

## Different functions of vicilin and legumin are reflected in the histopattern of globulin mobilization during germination of vetch (*Vicia sativa* L.)

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**Abstract.** The temporal and spatial patterns of storage-globulin mobilization were immunohistochemically pursued in the embryonic axis and cotyledons of vetch seed (*Vicia sativa* L.) during germination and early seedling growth. Embryonic axes as well as cotyledons of mature seeds contain protein bodies with stored globulins. Prevascular strands of axes and cotyledons, the radicle and epidermal layers of axis organs were nearly exclusively stained by vicilin antibodies whereas the cotyledonous storage mesophyll gave similar staining for vicilin and legumin. Globulin breakdown started locally where growth and differentiation commenced in the axis. There, vicilin mobilization preceded legumin mobilization. Thus vicilin represents the initial source of amino acids for early growth and differentiation processes in vetch. Legumin presumably only serves as a bulk amino acid source for subsequent seedling growth during postgerminative globulin degradation. During the first 2–3 d after the start of imbibition the axis was depleted of globulins whereas no decrease in immunostainability was detected in the cotyledons except in their vascular strands where immunostainability was almost completely lost at this time. Continuous vascular strands were established at the third day when globulin breakdown was finished in the axis but had just started in the cotyledon mesophyll. Protein mobilization proceeded in a small zone from the epidermis towards the vascular strands in the center of the cotyledons. In this zone the storage cells, which initially appeared densely packed with starch grains and protein bodies, concomitantly transformed into cells with a large central vacuole and only a thin cytoplasmic layer attached to the cell wall. These results agree well with the hypothesis that during the first 2 d after imbibition the axis is autonomous in amino acid provision. After the endogenous reserves of

the axis are depleted and the conductive tissue has differentiated, globulins are mobilized in the cotyledons, suggesting that then the amino acid supply is taken over by the cotyledons. For comparison with other degradation patterns we used garden bean (*Phaseolus vulgaris* L.) and rape (*Brassica napus* L.) as reference plants.

**Key words:** *Brassica* (germination) – Germination – Globulin breakdown – *Phaseolus* (germination) – Seedling growth – *Vicia* (germination)

### Introduction

Globulins are the major seed storage proteins of dicotyledonous plants. During seed maturation the globulins are deposited in protein bodies which belong to the vacuolar compartment. Major globulin deposition takes place in storage mesophyll cells of cotyledons, as in grain legumes, and in the endosperm, as in castor bean. In addition, globulin-containing protein bodies have been found in the embryonic axis of various plants including pea and soybean (Perner 1965; Yoo 1970; Alekseeva and Kobarskaya 1978; Alekseeva et al. 1989).

Numerous biochemical investigations have been performed on globulin mobilization in storage cotyledons of dicotyledonous plants during and after seed germination (for reviews, see Wilson 1986; Shutov and Vaintraub 1987; Müntz 1996), the results of which can be summarized as follows: Depending on the plant species, globulin mobilization only becomes measurable 2–3 d after the start of imbibition (dai) when the radicle has already broken through the seed coat and the period of germination has ceased. Globulins are broken down in the protein bodies. If protein bodies of mature seeds contain proteinases, these cannot degrade the stored globulins. Proteinases which trigger globulin breakdown are synthesized *de novo* on the rough endoplasmic reticulum (rER) and transferred via the secretory pathway into the protein bodies (e.g. mung bean; Baumgartner

Abbreviations: dai = days after imbibition; hai = hours after imbibition

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and Chrispeels 1979). So far, the only known exception seems to be buckwheat, where a stored endopeptidase was shown to start globulin mobilization in the cotyledons (Belozersky et al. 1990).

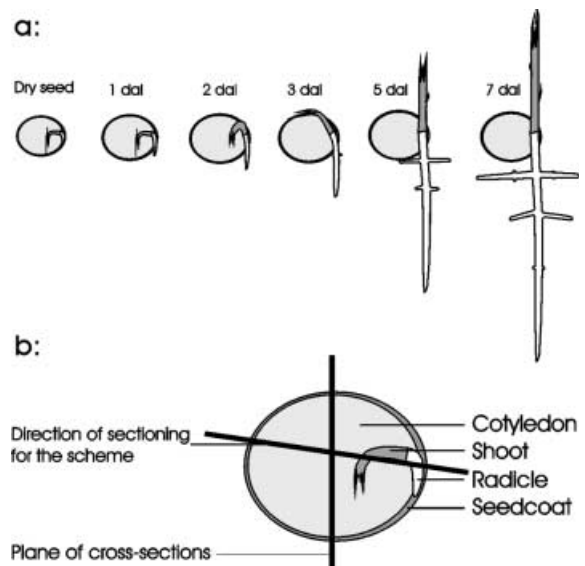
In the embryonic axis, protein biosynthesis and growth start much earlier than globulin mobilization in storage tissue. Since no decrease in total nitrogen of pea cotyledons could be measured during germination it was assumed that during this period amino acids for protein biosynthesis are supplied from endogenous reserves of the axis (Bain and Mercer 1966). Only a few authors reported on biochemical analysis of protein mobilization in embryonic axes. Recently, Vigil and Fang (1995a,b) showed that during the first 24 h after starting imbibition (hai), isolated cotton embryonic axes, like those remaining attached to the cotyledons, grew without any external nutrient supply. Total endogenous proteolytic activity increased and concomitantly proteins were broken down in the axis.

Globulin mobilization has been almost exclusively studied by biochemical analysis, sometimes combined with cell fractionation based on tissue homogenization. Temporal patterns of proteolytic activity and storage-protein breakdown that were determined in this way do not reflect the fact that storage tissue is composed of cells at various stages of proteinase activity and globulin breakdown. In an early approach the histochemical pattern of proteinase activity and protein degradation was pursued in mung bean cotyledons (Harris and Chrispeels 1975), and indicated that protein breakdown started in the cells farthest from the vascular strands where the first marked increase in proteolytic activity was also detected 2–3 dai. Patterns different from that of mung bean have been reported for the cotyledons of several plant species from different families (Smith 1981). Immunoprobes for different storage proteins have only once been used for a histochemical analysis of the temporal and spatial pattern of napin and cruciferin mobilization after rape seed germination (Höglund et al. 1992). A more rapid storage-protein mobilization was detected in the axis than in the cotyledons after germination. Unfortunately, the germination period itself was not investigated.

Here, we report on the temporal and spatial pattern of storage-protein mobilization in embryonic axes and cotyledons of vetch (*Vicia sativa* L.), garden bean (*Phaseolus vulgaris* L.) and rape (*Brassica napus* L.) during germination and seedling growth, as analyzed by immunohistochemical and ultrastructural methods using storage-globulin-specific polyclonal antibodies.

## Materials and methods

**Plant material.** Dry seeds of *Vicia sativa* L. cv. Consentini (Guss.) Arcang. (obtained from the Gatersleben Genebank, IPK Gatersleben, Germany; accession No. VIC133) were imbibed for 1 h in distilled water and grown in the dark at 23 °C on wet tissue paper in covered petri dishes. Embryonic axes and cotyledons were harvested for fixation at the developmental stages shown in Fig. 1a. These stages were chosen in correspondence to parallel biochemical investigations (data not shown). In a similar way, seeds of garden



**Fig. 1.** **a** Morphology of vetch seed during germination and seedling growth in the dark. **b** Planes corresponding to sections used for histological analysis and the projectional schemes of Figs. 12 and 13

bean [*Phaseolus vulgaris* L. var. Nanus (Jusl. Aschers.) “Imuna”, obtained from the Gatersleben Genebank, IPK Gatersleben, Germany, accession No. PHA6017] and rape (*Brassica napus* L. “Karat SV751516”, obtained from the Gatersleben Genebank, outstation Malchow, Germany; accession No. CR999/96) were germinated and seedlings grown to analyze embryonic axes and cotyledons at appropriate stages. The earliest stages analysed were 3 hai for plumules and 6 hai for cotyledons. Due to technical difficulties it was impossible to obtain undamaged sections earlier after starting the imbibition.

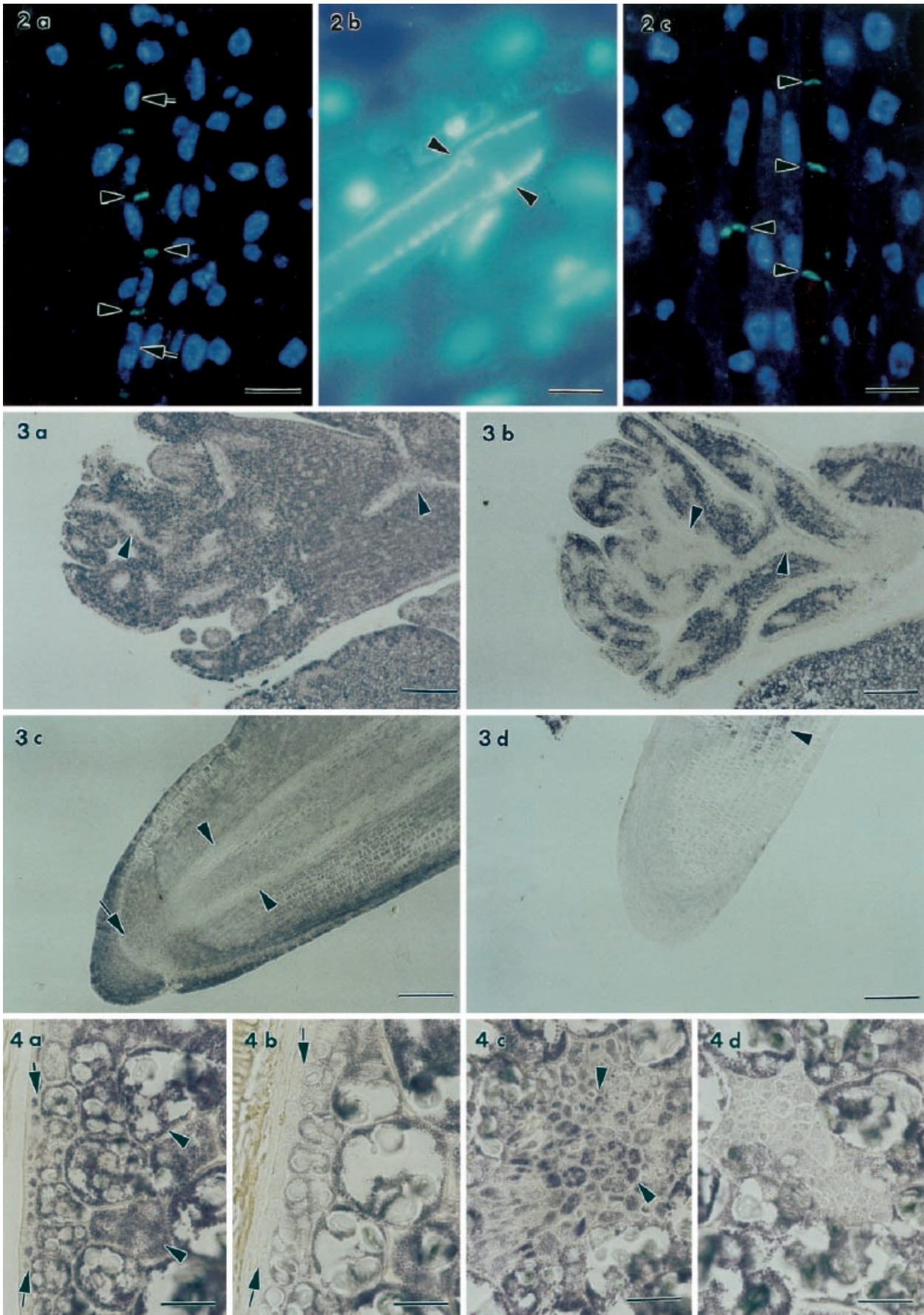
**Fixation for light microscopy.** Hand-sections of whole seedlings (longitudinal sections through the mid-region including the petiole and cross-sections, respectively, as indicated in Fig. 1b) as well as of total preparations of excised embryonic axes were fixed with 3% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M cacodylate-buffer (pH 7.3) for 3 h on ice. After dehydration in a graded

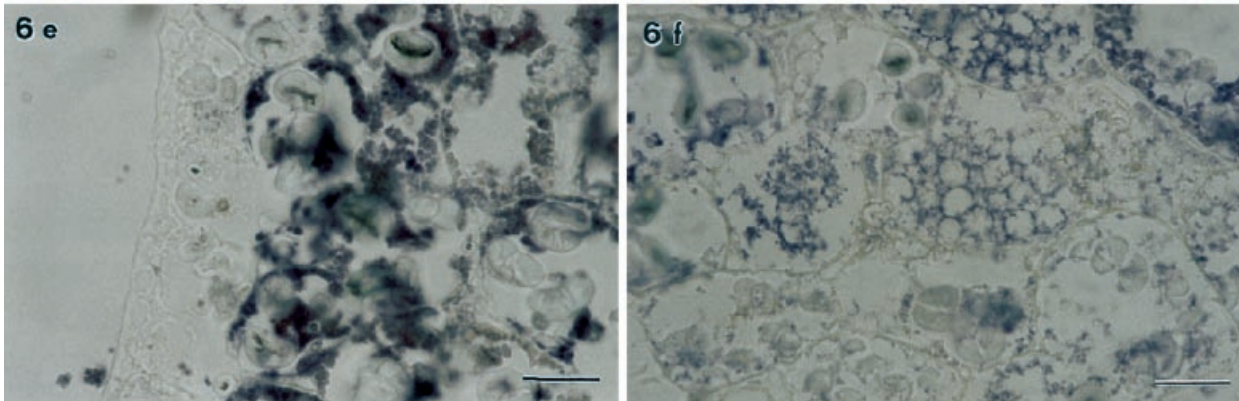
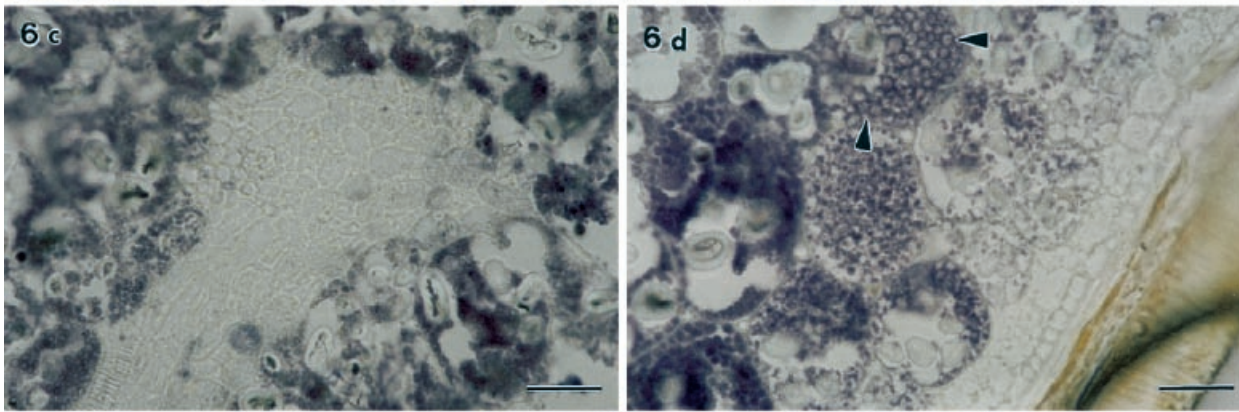
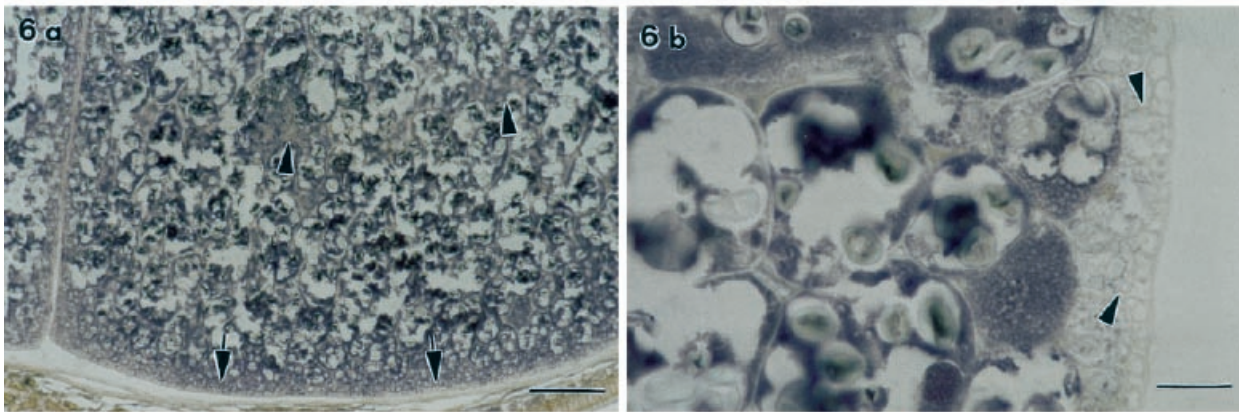
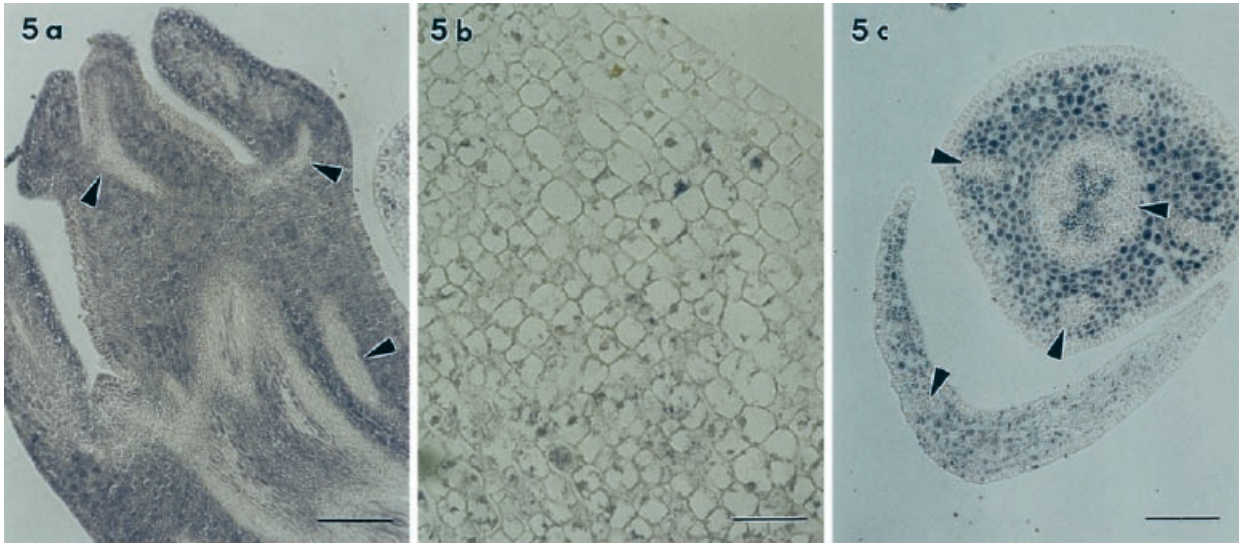
**Figs. 2–4.** Histochemical analysis of the embryonic axis and cotyledons of vetch seed (*Vicia sativa* L.) at the start of germination. *Arrows* and *arrowheads* indicate substantial immunostaining characteristics discussed in the text

**Fig. 2a–c.** Aniline-blue/DAPI double staining of petioles during early germination showed a late appearance of functional conductive connections between axes and cotyledons. **a** Undifferentiated sieve elements at 6 hai; *arrowheads* and *arrows* label sieve plates and nuclei, respectively. **b** 24 hai; the first functional vessels with opened transversal walls (*arrowhead*). **c** Fully differentiated sieve elements without nuclei at 2 dai, sieve plates stained for callose (*arrowheads*). Bars = 20 µm

**Fig. 3a–d.** In embryonic shoots (**a, b**) and radicles (**c, d**) immunostaining specific for vicilin (**a, c**) and legumin (**b, d**) revealed a tissue-specific localization of storage globulins at the start of germination (axes 6 hai). *Arrows* and *arrowheads* indicate the substantial immunostaining characteristics discussed in the text. Bars = 200 µm

**Fig. 4a–d.** Immunostaining specific for vicilin (**a, c**) and legumin (**b, d**) in cells of the abaxial part of vetch cotyledons at the start of germination. **a, b** Abaxial epidermis, and first and subsequent subepidermal cell layers of cotyledons. **c, d** Provascular strands. Bars = 50 µm





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**Figs. 5–6.** Immunohistochemical analysis of vicilin degradation in axis and cotyledons of vetch (*Vicia sativa* L.) seeds during germination and seedling growth. *Arrows* and *arrowheads* indicate substantial immunostaining characteristics discussed in the text.

**Fig. 5a–c.** Vicilin breakdown in the embryonic axis. **a** Vicilin staining of the embryonic shoot at 24 hai. Staining strongly decreased in the provascular strands as compared to 6 hai (Fig. 3a). **b** Vicilin staining of the radicle at 24 hai. **c** Vicilin staining of the embryonic shoot at 2 dai. Bars = 200 µm (**a**, **c**), 100 µm (**b**)

**Fig. 6a–f.** Vicilin degradation, as proceeding from the abaxial surface of the cotyledons at 1 (**a**) 3 (**b**) and 5 (**c**, **d**) dai. **e** Vicilin breakdown at the adaxial side of the cotyledons at 7 dai. **f** At 14 dai vicilin degradation was nearly completed. Bars = 200 µm (**a**), 50 µm (**b–f**)

ethanol series and substitution of ethanol for Rotihistol (Roth, Karlsruhe, Germany) the sections became infiltrated and embedded in Paraplast within 5 d at 61 °C with changing of pure Paraplast (Sherwood Medical, St. Louis, Mo., USA) every 12 h.

**Sectioning and immunostaining.** The Paraplast-embedded specimens were sectioned with a sledge microtome (Microm, Heidelberg, Germany) by means of “C”-type steel knives. The thickness of the sections was about 10 µm. The sections were floated on distilled water at 45 °C for approx. 0.5 h and transferred to poly-L-lysine coated slides. The Paraplast was removed from the sections with Rotihistol and the sections were rehydrated with a graded ethanol series. Subsequently, they were blocked with 10% horse-serum and 5% low-fat milk powder, 0.05% Tween 20 in Tris-Buffered saline (TBS; 15 mM NaCl, 10 mM Tris-HCl, pH 7.4) overnight.

Legumin was detected using polyclonal antibodies raised against recombinant legumin B1 from *Vicia sativa* L. (EMBL Data Library accession No. Z32796). The cDNA-deduced amino acid sequence of legumin B1 from vetch is 88.2% identical to that of legumin B4 from *V. faba* L. (Bäumlein et al. 1986). In immunoblots the sensitivity of the vetch-legumin B1-specific antibody against the different polymorphic legumin subunits was similar to that of our previously used faba bean-legumin antibody which had been raised against legumin prepared from *V. faba* cotyledons (zur Nieden et al. 1984). It detected legumin A as well as legumin B subunits and did not cross-react with the antibody used to detect vicilin. These vicilin-specific polyclonal antibodies had been raised against vicilin purified from cotyledons of *V. faba* L. Strict vicilin-specificity and use of the antibodies were previously described (Saalbach et al. 1995). The polyclonal phaseolin antibody (Senyuk et al. 1998) and the napin and cruciferin antibodies (Ericson et al. 1986; Rödin and Rask 1990) were kindly provided by V. S. Senyuk (Laboratory of Protein Chemistry, University of Moldova, Kishinev, Republic of Moldova) and Lars Rask (Department of Cell Research, Uppsala Biomedical Center, Swedish University of Agricultural Sciences, Uppsala, Sweden), respectively. Sites of antibody binding were visualized using a biotinylated secondary antibody and streptavidin-alkaline-phosphatase conjugates with 4-nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt (BCIP) as substrate (all chemicals Boehringer, Mannheim, Germany). The immunostained sections were dehydrated and embedded under coverslips using Entellan (Merck, Darmstadt, Germany).

**Investigation of vascular differentiation.** In order to differentiate developing and functional sieve elements, Paraplast-embedded sections of *Vicia sativa* L. were stained by the aniline-blue/4';6-diamidine-2'-phenylindole dihydrochloride (DAPI) double-staining method according to Schulz (1987) as modified by Pancke (1995).

**Photomicroscopy.** Light-microscopy was performed using a Zeiss (Oberkochen, Germany) Axiovert microscope. For fluorescence detection an appropriate filter combination was chosen (365, 395,

420 nm). Photographs were taken on Kodak EPY 64T and for fluorescence microscopy on Fuji Sensia 400 ASA color slide films.

**Electron microscopy.** Small pieces from the abaxial side of 5-dai *Vicia sativa* cotyledons were immersed in a mixture of freshly prepared 3.7% paraformaldehyde, 0.5% glutaraldehyde, in 50 mM cacodylate buffer (pH 7.4), containing 5 mM CaCl<sub>2</sub> for 2.5 h at room temperature. After renewing the fixative the tissue was incubated for an additional 18 h at 4 °C. Then, the sections were successively washed with buffer and distilled water (3 × 10 min each) at 4 °C. Dehydration was performed using a modified progressive lowering of the temperature scheme according to Villinger (1991) as follows: 12.5% ethanol for 10 min on ice, 25% ethanol for 10 min at –10 °C, 0.5% OsO<sub>4</sub> in 25% ethanol for 30 min at –10 °C, 38% ethanol for 15 min at –10 °C, 60% ethanol for 20 min at –20 °C, 75% ethanol for 40 min at –35 °C, 90% ethanol for 40 min at –35 °C, 100% ethanol for 40 min at –35 °C, 100% ethanol for 20 min while slowly raising the temperature to –10 °C, 100% ethanol for 10 min at –10 °C, 100% ethanol for 10 min on ice. After infiltration with LR White resin (London Resin Co.; hard grade) the specimens were polymerized for 24 h at 60 °C. Ultrathin sections were picked up on Formvar-coated nickel grids, blocked for 10 min on 50 mM glycine and for 30 min on a mixture of 5% BSA, 5% normal goat serum and 0.1% cold-water fish gelatine TBS. The grids were incubated for 1 h in a 1:200 dilution of polyclonal anti-legumin in washing buffer (TBS with 0.1% BSA), washed with washing buffer 6 × 5 min, and incubated in a 1:30 dilution of goat anti-rabbit antibodies with 15-nm gold (GAR15; Biocell) in washing buffer. After repeated washing steps with washing buffer, TBS and water the grids were stained with uranyl acetate and lead citrate.

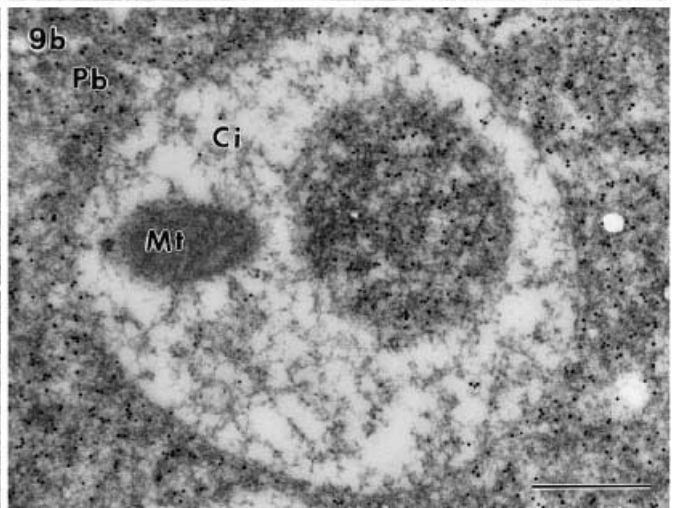
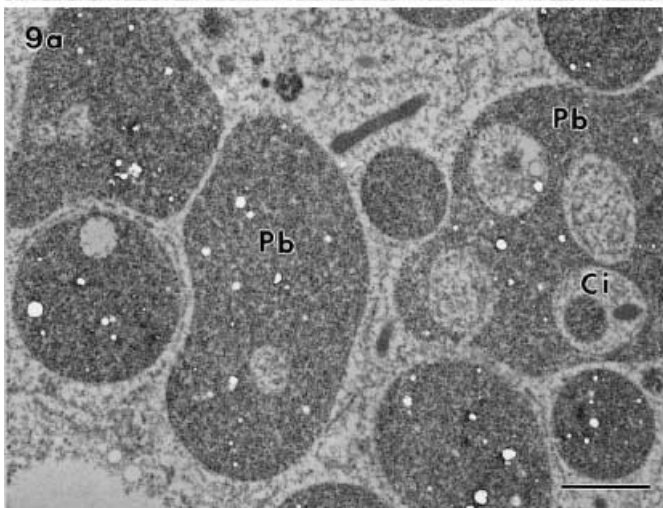
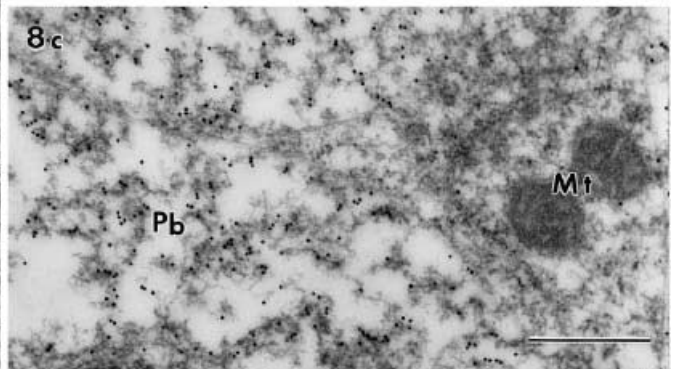
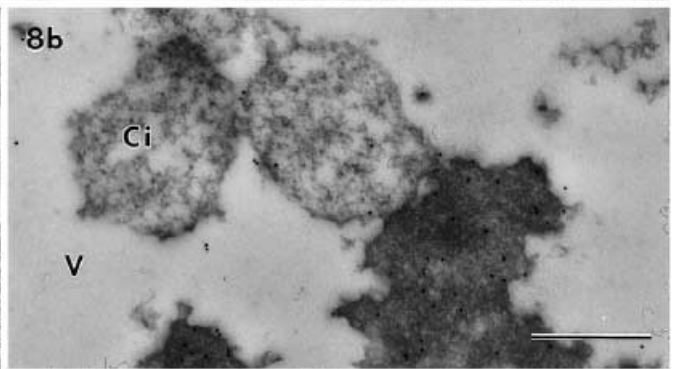
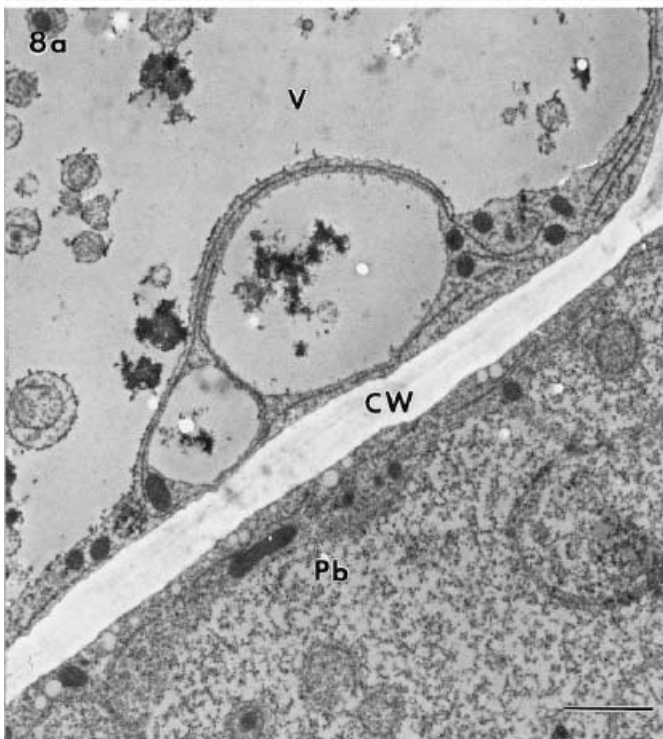
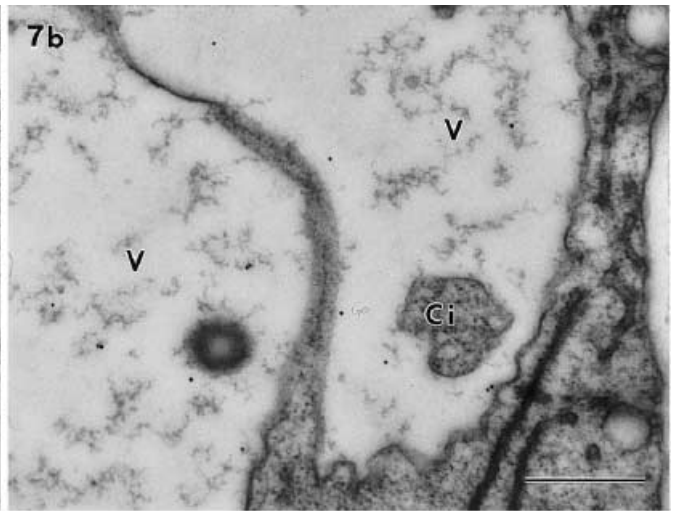
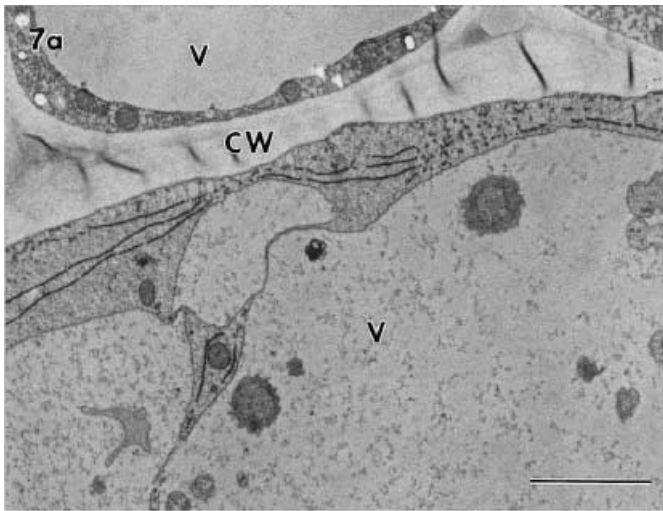
## Results

### *Storage-protein degradation in seeds of Vicia sativa* L. during germination and seedling growth

**Germination and seedling growth.** As indicated by the protrusion of the radicle, vetch seeds germinated 24 hai under the applied conditions. The shoot emerged within the following 2 d after germination. Typical etiolated seedlings developed during the subsequent days (Fig. 1a).

**Vascular tissue differentiation.** Only prevascular strands were observed in the axis, in the petioles and cotyledons of germinated seeds after 3 h. Seeds of this germination stage were still taken to reflect the situation in dry seeds since for technical reasons no undamaged sections could be obtained from dry embryos. The section level is indicated in Fig. 1b. It was directed in such a way that the embryonic shoot with axis and leaf primordia, the radicle, petioles and cotyledons were hit at characteristic levels. The disappearance of cell nuclei from the sieve tubes and callose staining according to Schulz (1987) were used as indicators of phloem differentiation, which is exemplified for petioles connecting axis and cotyledons of germinating vetch seeds in Fig. 2a–c.

At 6 hai, all sieve elements found in the petioles, axis and cotyledons were undifferentiated and still not functioning, as indicated by the presence of nuclei (Fig. 2a). At least in the petioles, xylem differentiation preceded phloem differentiation (Fig. 2b, c). Functional ring and spiral vessels became visible 24 hai. In Fig. 2b, the section through a vessel shows an opened transverse



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**Figs. 7–9.** Ultrastructural changes that cotyledon cells of vetch (*Vicia sativa*) seedlings undergo in the zone of storage-globulin breakdown. Sections from the abaxial side of 5-dai cotyledons were prepared and immunogold labeled as described in *Materials and methods*

**Fig. 7.** Ultrastructure (a) and legumin-specific immunogold labeling (b). a Epidermal cell (upper cell) and cell of the first subepidermal layer (lower cell). b Legumin labeling in a cell of the first subepidermal layer

**Fig. 8.** Ultrastructure (a) and legumin-specific immunogold labeling (b, c) of cells from the second (upper cell in a b), third (lower cell in a c) subepidermal cell layers

**Fig. 9** Ultrastructure (a) and legumin-specific immunogold labeling (b) of cells from the fifth subepidermal cell layer. Whereas at 5 dai storage globulin breakdown and vacuolation had already proceeded far in the abaxial epidermis as well as in the first and second subepidermal cell layer only partial globulin erosion was observed in cells of the third subepidermal layer; moreover, whereas intact protein bodies still filled cells of the fifth layer. The storage-protein character of electron dense material of vacuoles and protein bodies was proven by immunolabeling with legumin-specific polyclonal antibodies. For details see text. *Ci*, cytoplasmic inclusion; *CW* cell wall; *Mt*, mitochondrion; *PB*, protein body; *V* vacuole. Bars = 2  $\mu\text{m}$  (Figs. 7a, 8a, 9a), 0.5  $\mu\text{m}$  (Figs. 7b, 8b, 8c, 9b)

wall revealing functionality of the vessel. By 48 hai the first functional sieve elements were detected (Fig. 2c). The nuclei had vanished and the sieve elements formed functional sieve tubes. A similar differentiation of the conductive tissue was observed in the embryonic axis and in the cotyledons (data not shown).

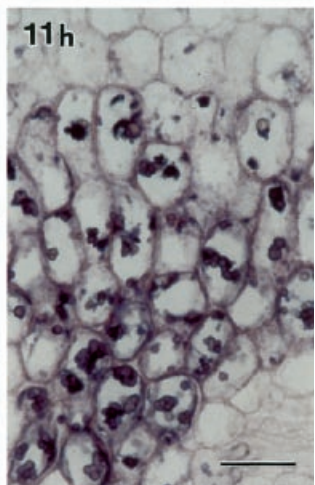
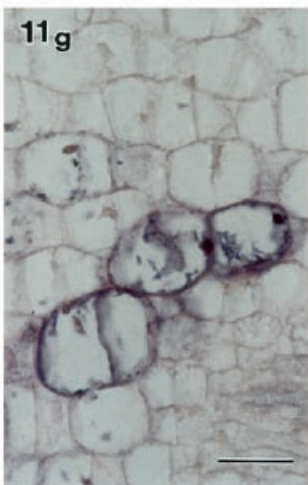
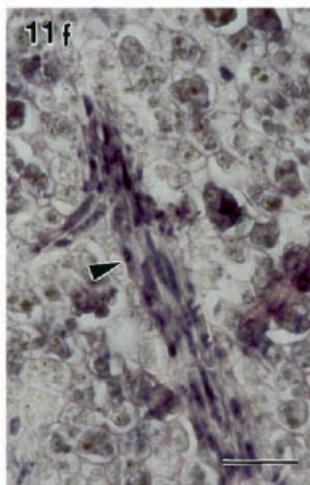
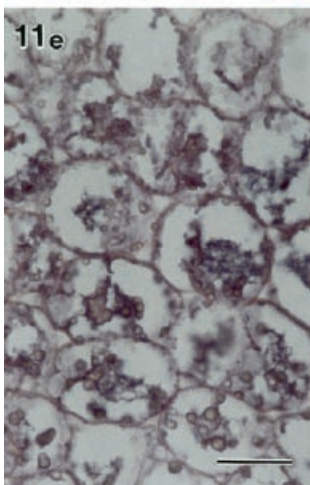
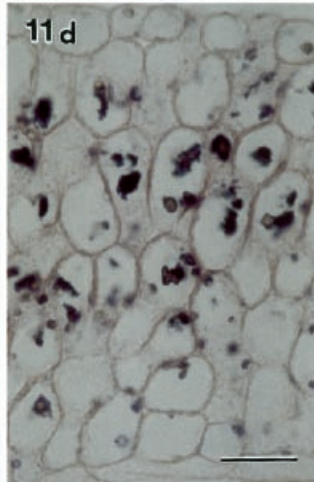
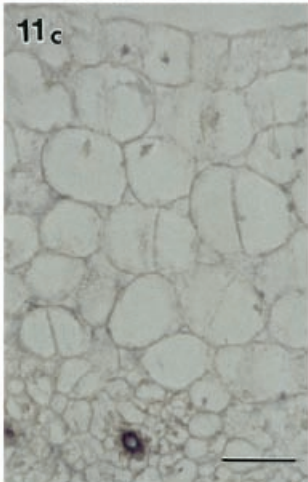
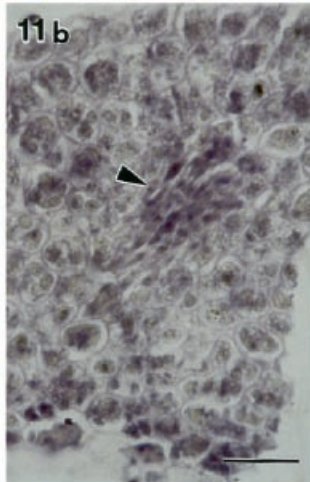
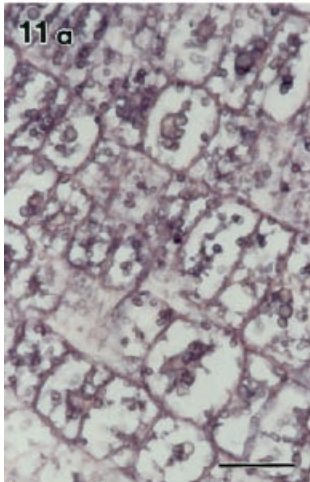
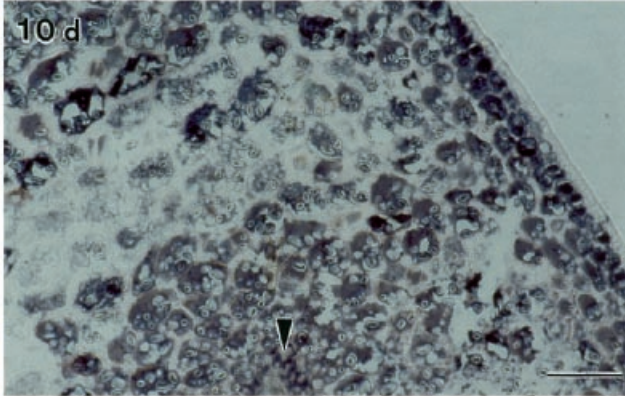
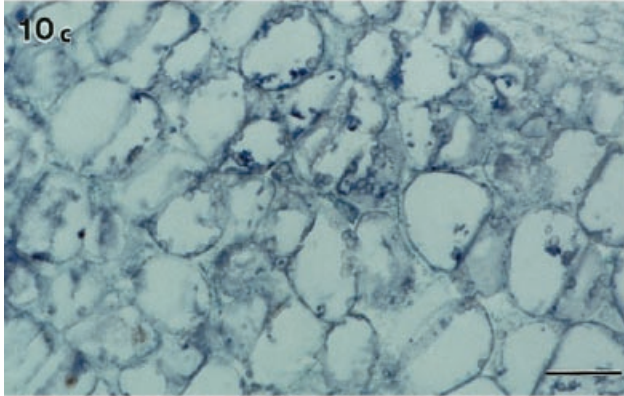
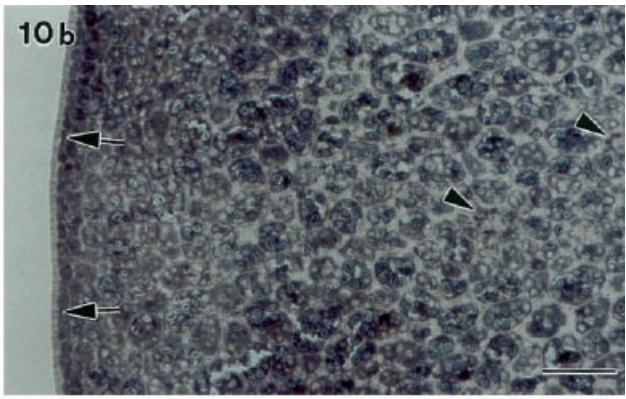
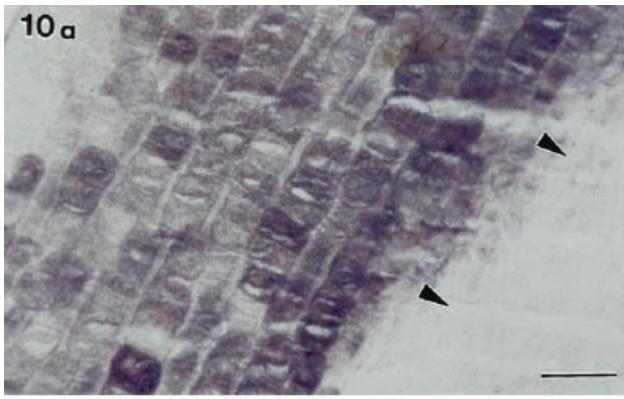
*Tissue-specific localization of storage globulins in embryonic organs of dry seeds.* Axis organs were homogeneously marked by the vicilin antibodies while legumin antibodies revealed a highly differentiated histological pattern (Fig. 3a–d). Vicilin was detected in all axis tissues including epidermal cell layers, prevascular strands and early calyptra, even if these gave only weak immuno signals like in the prevascular strands, e.g. the prevascular strands of the young radicular central cylinder, and cells close to the radicular meristem (arrows and arrowheads, respectively, in Fig. 3a, c). Legumin was present within the mesophyll but seemed to be nearly absent from epidermal cells in the shoot (arrows in Fig. 3b), and was completely lacking in the prevascular strands and their surrounding tissues (arrowheads in Fig. 3b), especially in the radicle (Fig. 3d). Only at the neck of the radicle were a few cells with legumin staining observed (Fig. 3d, arrowheads).

Cotyledon mesophyll cells were similarly stained by vicilin and legumin antibodies (Fig. 4a–d). Vicilin was found to be present in almost all cells of the cotyledon epidermis (arrows in Fig. 4a) where it was located in granular structures corresponding to protein bodies (arrowheads in Fig. 4a). Legumin staining was restricted to some sites in the epidermis but was largely absent (arrows in Fig. 4b). Legumin staining was weaker in the subepidermal cell layer than in the main storage parenchyma deeper in the cotyledons. Vicilin was also present in protein bodies of the prevascular strands of cotyledons (arrowheads in Fig. 4c) where hardly any legumin signal could be detected (Fig. 4d).

*Globulin mobilization in the embryonic axis.* Due to the omnipresence of vicilin the temporal and spatial pattern of its degradation is documented in detail here. The breakdown of legumin followed a similar pattern as indicated in the schematic survey (see Fig. 13).

In tissues of the axis, globulin staining could only be detected until 2–3 dai, indicating that the globulins had been almost completely degraded (Fig. 5a–c). Globulin degradation was always initiated in the radicle, in epidermal layers, and in prevascular strands where predominantly vicilin had been stored. While at 24 hai these tissues (prevascular strands and their surrounding tissue, see arrowheads in Fig. 5a, for radicle, see Fig. 5b) were already devoid of storage globulins, the leaf primordia still contained high amounts of vicilin (Fig. 5a). Even at 2 dai, vicilin was found in leaf primordia, although the staining intensity had decreased (Fig. 5c), but their vascular strands were free of vicilin immunostaining (arrowheads in Fig. 5c). Subsequently, as the axis grew and developed, globulins also became undetectable in leaf primordia.

*Globulin mobilization in the cotyledons.* At 1 dai the complete cotyledon was stained by vicilin antibodies, including the prevascular strands and epidermal cells (arrowheads and arrows, respectively, in Fig. 6a). Vicilin and legumin mobilization was initiated in the abaxial epidermis and subepidermal cell layer at 3–5 dai. The arrowheads in Fig. 6b indicate the decreased vicilin staining in epidermal cells at 3 dai. In cotyledons, as in axes, globulins were first degraded in the prevascular strands during their differentiation. This degradation was completed 4–5 dai at the latest (Fig. 6c). From the abaxial epidermis, vicilin degradation proceeded towards the conductive tissue in the center of the organ. By 5 dai the front of vicilin breakdown had already reached the fifth subepidermal cell layer where typical dilations of protein bodies were visible (arrowheads in Fig. 6d) and vicilin lumps were attached to the inner protein-body membranes. In the adaxial cotyledon epidermis, globulin degradation started approximately 2 d later than on the abaxial side. It similarly proceeded towards the central vascular strands, although more slowly than from the abaxial cotyledon surface. Vicilin breakdown reached the third subepidermal cell layer from the adaxial side at 7 dai (Fig. 6e). At 14 dai vicilin degradation was nearly completed and only remnants of vicilin were stained in dilated protein-body-like-structures of the cotyledon cells (Fig. 6f). This temporal and spatial pattern of globulin mobilization was similarly observed regardless of whether longitudinal or transversal sections were analyzed. Thus protein mobilization moved in an approximately shell-shaped zone from the surface towards the inner vascular bundle of the cotyledons. Behind the progressing protein-degradation zone, cells remained depleted of storage globulins, whereas cells in front of this moving zone were still densely packed with storage organelles and exhibited an immunoreactivity similar to that at the beginning of germination. The spatial and temporal pattern of the degradation of both storage globulins as derived from

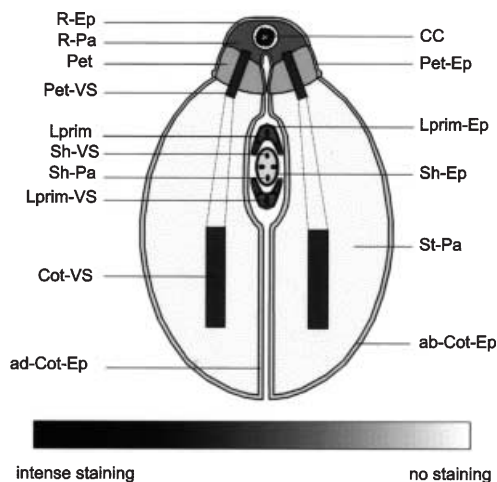




**Figs. 10–11.** Histochemical patterns of storage-protein degradation in garden bean (*Phaseolus vulgaris* L.) and rape (*Brassica napus* L.) during germination and seedling growth

**Fig. 10a–d.** *Phaseolus vulgaris* L. after phaseolin staining. **a** Phaseolin staining of a garden bean radicle at 6 hai shows high amounts of phaseolin only within the cortex tissue. **b** The cotyledon is homogeneously filled with protein bodies containing phaseolin. **c** The hypocotyl is completely emptied at 5 dai. **d** Patches of globulin degradation become visible in the cotyledons at 7 dai equidistant from vascular strands. Bars = 50  $\mu$ m (**a, c**), 200  $\mu$ m (**b, d**)

**Fig. 11a–h.** *Brassica napus* L. Staining of napin (**a–d**) and cruciferin (**e–h**) reveals nearly identical histopatterns. From 6 hai (**a, e**) to 24 hai (**c, g**) the radicle tissues become completely depleted. **g** Only remnants of cruciferin remain visible in a more or less diffuse manner. **b, f** Napin and cruciferin both stain the complete cotyledons at 6 hai. **d, h** At 5 dai, both storage globulins are highly degraded, *arrowheads*, prevascular strands. Bar = 50  $\mu$ m



**Fig. 12.** Histological scheme of a vetch seed resulting from a projection of the longitudinal section plane described in Fig. 1. *ab*, abaxial; *ad*, adaxial; *CC*, central cylinder; *Cot*, cotyledon; *Ep*, epidermis; *Lprim*, leaf primordia; *Pa*, parenchyma, *Pet*, petioles, *R*, radicle; *Sh*, shoot; *St-Pa*, storage parenchyma; *VS*, vascular strand. The *bar's* grey scale gives an approximate measure of the relation between the immunohistochemical staining intensity, the distribution within the sections and the shadowing in the schemes of Fig. 13. *White*, no staining; *black*, intense staining and protein bodies filled with globulin

the immunohistochemical analysis is schematically summarized in Fig. 13 (for an explanation, see also Fig. 12).

**Ultrastructural changes of cells during storage-protein mobilization.** In the zone of globulin degradation which moved from the organ's surface towards the vascular strands inside the cotyledons, the cells that initially were densely packed with starch grains and protein bodies underwent transformation into cells devoid of proteins having large central vacuoles and only a thin cytoplasmic layer attached to the cell wall. The cells behind the progressing front of storage-protein mobilization were alive as indicated by vital staining, and by plasmolysis and deplasmolysis, in hypertonic and hypotonic sucrose solutions respectively (data not shown). During the transformation process, profound ultrastructural changes

took place in the cotyledon cells (Figs. 7–9). In the epidermal cells, protein bodies were less densely packed than in storage-parenchyma cells. These organelles became translucent like vacuoles at only 3 dai, but were still embedded in lots of cytoplasm in epidermal cells (data not shown). Two days later (5 dai) the epidermal cells contained large vacuoles which were presumably generated by protein-body fusion (Briarty et al. 1970; Bewley and Black 1994). These vacuoles were devoid of any storage globulin (Fig. 7a,b). Deeper in the storage parenchyma more electron-dense proteinaceous material was present in the protein-storage vacuoles and protein bodies, respectively, than in the surface layers (Figs. 8 and 9). Intact protein bodies without evident globulin erosion were still found in cells of the fifth and deeper subepidermal cell layers at this stage of seedling growth. In the vacuoles, membrane-bounded inclusions were detected, e.g. mitochondria-like structures (Figs. 8c and 9b), which can be interpreted as the result of an autophagy-like process. Degradation of storage proteins was accompanied by a dilation of the protein bodies while their contents appeared less and less dense. This increase in size seemed to result from protein-body fusion. Portions of cytoplasm between the protein bodies could thereby be incorporated into the lumen of the generating vacuoles where they could be seen as cytoplasmic inclusions (Herman 1994).

#### Storage-protein mobilization in garden bean (*Phaseolus vulgaris* L.) and rape (*Brassica napus* L.)

A taxonomically distant legume and a representative of the *Brassicaceae* were included into our analyses to verify whether or not the histochemical patterns of storage-globulin mobilization differ from that found in vetch.

***Phaseolus vulgaris.*** Phaseolin, a 7S globulin homologous to vicilin, strongly predominates in the embryonic organs of *Phaseolus vulgaris*, which revealed a completely different histopattern of globulin degradation than vetch. In the prevascular strands of the embryonic axis, especially within the hypocotyl, no phaseolin was detected (arrowheads in Fig. 10a). In the cotyledons, phaseolin was found to be present in all tissues including the prevascular strands (arrowheads in Fig. 10b). Only a weak phaseolin immunostaining was observed in the epidermis cells (arrows in Fig. 10b). Since *Phaseolus vulgaris* seeds germinate epigeously the shoot only grew negligibly (with exception of the expanding primary leaf) during the period of our investigation whereas the radicle and the hypocotyl were the main growing parts of the axis up to 7 dai. After 2–3 dai the radicle broke through the seed coat, marking the end of the germination period. At 5 dai the hypocotyl was nearly completely devoid of phaseolin (Fig. 10c). In the cotyledons, phaseolin degradation started at 5 dai. Patches of degradation were visible in the cotyledons at 7 dai (Fig. 10d). These areas of globulin degradation were equidistantly located from the differentiating prevascular strands (arrowhead in Fig. 10d), which also became

depleted of their protein-reserves during this time (5–7 dai). The circum-vascular cell layers lost their immunostainability for phaseolin later during seedling growth.

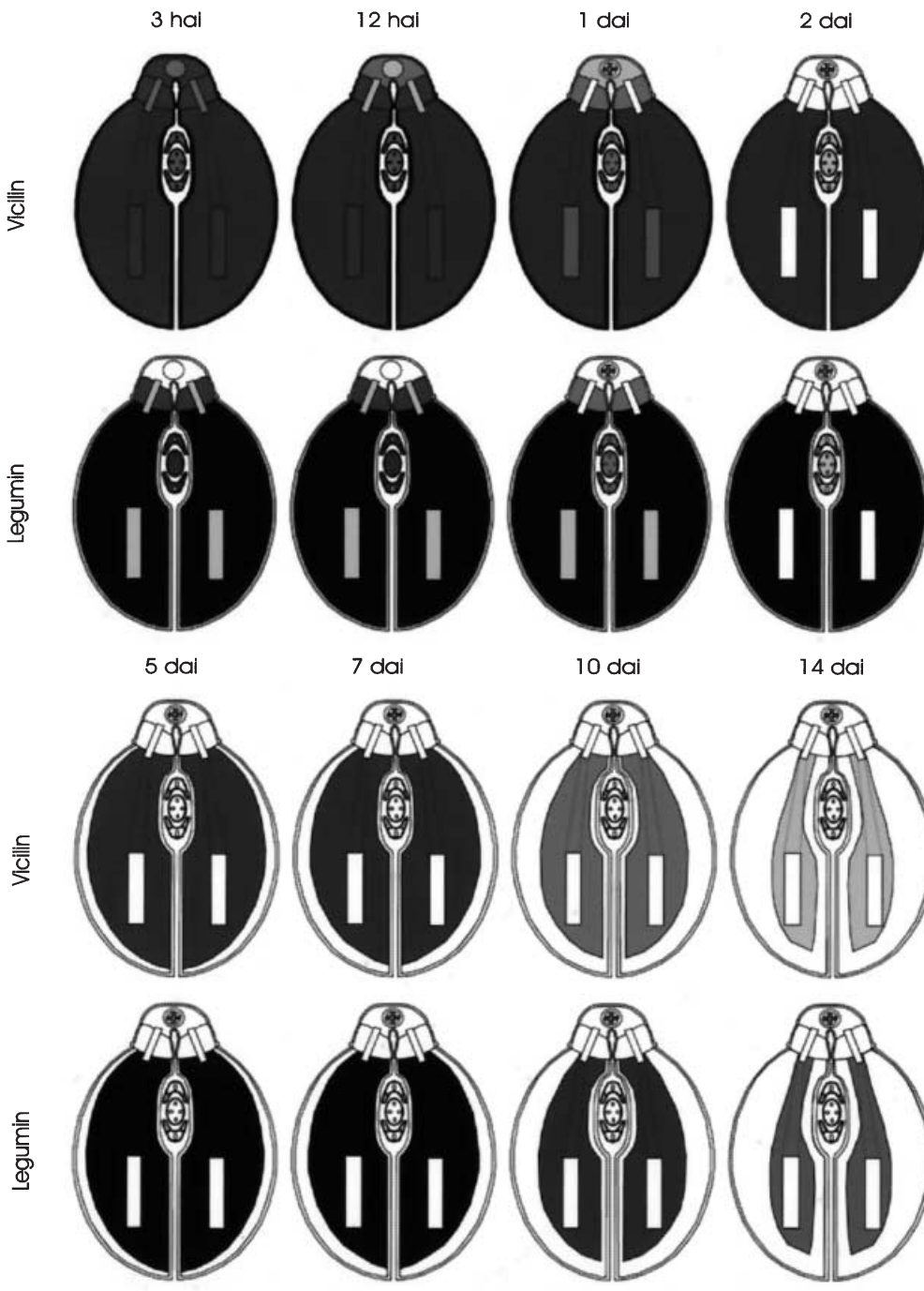
*Brassica napus*. Rape seeds contain 2S and 11S globulin, called napin and cruciferin (homologous to legumin), respectively, as major storage proteins. Both are present in all organs and tissues of the mature embryo. At 6 hai, napin (Fig. 11a,b) as well as cruciferin (Fig. 11e,f) were found in the axis and the cotyledons. The prevascular strands were stained for both storage proteins in all embryonic organs (for cotyledons shown by arrowheads in Fig. 11b and f). Concomitant with the fast germination of rape, which is finished between 12 and 18 hai, the axis became nearly completely depleted of storage globulins at about 1 dai. Only remnants showing diffuse immunostaining were detectable at this time in the radicle (Fig. 11c and g). In the conduplicately folded rape cotyledons, which later become photosynthetic organs during seedling growth, mobilization of the storage globulins started concomitantly from the epidermis on both sides. Due to their thinness, a gradient of globulin degradation was not as clearly visible as in vetch cotyledons. Napin was degraded slightly faster than cruciferin. Thus in cotyledons at 5 dai hardly any napin was found (Fig. 11d) whereas cruciferin still was present in considerable amounts (Fig. 11h).

## Discussion

The results of our immunohistochemical analysis agree with the finding that vetch seed embryonic axes contain stored vicilin and legumin in protein bodies (Fig. 3) and that in the dry axis more vicilin than legumin is stored (data not shown). In addition, it was shown that in the axis globulins were mainly degraded during and immediately after germination and had completely disappeared by 2–3 dai, whereas in this period no globulin breakdown could be observed in the cotyledons except for those amounts of vicilin that were immunohistochemically detected in the prevascular strands of cotyledons where they disappeared before globulin breakdown started in the main storage mesophyll of this organ (Fig. 13). Early tissue-specific vicilin degradation had so far not been recorded by biochemical analysis of cotyledons. The percentage of vicilin present in vascular strands was too small to be detectable in the total amount of cotyledonous vicilin. According to its immunostaining, vicilin was also the predominating globulin in those axis tissues where storage protein degradation started, as in the radicle, in the prevascular strands, and epidermal layers. Therefore, a role as the preferential amino acid source at the beginning of germination can now be ascribed to this globulin. So far, no differential deposition and functions have been attributed to vicilin and legumin. It is still unclear which enzymes trigger globulin breakdown in vetch (Becker et al. 1997) but it has to be expected that they first become active in those predominantly vicilin-containing tissues where globulin mobilization starts.

Globulin degradation started where growth and differentiation were initiated in the axis. In the cotyledons only the prevascular strands undergo differentiation during seed germination and here globulin breakdown first takes place. This means that local storage-protein breakdown supplies amino acids for protein biosynthesis and that during germination, when no continuous vascular strand connections exist, the embryonic axis does not depend on long-distance transfer of amino acids from the cotyledons. Differentiation of conductive tissue was an early event in the axis, petioles and cotyledons during and after germination. Vessel formation preceded sieve-tube differentiation. Vascular strand connections were established at 3 dai when the axis was already completely depleted of globulin reserves and globulin breakdown could first be detected in the cotyledons. This indicates that, at this time, long-distance amino-acid transfer starts from the cotyledons into the embryonic axis. Thus the cotyledons take over as a source of nitrogen compounds for the growing axis, which acts as a sink, in good agreement with the results of the biochemical analysis. Diffusion of free amino acids from the cotyledons into the axis cannot be excluded, but should be of minor importance since no decrease in globulin contents could be detected in the cotyledons during germination and early post-germinative growth. No dry-matter changes were detected either in cotyledons or in the embryonic axes until 48 hai (data not shown). Afterwards the per-organ dry weight of the axis increased progressively whereas the cotyledon dry weight decreased. No changes in the protein quantities per cotyledon and per axis were measured during the first 2 dai. In accordance with the observations of Harris and Chrispeels (1975) in mung bean cotyledons, globulin degradation was initiated in the abaxial epidermis of the cotyledons after vetch seed germination. The front of globulin degradation which proceeded towards the vascular bundle was restricted to a small layer of cells. Cells behind this front were depleted of globulins whereas in front of the proteolytically active zone no globulin degradation was histochemically detectable in the cells. Our results agree with the hypothesis (e.g. Shutov and Vaintraub 1987) that in cotyledon mesophyll major storage-globulin mobilization occurs only after increasing amounts of proteinases have been synthesized from newly transcribed mRNA (Becker et al. 1994, 1995, 1997; Müntz et al. 1998; Fischer et al. 2000). Thus, a shell-shaped wave of proteinase gene expression obviously moves through the cotyledons from the epidermis towards the central vascular bundle. This process should be further investigated by immunoanalysis using proteinase-specific antibodies. Cells between this layer of proteolytic activity and the central vascular strand should mediate the amino acid transfer into the sieve tubes.

In all three analyzed species, protein mobilization started much earlier in the embryonic axis than in the cotyledons where it only began after protein reserves of the axis were depleted. At first glance, this agrees well with the suggestion that the lack of amino acids as building blocks for protein biosynthesis in the axis



**Fig. 13.** The scheme summarizes the temporal and spatial pattern of storage protein degradation during the first 2 weeks of seed germination and seedling growth of *Vicia sativa* L. The shading intensity roughly reflects the immunostaining of semithin tissue sections (see the legend of Fig. 12) and it gives no absolute quantitative information on the protein content but only an impression of the degree of storage globulin mobilization. For histological explanation see Fig. 12

might represent the signal for starting globulin degradation in the cotyledons. But there, transcription of proteinase mRNAs had already started roughly 1 d before proteinases could be detected and reserve-protein breakdown was registered (Fischer et al. 2000). In cotyledons, transcription initiation roughly coincided with radicle breakthrough, with the transition from cell elongation to the cell division and differentiation period, and with the depletion of storage proteins in the radicle. These events might impose a rapid increase in the demand for amino acids in the axis and strengthen the metabolite gradient. Nevertheless, as shown for other plants, hormonal signaling from the axis to the cotyledonous storage tissue also has to be

taken into consideration, and it remains controversial how the axis controls the temporal pattern of protein mobilization in cotyledons or endosperm during germination of dicotyledonous seeds (Bewley and Black 1994). Early protein mobilization occurs in restricted tissue regions. There, first proteinase molecules have either to be synthesized from free amino acids or from those derived from protein degradation by stored proteinases. Once proteinase molecules have been formed these can induce an “autocatalytic” increase in proteolytic activity. Whereas most published results indicate *de novo* formation of triggering proteinases in cotyledons and endosperm of dicotyledonous seeds, with the exception of buckwheat (Belozersky et al.

1990), the initiation of protein mobilization has so far not been analyzed in embryonic axes. Recently, we found strong indications that, at least in the latter organs, stored proteinase might initiate early protein mobilization (data not shown).

The spatial pattern of storage-protein mobilization in vetch was different from that in garden bean and rape. Independent of these differences, globulin degradation started locally in the embryonic axis of all three species whereas in the cotyledons globulin mobilization occurred only after seeds had germinated. There, it proceeded in small cell layers and never took place simultaneously in the whole tissue. In vetch and garden bean cotyledons the observed different histo-patterns of vicilin and legumin breakdown agreed well with patterns of general protein degradation described by Smith (1981) for these species. It has to be assumed that these patterns mainly reflect patterns of proteinase gene expression (Fischer et al. 2000) which must be controlled in a finely tuned manner and thus generate the histo-pattern of globulin breakdown.

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