

Acid phosphatase activity in the testa was localized near walls in the meso- and endostrata, shown blue in the mesotesta (Fig. 8b) and also visible outside the cells in the meso- and endotesta (Fig. 8c). In the vascular bundles (ph, x) there was very little or no AcP activity (Fig. 8c). Although the cotyledon was not the object of this study, in Figure 8c it is also clear that there was no AcP activity.

DISCUSSION

My results clearly show that the testa plays a very active role during the first step of imbibition. The complex structure of the meso- and endotesta and dye tests support this suggestion. The structure of the testa matched the information reported in detail in our previous study (Aniszewski, 2006).

The vascular system is located in the endostrata, which is also a site of dehydrogenase activity. This activity is strong in the endotesta. Parenchymal cells are living cells capable of cell division, and exhibit dehydrogenase activity. Dehydrogenases transform colorless tetrazolium into colored nondiffused formazan (Serva, 2004; Oritani et al., 2004). Dehydrogenase activity indicates metabolic activity on the part of cells. Although not shown in the results, dehydrogenase activity was also recorded in the surface zone of the cotyledon below the endotesta after 24 h incubation. This is evidence of the active role of the testa in the first phase of imbibition, when oxygen consumption and protein synthesis begins (Freeman, 2002). Supply of water to the seed is mediated via the testa. Therefore the testal cells have some regulatory role in imbibition, the step before germination. This idea is supported by acidic phosphatase (AcP) activity, which I identified near cell walls in the meso- and endotesta. Plant AcPases are present in a variety of plant tissues and frequently occure in multiple forms differing in molecular mass. Generally they occur in very small quantities in plant tissues and are unstable in dilute solutions (Čirkovič et al., 2002). The biological role of AcPases in plants is believed to be specifically connected with germination (Čirkovič et al., 2002). In plant cells, AcP is present in small vesicles (spherosomes) and other hydrolase-containing vacuoles (Kleinsmith and Rish, 1995). AcP was also

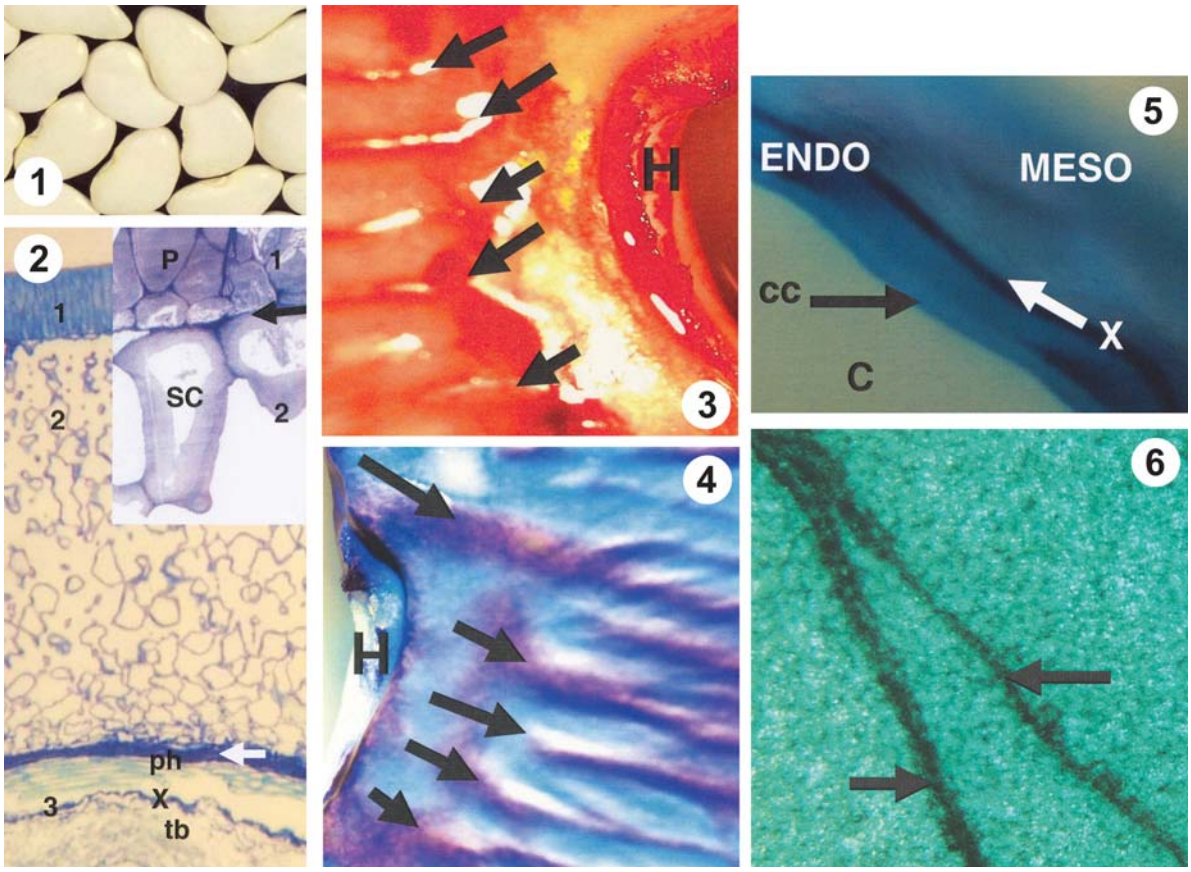

Fig. 1. White seeds of *Phaseolus lunatus* genotype AC 2441. × 1.5. **Fig. 2.** General scheme of white seeds of *Phaseolus lunatus* genotypes AC 2441. Testa constructed of the exotesta (1), mesotesta (2) and endotesta (3). Endotesta contains support tissue (white arrow), phloem (ph), xylem (x) and testa bottom (tb). × 500, Inset fragment of exo- (1) and mesotesta (2). Stape cells (SC) are visible in the mesotesta, and palisade (P) cells in exotesta. Black arrow shows crushed cell layer. × 8800. **Fig. 3**. Distribution of Congo red dye after 24 h incubation. Staining is strongest in hilum (H) and vascular bundle areas in testa (black arrows). × 14. **Fig. 4.** Methylene blue incubation. Staining similar to that shown in Figure 3. Black arrows show vascular bundles conducting methylene blue dye from hilum (H). × 10. **Fig. 5**. Transverse section of testa and cotyledon after 24 h incubation in methylene blue. Staining is strongest in the area of the endo- and mesotesta, especially in transverse sections of xylem bundles (x, white arrow) and in crushed cell (cc) layer (black arrow). c – cotyledon; cc – crushed cells; x – xylem bundle region; endo – endotesta; meso – mesotesta. Cotyledons are still not dyed after 24 h incubation. × 250. **Fig. 6**. Vascular bundles in endotesta. Black arrows show two smaller vascular bundles extending from one. Bundles indicated by arrows are deeper in the endotesta than the bundle from which they lead. × 250.

established in plant cell walls (Ferte et al., 1993). The sperosomes and vacuoles are involved in digestion and recycling of intracellular constituents in a manner analogous to animal cell lysomes. During imbibition, AcP and other hydrolytic enzymes are also important for cytolysis and cell wall degradation.

The generally accepted opinion in the literature is that water cannot enter the seed unless the testa is broken (Freeman, 2002). I have explicity shown that water (and dye) can penetrate the testa protection system without the testa needing to be broken. The water (and dye) pathway, defined here as a route from the hilum to the endotesta, involves a controlled process. The exo-, endo- and mesotesta and hilum seem to be regulators of this process. The testa protection mechanism has been observed in a number of legumes and reported by many authors (Duke and Kakefuda, 1981; Tully et al., 1981; Duke et al., 1983; Powell and Matthews, 1978; Powell et al., 1986; Powell, 1990; Legesse and Powell, 1992; Hahalis et al., 1996). The controlled process of water uptake and distribution by the seed testa strata is finished when the stable arrangement of palisade cells is ruptured by the forces born of the expansion of cotyledon storage cells during the process of embryo growth. The activity of AcP also seems to influence cell wall degradation and by this

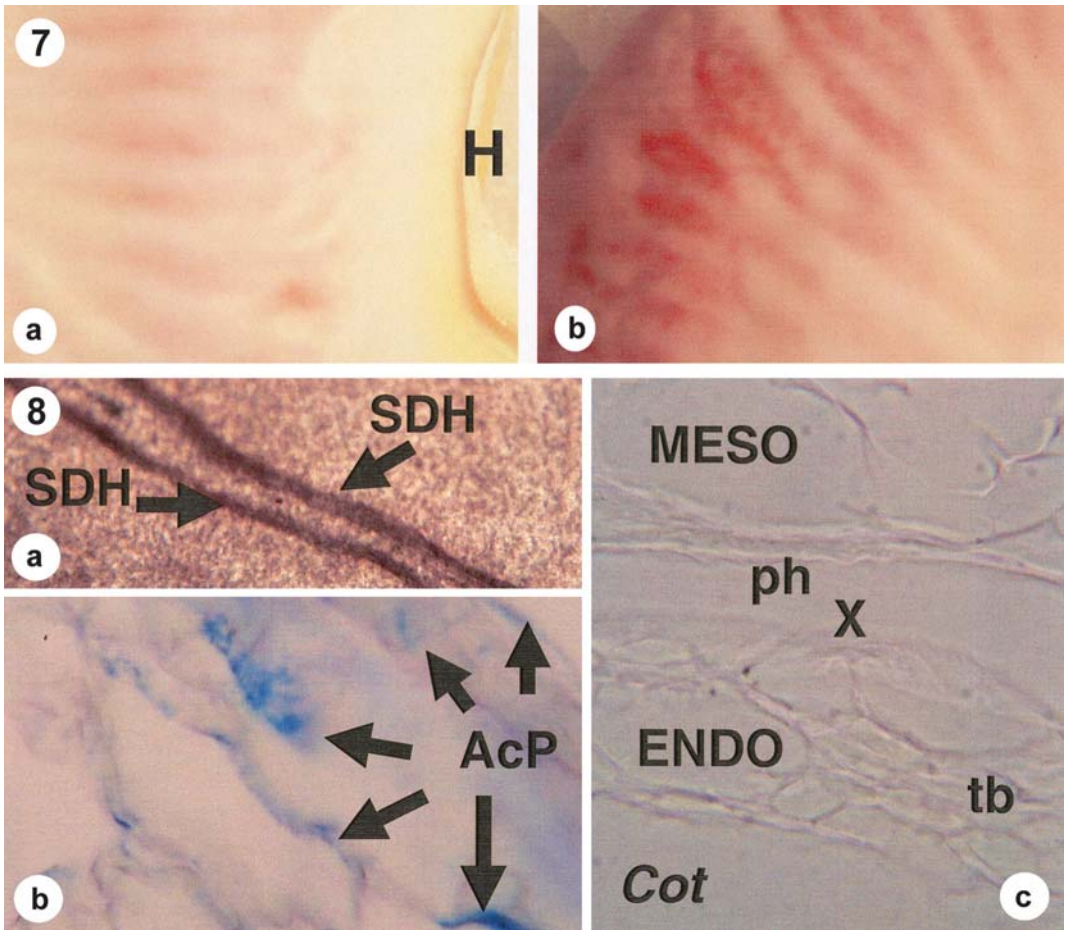

Fig. 7. Dehydrogenase activity. Dehydrogenase activity in the beginning of incubation (**a**). Activity is strongest in the endo- and mesotesta near the xylem and phloem bundles (red color). There is no enzyme activity in the exotesta (hilum, H), (**b**) dehydrogenase activity after 24 h incubation. Strong activity of dehydrogenase indicated by TZ red. × 10. **Fig. 8.** Activity of enzymes in the testa. (**a**) Succinic dehydrogenase (SDH) indications (arrows). × 300, (**b**) Acidic phosphatase (AcP) indications in mesotesta (arrows). × 300, (**c**) Acidic phosphatase indications in meso- and endotesta. Enzyme activity is visible as alteration of the surface of the testa cut. The outlines in the figure are the result of enzyme activity. Even surface indicates no enzyme activity. × 220. MESO – mesotesta; ENDO – endotesta; tb – testa bottom; ph – phloem; x – xylem; Cot – cotyledon.

means makes this process easier. With the destruction of the stable arrangement of the exotesta, the testa can be broken by the seed cotyledon (Aniszewski et al., 2006).

The strategy of seed survival is based on controlled water uptake. Hydration of the testa strata lead to mobilization of nutrients and cellular vesicles in the intercellular spaces (Aniszewski et al., 2006). The source of the nutrients and vesicles is possibly the endo- and mesostrata cells. It is known that the fully matured seed testa is a reserve tissue (Werker, 1997). In the stage of embryo dormancy during the first phase of imbibition, this process of nutrient mobilization seems to be possible only in the testa cells. Clear evidence of this is presented in Figure 5, in which MB dye is visible in the endo- and mesotesta but still not in the cotyledon. The activity of AcP can be connected with this mobilization. The testa can contain a significant amount of nutrients. In some beans, even 4.8% of the seed protein is in the testa (Bressani and Elias, 1980; Werker, 1997).

My results present data useful in discussing the role of the hilum in the testa. The hilum is like a door for water (dye). It is located in the exotesta, with direct connections to the meso- and endotesta and across these compartments to the embryo and cotyledon. From the hilum some water is distributed to the embryo, and by diffusion to the storage cells of both cotyledons. More water (dye) from the hilum is distributed to the xylem bundles in the endostrata and thence to all over the testa area, including the intercellular spaces of the mesotesta. After 24 h of

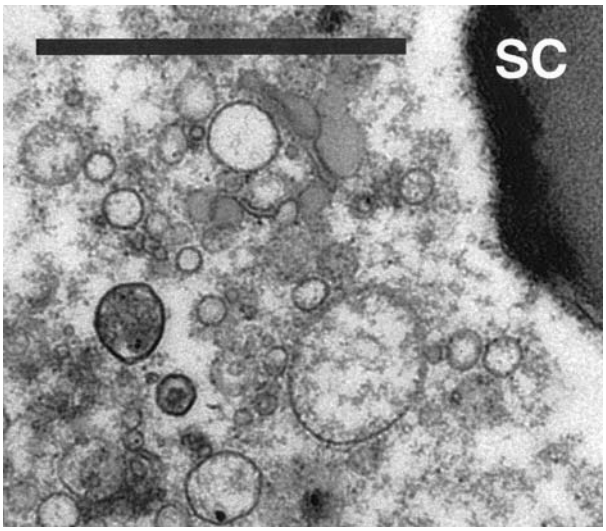

Fig. 9. TEM image showing membranous vesicles and mobilized nutritional material in intracellular spaces in the mesotesta just above the endotesta. SC – stapes cell. Bar = 20 nm.

hydration, cellular vesicles and some nutrients were mobilized in the mesotesta. It can be suggested that the *Phaseolus lunatus* testa conducts water (dye) very speedly. I suggest that the strong enzyme activity in the meso- and endotesta is indicative evidence of a signal which starts the initial nutritional stage of seed germination. Each testa stratum seems to have an active role in these processes.

ACKNOWLEDGEMENTS

The author thank Emeritus Professor Heikki Hyvärinen and Anna-Liisa Karttunen for their contribution to this research. Thanks are also due to Martti Varhimo, Matti Savinainen, Eija Ristola, Mervi Kinnunen, Ilkka Konttinen and Kate Lessey for their technical assistance, and Reinhard Lieberei for donating *Phaseolus lunatus* seeds.

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