Comparison of globulin mobilization and cysteine proteinases in embryonic axes and cotyledons during germination and seedling growth of vetch (Vicia sativa L.)

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Received 25 January 2000; Accepted 11 May 2000

Abstract
Vicilin and legumin, the storage globulins of mature dry vetch (Vicia sativa L.) seeds, are found in protein bodies which are present not only in the cotyledons, but also in the radicle, axis and shoot (together, for reasons of simplicity, here called axis). When at 24 h after the start of imbibition (hai) the radicle breaks through the seed coat a major part of the globulins in the axis has already been degraded, whereas in the cotyledons globulin breakdown cannot yet be detected. Globulin mobilization starts with the degradation of vicilin. At 48 hai when globulin mobilization in the cotyledons just begins, the axis is already nearly depleted of globulins. Mobilization of storage globulin is probably brought about by a complex of different cysteine proteinases (CPRs). The papain-like CPR2 and CPR4, and the legumain-like VsPB2, together with their mRNAs, are already present in axes and cotyledons of dry seeds. This means that they must have been formed during seed maturation. Additional papain-like CPRs are formed later during germination and seedling growth. CPR4 and VsPB2 together with their corresponding mRNAs become undetectable as germination and seedling growth proceed. VsPB2 and VsPB2-mRNA are substituted by the homologous legumain-like proteinase B and its mRNA. The composition of stored and newly formed CPRs undergoes developmental changes which differ between axes and cotyledons. It is concluded that storage globulin mobilization in germinating vetch seeds is started by stored CPRs, whereas the mobilization of the bulk of globulin is predominantly mediated by CPRs which are formed de novo.

Key words: Cotyledons, cysteine proteinases, embryonic axis, germination, globulin mobilization, vetch.

Introduction
During seed maturation protein reserves are not only deposited in classic storage tissues like the cotyledon mesophyll of dicotyledons or the endosperm of cereals, but also in the embryonic axis. In all these seed organs protein storage takes place in protein bodies which belong to the vacuolar compartment (Müntz, 1998). Protein bodies and storage proteins were detected in the embryonic axes of taxonomically distant plants such as maize (Tsai, 1979), pea and soybean (Alekseeva et al., 1977, 1978, 1979a, b, 1989), and cotton (Vigil and Fang, 1995). In cereal endosperm and legume cotyledons significant mobilization of storage protein starts after the radicle has broken through the seed coat, i.e. when germination switches to seedling growth (Bewley and Black, 1994). However, protein biosynthesis in the axis organs and cotyledons of dicotyledonous seeds commences much earlier (for reviews see Bewley, 1982, 1997).

The mechanisms behind the temporal gap between the start of protein biosynthesis during early seed germination and the beginning of reserve protein mobilization in storage tissue after germination, are still not fully understood. Already 40 years ago nitrogen balance determinations led to the suggestion that in pea embryonic axes endogenous protein mobilization must supply amino acids for early protein biosynthesis during germination (Lawrence et al., 1959; Lawrence and Grant, 1963). Murray and coworkers hypothesized that protein breakdown in the embryonic axis precedes storage protein mobilization in cotyledons of pea (Murray et al., 1979a,

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b). Recently, Vigil and Fang demonstrated that when cultivated in the absence of any external nutrient supply, detached embryonic axes of cotton exhibit similar elongation growth to the axes of intact seeds during 24 hai (Vigil and Fang, 1995a). They also found that the increase in proteolytic activity and protein breakdown showed a more or less similar pattern in both detached and attached cotton embryos (Vigil and Fang, 1995b). Similar growth characteristics have been described for isolated axes of Phaseolus vulgaris L. (Walton, 1966).

Cysteine proteinases (CPRs) are the favoured candidates for initiating and mediating storage protein degradation in storage tissues of cereals (Bewley and Black, 1994) and dicotyledonous plants (Wilson, 1986; Shutov and Vaintraub, 1987; Müntz, 1996). However, a specific analysis of cysteine proteinases and storage globulin mobilization in embryonic axes has never been done, nor have they been compared with similar enzymes and processes in the cotyledons of dicotyledonous seeds.

In previous publications the families of papain- and legumain-like CPRs from Vicia species were described (Becker et al., 1994, 1995, 1997; Fischer et al., 2000). CPR1, CPR2 and CPR4 are papain-like CPRs from vetch. VsPB2 and proteinase B are legumain-like vetch CPRs with specificity for Asn in P1 position at the peptide bond to be cleaved. Whereas proteinase B is an enzyme of globulin mobilization in cotyledons of seedlings, VsPB2 belongs to the group of vacuolar processing enzymes (VPE) which are responsible for propolypeptide processing during seed maturation (Hara-Nishimura et al., 1982, 1998). CPR1, CPR2 and VsPB2 have already been found in protein bodies, and CPR1, CPR2 and proteinase B were shown to degrade storage globulins in vitro (Fischer et al., 2000).

This study reports on the temporal gene expression pattern of several papain- and legumain-like CPRs in axis and cotyledons during seed maturation, germination and seedling growth of vetch, Vicia sativa L. Parallel to this, the patterns of vicilin and legumin mobilization were followed with immunoprobes. The results suggest that internal globulin breakdown starts the axis independent from amino acid import during germination. Later on, amino acid supply to the axis is provided by the storage cotyledons. CPR1, CPR2, CPR4, and VsPB2 probably start the globulin mobilization in embryonic axes and cotyledons during the early germination of vetch. At a later stage, de novo formed CPRs contribute to or may even take over completely the function of mobilizing the bulk globulin.

Materials and methods

Plant material

Dry seeds of Vicia sativa L. cv. consentini (Guss.) Arcang. (obtained from the Gatersleben Gene Bank, IPK Gatersleben, accession no.: VIC 133) were imbibed in distilled water for 1 h and grown in the dark at 23 °C on wet tissue paper in covered photo trays. Developing seeds were harvested at different intervals after flowering. After removal of the seed coats the embryonic axes were carefully dissected from the cotyledons. If not immediately used for analysis, harvested organs were shock frozen in liquid nitrogen and stored at −80 °C.

Homogenization and protein extraction

Freshly harvested or frozen organs were homogenized with quartz sand in a precooled mortar and pestle at 4 °C. Total proteins were extracted with 100 mM TRIS-HCl buffer (pH 8.0) containing 150 mM NaCl and 1 µl ml⁻¹ 2-mercaptoethanol. After the buffer was added, the homogenate was sonicated twice at 10 W for 30 s to disintegrate remaining organelles. Extraction took place under shaking in the dark for 15 min followed by centrifugation at 15 000 g for 20 min. The supernatant was used for subsequent analyses.

Albumins and globulins were separated by stepwise extraction. In the first step the albumins were solubilized. For this the homogenized tissue was resuspended in a buffer according to Sluyterman and Wijdens (Sluyterman and Wijdens, 1970) and Lichtenfeld et al. (Lichtenfeld et al., 1979) adjusted at pH 4.5 (v/w = 5:1 or 10:1). The homogenate was kept on a shaker at 4 °C for 30 min followed by centrifugation at 15 000 g for 20 min. The procedure was repeated twice more and the pooled supernatants referred to as the albumin fraction. Immunoblots showed globulins to be absent from the albumin fraction (results not shown). The remaining pellet was then resuspended in 0.1 M phosphate buffer, pH 7.0, containing 0.4 M NaCl to isolate globulins. The pooled extracts of three formations formed the globulin fraction. The final pellet was found to contain only neglectable amounts of non-extracted globulins (data not shown).

Protein quantitation

Protein content was determined by dye binding assays (Bradford, 1976) with protein dye reagent commercially available from BioRad Laboratories (Hercules, California, USA).

Protein electrophoresis and immunoblotting

Proteins were analysed by SDS-PAGE (11% or 12% polyacrylamide, 0.75 mm thickness) according to the method of Laemmli (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue G250 or with the Gel Code Blue Stain Reagent of Pierce Chemical Company (Rockford, IL, USA). Gelatine-containing proteinase activity gels were stained with Coomassie Brilliant Blue R250.

For immunoanalysis gels were blotted onto nitrocellulose membrane and incubated with the appropriate primary and secondary antibodies according to Towbin et al. (Towbin et al., 1979). Labelled proteins were visualized with the BCIP/NBT Color Substrate from Promega Corporation (Madison, WI, USA) or the Super Signal Chemiluminescent System of Pierce. Antibodies against polypeptides of CPR1, CPR2, VnPB2, and proteinase B (for cDNA sequences see EMBL Data Library accession no. X75749, Z30338, Z91954 and Z34899) were raised in rabbits or chicken as described earlier (Becker et al., 1994). Antigenes were produced in Escherichia coli. Initially proteinase B turned out to be sensitive to autocatalytic degradation. Mutation of the codon for His in position 174 into a Gly yielded a stable product. According to Chen et al. et this His belongs to the active centre of legumain-like CPRs (Chen et al., 1998). Legumin- and vicilin-specific antibodies that had been raised against...
protein preparations from cotyledons of mature *Vicia faba* seeds were used as described by zur Nieden et al. (zur Nieden et al., 1984) and Saalbach et al. (Saalbach et al., 1995), respectively.

CPR4 (for cDNA-deduced polypeptide sequence see EMBL Data Library accession no. Z99172) was labelled with polyclonal antibodies raised against Cys-EP from castor bean which had been provided by Dr. C Gietl from the Botanical Institute of the Technical University of Munich, Germany (Schmid et al., 1998). The Cys-EP antibody proved to be more sensitive and more specific than the CPR4 antiserum previously used (Fischer et al., 2000).

All antibodies used were tested for their specificity. Antibodies against the papain-like CPR1, CPR2 and CPR4 did not cross-react with other CPR antigens (Fischer et al., 2000). VsPB2 from *V. sativa*, (for cDNA see EMBL accession no. AJ007743) was detected with antibodies raised against the homologous VnPB2 from *V. narbonensis* which did not cross-react with the related protease B. On the other hand antibodies against protease B also recognized VsPB2.

**mRNA blotting**

Total RNA was extracted with the QIAGEN system (RNeasy Plant mini kit, Qiagen GmbH, Hilden, Germany). After glyoxal denaturation and agarose gel electrophoresis using the Ambion system (Northern Max™, Ambion Inc. Austin, Texas, USA) RNA was transferred to a positively charged nylon membrane (NyttranPlus, Schleicher &Schuell) and hybridized for 20 h with protease-specific radiolabelled single-stranded antisense cDNA fragments in Church hybridization buffer (Church and Gilbert, 1984). After successive washings with 6 ×, 4 ×, and 2 × SSC plus 0.1% SDS the membrane was exposed on X-ray film (Hyperfilm™ ECL™, Amersham) at −70 °C. The use of single stranded DNA strongly improved the sensitivity of mRNA detection as compared to conventional methods of mRNA-blotting.

All cDNA probes used for mRNA blotting were from *Vicia sativa* L. (Becker et al., 1994, 1995; Fischer et al., 2000) and corresponded to the above-described immunoprobes (for database accession numbers see above) except VsPB2-cDNA. Whereas an antibody specific for the homologous enzyme from *V. narbonensis* was used to detect VsPB2 the *V. sativa*-specific VSPB2-cDNA (EMBL Data Library accession no. AJ007743) was used for mRNA blots.

**Proteinase activity staining in gelatin-containing SDS gels**

Protein samples were dissolved in SDS-probe buffer without heating and loaded on SDS-polyacrylamide gels containing 0.04% gelatin. The basic pH of the gels maintained the acidic proteinases inactive. In addition, the gels were run at 0 °C to inactivate proteinases. Following this the gels were transferred into an appropriate incubation buffer to study proteinase activity. CPR activity was analysed in 0.1 M McIlvaine buffer pH 4.8 or 5.5 unless otherwise indicated. TRIS-HCl buffers were used in the basic pH range. Gels were gently shaken during incubation at 37 °C in the dark. Local gelatin degradation, visible as clear bands on a dark background after Coomassie staining (Becker et al., 1995, 1997), revealed the sites of active proteinases.

**Polypeptide sequencing**

N-terminal Edman degradation of blotted polypeptides was performed with a Beckman sequencer LF3400, using a special procedure for sequencing samples on poly(vinylidene difluoride)-type membranes (Beckman protocols).

**Results**

**Germination, seedling growth, and protein balance**

Under the conditions used here, germination ended after 24 hai when in more than 50 per cent of the seeds the radicle had broken through the seed coat thus marking the start of seedling growth (Fig. 1). The dry matter content of axis and cotyledon remained unchanged during germination (Fig. 1A). In the next 24 h, however, the dry matter of the axis slowly increased whereas that of the cotyledons slowly decreased. From 48 hai on, these dry matter changes in axes and cotyledons became more profound. Analysis of fresh weight increase showed that in both organs water uptake was nearly finished at 24 hai (data not shown).

Except for a small increase in the protein content of the axis during the first 4 hai (Fig. 1B), protein content of axis and cotyledon remained unchanged in the first 24 hai. From 24 hai onwards protein content started to increase in the axis and to decrease in the cotyledons, first slowly, but after 48 hai this development became much stronger. The temporal changes in protein content per organ followed a pattern basically similar to that of dry matter changes until 72 hai. After 72 hai, the protein content in cotyledons continued to decrease whereas after the initial increase in the axis a decrease in the protein content could now be observed. This can be explained by compositional changes in axis proteins which lead to decreased solubility in the phosphate-buffered saline that was used for protein extraction.

The amount of soluble amino compounds in the axis remained constant during germination (Fig. 1C), rose

**Amino acid analysis**

Free amino compounds were extracted from tissue homogenates according to the procedure described by Shaul and Galili (Shaul and Galili, 1992). Total amino compound was quantified by ninhydrin staining according to Mertz et al. (Mertz et al., 1974). Aliquots of the extract were pipetted in Eppendorf tubes and dried in a SpeedVac. After the solid residue was redissolved in 10 mM Na-bicarbonate (pH 9.0) the amino compounds were transformed into dabsyl-derivatives (Krause et al., 1995) which were fractionated and quantified by HPLC.

**Protein body isolation**

Protein bodies were isolated according to Mader and Chrispeels (Mader and Chrispeels, 1984). After homogenization of the tissue in a buffer containing 100 mM MES (pH 5.5), 1 M EDTA, 600 mM mannitol, the extract was filtered through Miracloth and centrifuged (4 min, 100 g) to remove starch grains. The supernatant was layered onto a solution of 5% Ficoll in the same buffer and centrifuged (20 min, 100 g). The resulting pellet was washed twice and resuspended in 100 mM TRIS (pH 8.0) containing 150 mM NaCl. Pellets (protein body fraction) and supernatants (proteins from the cytosol and other cellular compartments) were used for further analysis.
Fig. 1. Changes in total amounts of dry matter (A), salt-soluble protein (B), and soluble amino compounds (C) in embryonic axis and cotyledon during germination and seedling growth. Germination ended 24 hai when the radicle broke through the seed coat. Size increase of the seed and seedling growth are schematically indicated in Fig. 1B.

slowly between 24 and 48 hai followed by a strong increase between 48 to 72 hai.

The amount of soluble amino compounds in cotyledons hardly changed until 8 hai. From then on it rose slowly between 8 and 24 hai, very strongly between 24 and 72 hai and somewhat less strongly between 72 and 96 hai (Fig. 1C).

Breakdown of specific proteins

The abundance of several proteins in the albumin fraction changed with time (white and black arrowheads in Fig. 2A). After microsequencing it was possible to identify some of these proteins (Fig. 2A, E).

Vicilins were the most abundant proteins in the globulin fraction (Fig. 2B) extracted from the embryonic axis, but not in that of the cotyledons (Fig. 2F). Immunoblotting indicated that vicilin (Fig. 2C) and legumin (Fig. 2D) were nearly completely degraded in the axis at 72 and 96 hai, respectively. The legumin antibody used was raised against non-denatured total legumin and reacted much stronger with $\alpha$-legumin than with $\beta$-legumin chains. $\alpha$-Chains of legumin were preferentially degraded whereas the unchanged strength of $\beta$-chain bands indicated its stability during this period which agrees with similar findings for legumin breakdown in other plants (Vicia faba L., Lichtenfeld et al., 1979; Fagopyrum esculentum L., Dunaevsky and Belozersky, 1989; Glycine max (L.)
Cysteine proteinases activity

In the extracts of both embryo axis and cotyledons, proteinases with an acidic pH optimum were predominantly found between 25 to 50 kDa (Fig. 4B, F). A further set of high molecular weight (>70 kDa) proteinases with a basic pH optimum were common in the embryonic axis but only sparsely present in the cotyledons (Fig. 4A, E). Basic proteinases all had molecular weights of 70 kDa or more. Weak acidic proteinase activity (arrowheads) was already present in extracts from dry seed organs (Fig. 4B, F at 0 h). In the axis number and activity of these acidic proteinases started to increase during germination between 12 and 24 hai. In cotyledons, a similar increase in number and activity became apparent only after germination from 48 hai onwards. When extracts were preincubated with E-64, an inhibitor of papain-like CPRs, all acidic proteinases activity disappeared but for two bands with molecular weights of 37 and 42 kDa (Fig. 4C, G). These bands probably correspond to legumains which are not affected by E-64 inhibition (Becker et al., 1995). Treatment with iodoacetic acid, which inhibits papain- as well as legumain-like CPRs, eliminated all proteinase activity in the lower molecular weight range (Fig. 4D, H). Activity in the higher molecular weight range (>70 kDa) was not affected by E-64 (Fig. 4C, G), but at least partially affected by iodoacetic acid (Fig. 4D, H). This was probably due to effects of iodoacetic acid on SH-groups which are important for the correct folding of the enzyme proteins. Proteinases with molecular weight >70 kDa were not inhibited by serine and aspartate proteinase inhibitors, but were affected by EDTA, a known inhibitor of metallo-proteinases (data not shown). The proteinase inhibitor tests thus indicated that acidic papain- and legumain-like CPRs are already present in axis and cotyledons of dry seeds.
CPR activity was much stronger in axis extracts than in cotyledon extracts (Fig. 4B versus 4F). Since similar amounts of protein were applied to the gel lanes the differences in CPR activity must reflect differences in the ratio between proteinases and other proteins. That this ratio is much larger in the embryonic axes as compared to that in cotyledons, is presumably due to the larger amount of globulins in the latter organ.

Cysteine proteinase mRNAs and polypeptide patterns during development
The mRNAs of CPR2-, CPR4- and VsPB2- were already found in embryonic axes and cotyledons of developing seeds at mid and late maturation stage (Fig. 5A). They were similarly detected in mature dry seeds. The CPR4- and VsPB2-mRNAs label declined after late maturation and in embryonic axis they were no longer...
Cysteine proteinases of germinating vetch

CPR1- and proteinase B-mRNAs label was found in the embryonic axes and cotyledons during the maturation stages and in dry seeds. In the axis CPR1-mRNA first appeared at 12 hai. After an initial increase until 48 hai, the label decreased again, but remained present until 144 hai. In cotyledons CPR1-mRNA label was first detected at 48 hai from which time on it continued to increase in strength. The first traces of proteinase B-mRNA label appeared simultaneous in axis and cotyledon at 48 hai and markedly increased in strength until 144 hai. Typically, for all enzymes tested mRNA label was much stronger in the cotyledon than in the embryonic axis.

Albumin and globulin extracts used to register protein degradation (Fig. 2) were also analysed by immunoblotting to study the presence of CPR polypeptides (Fig. 5B). Whereas CPR1, CPR2, VsPB2, and proteinase B were found in albumin extracts, CPR4 was detected in the globulin fractions. Significant amounts of CPR2, CPR4 and VsPB2 were already present in axes and cotyledons of dry seeds (0 hai). Trace amounts of CPR1 were also found in the embryo axis at this time. In the cotyledon, however, CPR1 was absent until 48 hai. At 48 and 72 hai proteinase B first appeared in the axis and cotyledon, respectively. The abundance of CPR1, CPR4 and VsPB2 and proteinase B followed patterns similar to those found for their corresponding mRNAs (compare Fig. 5A and B). In the case of CPR2, however, a strong decrease occurred in the embryo axis after 48 hai, i.e. at a time when the strength of the corresponding mRNA band still increased (compare Fig. 5B and A).

Free amino compounds in axes and cotyledons

HPLC analysis of free amino acids was performed with extracts from embryonic axes and cotyledons harvested at different times during 96 hai. The quantitative changes of selected amino acids are summarized in Fig. 6. In the embryonic axes the amounts of Asp, Glu, Asn, and Gln hardly changed until 48 hai. Afterwards, a moderate increase in Gln and a strong increase in Asn was observed whereas the amounts of Asp and Glu remained basically the same. In cotyledons the amounts of Asp, Glu, Asn, and Gln slowly started to increase between 12 and 24 hai. This increase became much stronger between 24 and 48 hai and was more pronounced for Glu and Gln than for Asp and Asn. In axes as well as in cotyledons the quantitative changes of these amino acids corresponded to those of total soluble amino nitrogen shown in Fig. 1C.

Discussion

It has been demonstrated in this study that during germination and early seedling growth of vetch, globulins are first mobilized in the embryonic axis. This agrees with the results of immunohistochemical analysis which detectable from 72 hai onwards, whereas in the cotyledons traces remained visible until 144 hai. However, the CPR2-mRNA label strongly increased in both organs from 24 hai onwards, i.e. during seedling growth. No
showed that axis tissues were depleted of vicilin and legumin before the bulk of these storage proteins was mobilized in the cotyledons. It further revealed that vicilin predominated in those tissues where growth and differentiation in the axis begins, and that at the start of these processes vicilin was the main globulin to be mobilized. Vicilin was also prominent in provascular strands of cotyledons and was already mobilized here during germination (Tiedemann et al., 2000). These immunohistochemical data could not be confirmed by the biochemical analysis of cotyledon extracts presented here. The amount of vicilin in the prevascular strands is probably too small, in relation of the large amounts in the mesophyll of cotyledons, to allow its degradation to be detected by electrophoretic analysis of total globulin extracts.

While in both embryonic axis and cotyledon the amount of free amino compounds increased during late germination, no significant changes in protein quantities occurred. Still, globulins were broken down in the axis during germination. Stable protein levels can therefore only be explained by a simultaneous formation of new proteins in the axis. At the same time no degradation of storage protein and no change in the total amount of protein occurred in the cotyledon. According to Tiedemann et al. there is no continuous vascular bundle connection between axis and cotyledons in vetch seeds during germination (Tiedemann et al., 2000). This means that there is no net transfer of amino compounds resulting from storage protein breakdown from the cotyledon into the axis at this time. Consequently, the axis is largely independent of external supplies and relies mainly on its own reserves (Fig. 7, stage 1). In support of this view are the observations of Vigil and Fang who found that detached cotton embryonic axes without external nutrient supplies, grew just as well as those still attached to cotyledons (Vigil and Fang, 1995a, b). The changes in the amounts of total free amino compounds probably indicates a switch from internal amino acid supply for protein biosynthesis in the embryonic axis to its supply from globulin mobilization in the storage cotyledons.

Fig. 6. Scheme of amino acid supply for protein biosynthesis in embryonic axes and cotyledons during germination (stage 1: germination) and seedling growth (stage 2: seedling growth). pb, Protein bodies; v, vacuole.

Fig. 7. Total amounts of acidic amino acids and their amides in embryonic axes and cotyledons during germination and seedling growth.
This is characteristic for the time after germination (Fig. 7, stage 2).

A switch in sources of amino acid supply was not only reflected in total amounts but also in compositional changes of soluble amino compounds. An accumulation of acidic amino acids and amides which form major constituents of vicilin and legumin, is to be expected if they are more rapidly generated by globulin mobilization than used up for protein biosynthesis and other metabolic processes. Amides form the major nitrogen transport compounds in Vicieae (Murray, 1988). Gln which was absent in axes and cotyledons of dry seeds, appeared at 4 hai. This might indicate the beginning of globulin breakdown which at that time still cannot be registered at the protein level. In cotyledons, the major increase in acidic amino acids and amides precedes that in the axis and occurs from 24 to 48 hai. From that time on the cotyledons contain more soluble Glu and Gln than Asp and Asn. This corresponds roughly to the quantitative ratios of these amino acids in storage globulins (Münz et al., 1972). In the axis only Asn showed a strong increase which occurred between 48 to 72 hai. At this time functional conductive tissue connections have been established between storage organ and axis (Tiedemann et al., 2000). Timing and predominance of Asn thus reflects the start of an influx of Asn as the major compound for long-distance nitrogen transport from the cotyledons into the axis. Similar results have been published for cotyledons of germinating mung bean (Ericson and Chrispeels, 1973; Kern and Chrispeels, 1978). Vicilin, the major storage protein of mung bean, contains more Glu/Gln than Asp/Asn, but Asn is the main amino acid in the cotyledon exudate at 4–5 dai.

During germination and seedling growth globulins are broken down under acidic conditions. All acidic proteinase activity was subdued by iodoacetic acid which is an inhibitor of papain- and legumain-like cysteine endopeptidases. Differential inhibitor analysis revealed the presence of representatives from both families of CPRs. Acidic proteinase activity but not basic proteinase activity was also present in the dry seeds. In agreement with previous reports (Shutov and Vaintraub, 1987) these results strongly indicate that CPRs are responsible for globulin mobilization. Therefore the temporal patterns of CPR gene expression were followed during seed maturation, germination and seedling growth, using CPR-specific cDNA- and immunoprobes (Becker et al., 1994, 1995; Fischer et al., 2000).

With the exception of CPR2 in the embryonic axis the pattern of CPR polypeptides roughly paralleled that of their corresponding mRNAs. During early germination CPR1 was immunologically found to be restricted to a zone in the root tip but more distributed in the cotyledons (J Tiedemann et al., unpublished results). Whereas in the axis the local concentration of CPR1 polypeptide was high enough to permit immunodetection, the amount of CPR1-mRNA probably was below the limits of detectability. CPR1-mRNA becomes evident in axis extracts from 12 hai onwards. Generally, CPR-mRNAs were detected a little bit earlier than their corresponding polypeptides. This suggests that the presence of these enzymes was mainly controlled by the availability of their mRNAs for translation. The mRNAs and polypeptides of the papain-like CPR2, CPR4 and of the legumain-like VsPB2, were found to be generated during seed maturation and to be present in dry seeds. During germination and seedling growth the papain-like CPR4 and the legumain-like VsPB2 disappeared from the axis and cotyledon after 2 and 4 dai, respectively. On the other hand the amount of papain-like CPR1 increased whereas the legumain-like proteinase B now appeared. During early seedling growth CPR2 disappeared from the axis but became more abundant in cotyledons. This means that in cotyledons CPR1 and CPR2 must have been formed de novo in this period. It has already been shown that these two enzymes are present in protein bodies and that at least in vitro they are capable of degrading vicilin and legumin from vetch cotyledons (Fischer et al., 2000). So far nothing is known about the intracellular localization of CPR4 and its ability to digest globulins. Since the N-terminal signal peptide of its precursor indicates a vacuolar or cell wall localization, it is believed that CPR4 is also involved in the early globulin breakdown before a de novo formation of other CPRs takes place. This is underlined by the finding that CPR4 is contained in the globulin fraction which might reflect an association of enzyme and storage protein essential for globulin mobilization. The main function of CPR1 and proteinase B seems to catalyse bulk globulin mobilization in cotyledons after germination. Also CPR2 may contribute to the bulk globulin breakdown as indicated by the increased levels of CPR2-mRNA in cotyledons during seed maturation.

Shutov et al. and Jung et al. showed that, at least in vitro, legumain-like CPRs cannot digest mature unmodified legumin from vetch and field bean (Shutov et al., 1982; Jung et al., 1998). According to the hypothesis of Shutov and Vaintraub (Shutov and Vaintraub, 1987) globulin breakdown is triggered by an initial digestion by a papain-like CPR. This limited proteolysis makes the globulin available for further digestion by legumain-like CPR. This hypothesis is supported by the observation that representatives of both CPR-families were always present during early and bulk globulin mobilization. The storage of papain- and legumain-like CPRs in dry seeds raises questions. If these enzymes are indeed already formed during seed maturation and stored together with their globulin substrates in protein bodies, how then is a premature breakdown of globulin prevented? Also, do these stored proteinases degrade the globulins in protein bodies during early germination before new CPRs are formed at the end of the germination period? Answering these questions will be subject of future investigations.
Acknowledgements

The authors want to express their gratitude to Mrs Monika Wiesner for her excellent technical assistance and to Mrs Anett Kilian and Birgit Schäfer for the skillful drawing of the figures and preparing of the photos, respectively. Dr Manteufel kindly raised antibodies against the mutated proteinase B in rabbits, and the testing of the Cys-EP antibody against lysates of CPR4-expressing E. coli cells was kindly performed by Dr V Senyuk from the Laboratory of Protein Chemistry, State University of Moldova, Kishinev. The authors are especially grateful to Dr T Rutten (IPK, Gatersleben) for his excellent language editing of this paper. The project was supported by Deutsche Forschungsgemeinschaft (DFG) grants Mu 925/5-1, Mu 925/5-2, and Special Research Project 363 ‘Molecular Cell Biology of Plant Systems’ project No. A5 and B12.

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