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REVIEW ARTICLE

Stored proteinases and the initiation of storage protein mobilization in seeds during germination and seedling growth

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Abstract

Though endopeptidases and carboxypeptidases are present in protein bodies of dry quiescent seeds the function of these proteases during germination is still a matter of debate. In some plants it was demonstrated that endopeptidases of dry protein bodies degrade storage proteins of these organelles. Other studies describe cases where this did not happen. The role that stored proteinases play in the initiation of storage protein breakdown in germinating seeds thus remains unclear. Numerous reviews state that the initiation of reserve protein mobilization is attributed to *de novo* formed endopeptidases which together with stored carboxypeptidases degrade the bulk of proteins in storage organs and tissues after seeds have germinated. The evidence that the small amounts of endopeptidases in protein bodies of embryonic axes and cotyledons of dry seeds from dicotyledonous plants play an important role in the initiation of storage protein mobilization during early germination is summarized here.

Key words: Dicotyledonous seeds, germination, protein bodies, protein mobilization, stored proteases.

The controversy on the initiation of protein mobilization by stored or *de novo*-formed proteases during germination and seedling growth

Plants accumulate protein reserves in developing seeds. The major amount of these reserves consists of specific storage proteins (Shewry and Casey, 1999), like globulins, which predominate in dicotyledonous seeds, or prolamins, which are the major storage proteins of most cereals. Accumulation of protein reserves takes place during the middle and late maturation stages when seeds act as nitrogen sinks in the plant. They are deposited in membrane-bounded compartments, the protein bodies (PB), which are generated either directly from the endoplasmic reticulum (ER), as in maize, or from the protein storage vacuoles (PSV) as in the majority of species (Müntz, 1998). During this deposition period storage protein degradation and turnover are negligible (Madison *et al.*, 1981) indicating that the proteins are protected against premature proteolytic attack.

Storage proteins are mobilized during seed germination and subsequent seedling growth. Germination is regarded to be finished when the radicle breaks through the seed coat (Bewley and Black, 1994). This moment also marks the onset of seedling growth. The start of storage protein mobilization indicates that the mechanisms protecting storage proteins against degradation during middle and late seed maturation have been overcome. Storage protein mobilization has been predominantly investigated in seeds of cultivated plants such as grain legumes, rape, sunflower, and cereals which, during domestication, underwent selection for increased protein accumulation in the cotyledon storage tissues, as in dictoyledonous seeds, or in the endosperm, as in cereal grains. In these tissues, storage protein mobilization only becomes measurable after the radicle has protruded and germination is finished. Over the years the following picture has emerged of protein mobilization in storage tissues at this time. Inactive precursors of proteinases are

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Abbreviations: CP, carboxypeptidase; CPR, cysteine proteinases; dai, days after imbibition; ER, endoplasmic reticulum; MPR, metalloproteinase; PB, protein bodies; PSV, protein storage vacuole; VPE, vacuolar processing enzyme.

synthesized on the rER, from where they are transported through the endomembrane system into the PB. Here enzyme activation takes place and protein breakdown is initiated (Shutov and Vaintraub, 1987; Wilson, 1986; Bewley and Black, 1994; Müntz, 1996). The only alternative model has been presented for buckwheat cotyledons where a stored metalloendopeptidase starts globulin breakdown (Belozersky *et al*., 1990).

Storage proteins are not only accumulated and mobilized in specific storage tissues but also in axis organs like the radicle and the embryonic shoot (summarized by Tiedemann *et al*., 2000). Protein mobilization in storage tissues does not start in all regions simultaneously (reviewed by Smith, 1981). During germination small amounts of storage proteins are broken down in limited regions. This degradation might be mediated by stored proteinases. It is not known whether storage protein mobilization in embryonic axes is mediated by *de novo* formed or by stored proteinases, neither is it known to what extent early protein biosynthesis during germination in axis and storage tissue (Bewley, 1982, 1997) depends on free amino acids released by protein mobilization.

Reviewers always remark that endo- and exopeptidases are present in the PB from the dry seeds of many plants (Ashton, 1979; Matile, 1982; Wilson, 1986; Shutov and Vaintraub, 1987; Fincher, 1989; Bewley and Black, 1994; Müntz, 1996). This statement is based on a few studies in which, after the isolation and biochemical analysis of PB, both types of peptidases were found (Yatsu and Jacks, 1968; Nishimura and Beevers, 1978, 1979; Hara and Matsubara, 1980; Hara-Nishimura *et al*., 1982). Papain-like cysteine proteinases (CPRs) were found in PB of dry cucumber cotyledons (Hara and Matsubara, 1980; Hara-Nishimura *et al*., 1982). Recently, a new CPR family was detected with strict specificity for Asn in the P1 position at the cleavage site. These so-called legumains (reviewed by Hara-Nishimura, 1998) most probably have polypeptide processing functions in vacuoles. Vacuolar processing enzymes (VPE) from the PSV of maturing seeds are classified as βVPE. In contrast, α- and γVPE are found in lytic vacuoles of vegetative organs (Hara-Nishimura *et al*., 1995). Legumains of the latter group like proteinase B from vetch (Shutov *et al*., 1982; Becker *et al*., 1995) and legumain-like proteinase (LLP) from kidney bean (Senyuk *et al*., 1998) also participate in storage globulin breakdown in cotyledons of seedlings after germination. Both βVPE and proteinase B-like legumains are localized in PB of seeds (Hara-Nishimura *et al*., 1993; Becker *et al*., 1995, 1997). βVPEs are formed in the developing seeds at the time of storage protein deposition. They are present in PB of dry seeds and their activity declines only slowly following germination (Hiraiwa *et al*., 1993; Okamoto *et al*., 1996). The PB of dry buckwheat cotyledons contain both a metalloproteinase (MPR) as well as an aspartic proteinase and a carboxypeptidase(s) (CP) (Belozersky *et al*., 1990; Elpidina *et al*., 1990, 1991). An aspartic proteinase was also found in the PB of scutellum cells in dry barley grains (Marttila *et al*., 1995). Carboxypeptidases are the only exopeptidases found in PB whereas aminopeptidases are localized in the cytoplasm of storage tissue cells (Harris and Chrispeels, 1975; Van der Wilden *et al*., 1980; also reviewed by Ashton, 1979; Shutov and Vaintraub, 1987).

Although it is beyond doubt that PB of dry seeds contain proteinases, some of which have been characterized in detail, their function in storage protein mobilization has remained unclear for several reasons.

(1) According to Shutov and Vaintraub, a proteinase can only be considered as being involved in storage protein breakdown if it meets at least three criteria (Shutov and Vaintraub, 1987). (a) The proteinase and its substrate have to be found together in PB where pH, ion concentration etc. have to permit proteolytic activity. (b) The proteinase must degrade the corresponding unmodified or modified storage protein *in vitro*. (c) The temporal pattern of *in vitro*-determined proteolytic activity has to correspond to the time-course of storage protein mobilization. Proteinase patterns have often been followed by enzyme activity electrophoresis parallel to storage globulin mobilization without considering whether the different enzymes have access or not to these storage proteins in PB. Also, studies analysing whether proteinases of dry seeds were active *in vitro* against storage proteins did not show whether these proteinases were present in the PB (Hara and Matsubara, 1980, for cucumber; Wrobel and Jones, 1992, for barley; Mitsuhashi and Oaks, 1994, for maize; Duarte *et al*., 1996, for lupins). Even if a proteinase meets all three criteria, the evidence for its *in vivo* function remains circumstantial. Only a few experiments have been performed with isolated PB from dry seeds to show whether incubation under appropriate conditions leads to a breakdown of internal proteins that could be attributed to the action of stored proteinases.

(2) Contradictory results have made it difficult to assess the role of stored proteinases in the initiation of storage protein mobilization. Korolyova and coworkers claimed that stored proteinases extracted from dry vetch seeds did not degrade unmodified storage globulins *in vitro* from similar dry seeds (Korolyova *et al*., 1975). It was shown that at least some of the major globulins of dicotyledonous seeds have to undergo initial modification by limited proteolysis before they can be broken down (reviewed by Wilson, 1986; Shutov and Vaintraub, 1987). In vetch, the catalysis of this initial modification was attributed to a papain-like CPR, called proteinase A, which is formed *de novo* in cotyledon cells after germination (Shutov *et al*., 1984; Shutov and Vaintraub, 1987). However, this function of proteinase A has

recently been questioned (Becker *et al*., 1997). Harris and Chrispeels did not find autolysis in PB of mung bean although these contained proteinases (Harris and Chrispeels, 1975). In contrast to the finding with vetch and mung bean the proteinases present in quiescent soybean (Bond and Bowles, 1983) and cucumber seeds (Hara and Matsubara, 1980; Hara-Nishimura *et al*., 1982) were shown to attack internal storage proteins *in vitro*. At least one of the cucumber proteinases could be localized in PB (Hara-Nishimura *et al*., 1982).

(3) Single proteinases isolated and purified from post-germination seeds often meet the three criteria and are consequently considered as participating in or responsible for storage protein mobilization. Storage protein mobilization, however, involves several members of a proteinase family or members of different proteinase families. This makes it difficult to attribute specific functions to single enzymes if other members of the set of proteinases responsible for storage protein mobilization are unknown.

(4) The methods used were too insensitive for detecting those proteinases responsible for initiating storage protein breakdown in PB and to register this degradation in isolated PB under appropriate incubation conditions.

Current knowledge of *de novo* formed proteinases for protein mobilization in storage tissue is substantial (Müntz, 1996). According to the three criteria mentioned earlier, several of these proteinases participate in storage protein mobilization. These enzymes are mostly CPRs and include vicilinpeptidohydrolase from *Vigna radiata* (Baumgartner and Chrispeels, 1979), SH-EP from *V. radiata* (Okamoto and Minamikawa, 1998), serine endopeptidase from *Glycine max* (Wilson and Tan-Wilson, 1992), and the SH-proteinases from *Hordeum vulgare* and other cereals (Fincher, 1989; Koehler and Ho, 1990; Davy *et al*., 1999). Even in these cases the *in vivo* importance of the enzymes needs to be confirmed by experiments with transgenic plants, where corresponding genes have to be overexpressed or silenced, or by mutant analysis.

Experimental results and hypotheses have frequently been reviewed with respect to the initiation of storage protein mobilization by *de novo* formed endopeptidases (Shutov and Vaintraub, 1987; Wilson, 1986; Vierstra, 1993). Cereal grains, for example, store the bulk of their storage proteins in the starchy endosperm. During grain maturation the cells of the endosperm undergo controlled cell death. Storage protein mobilization is mediated by proteinases which are *de novo* synthesized in the living cells of the aleurone layer of the endosperm, and secreted into the starchy endosperm. (for reviews see Fincher, 1989; Koehler and Ho, 1990; Davy *et al*., 1998; Ritchie *et al*., 2000). Here the evidence is summarized for the start of protein mobilization in embryonic axes and cotyledons of dicotyledonous plants by stored proteinases. Only in a later stage during and after germination does the breakdown of the bulk of storage protein become mediated by *de novo* synthesized proteinases.

New evidence showing that stored endopeptidases start storage protein mobilization in germinating seeds of dicotyledonous plants

Stored proteinases must have been formed during late embryogenesis and they can therefore be traced back to the middle and/or late maturation stages. During seed maturation storage protein degradation has to be prevented. This can be brought about by maintaining the proteinases in an inactive form and/or by specific structural characters of the storage proteins which will prevent their premature breakdown by proteinases stored in the same organelle. At the onset of germination stored proteinases have to become active in order to initiate storage protein mobilization. Whereas the proteinases have to get access to their substrates, some of these substrates must undergo a structural change before they can be degraded by the enzymes.

Proteinases in maturing and dry seeds

In seeds of vetch (*Vicia sativa* L.) CPRs are responsible for storage globulin mobilization (Schlereth *et al*., 2000, 2001). Six members of two CPR families have been analysed (Fig. 1). These are CPR1, CPR2, proteinase A, and CPR4 belonging to the family of papain-like CPRs, and VsPB2 and proteinase B from the family of legumain-like CPRs (Becker *et al*., 1994, 1995, 1997; Fischer *et al*., 2000; Schlereth *et al*., 2000). All six are present in cotyledons but only five are found in embryonic axes, the missing one here being proteinase A. Whereas CPR1, CPR2, CPR4, VsPB2, and proteinase B all are localized in PB, proteinase A was not found in this organelle. The mRNAs of all six CPRs encode pre-propolypeptides that, in addition to the mature enzyme, comprise a signal peptide and propeptide(s). The precursors of the CPRs enter the secretory pathway and undergo co- and post-translational processing by limited proteolysis (Fig. 2). From other CPRs is known that proenzyme processing is related to enzyme activation (Okamoto and Minamikawa, 1998; Okamoto *et al*., 1999; Toyooka *et al*., 2000). *In vitro* CPR1, CPR2, proteinase A and B degrade storage globulin isolated from dry vetch seeds (Fischer *et al*., 2000). According to the three criteria proposed by Shutov and Vaintraub they can therefore be regarded as storage protein-degrading enzymes (Shutov and Vaintraub, 1987).

The mRNAs of CPR2, CPR4 and VsPB2 are present in embryonic axes and cotyledons of developing seeds (Fig. 1) from mid-maturation onwards until maturity

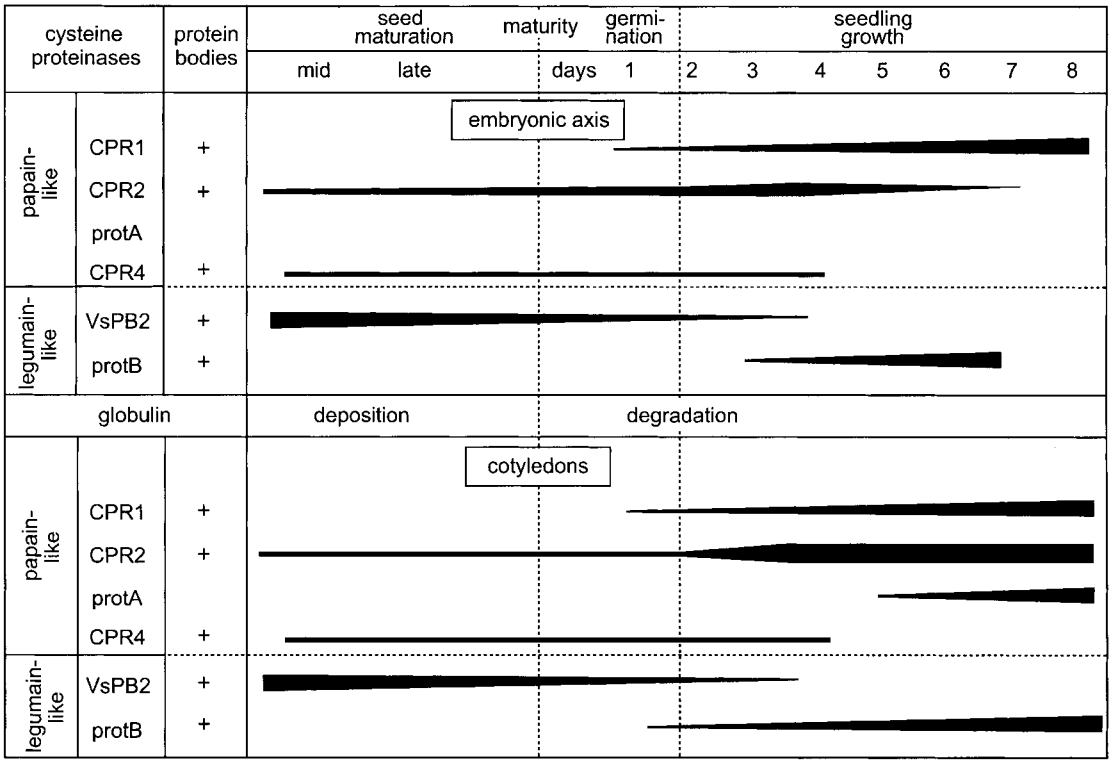

Fig. 1. Developmental regulation of cysteine proteinases (CPR) in relation to globulin deposition and mobilization during seed maturation, seed germination and seedling growth in vetch (*Vicia sativa* L.). The amount of enzyme polypeptides detectable on immunoblots is roughly reflected by the size of the bars. (+) Indicates presence of the enzyme in protein bodies (based on Müntz *et al.*, 1998. *Journal of Plant Physiology* **152,** 683–691, Fig. 4).

(Fischer *et al.*, 2000; Schlereth *et al.*, 2000). VsPB2 is the only legumain-like CPR found in PB of maturing cotyledons. It belongs to the group of βVPEs which *in vitro* process Asn-specific prolegumins into α- and β-legumin chains. This is accompanied by the transformation of prolegumin trimers into legumin hexamers which are deposited in PB (Hara-Nishimura *et al.*, 1993, 1998; Müntz *et al.*, 1993; Jung *et al.*, 1998). The processing function of βVPEs has still to be confirmed *in vivo*.

In mature dry seeds mRNAs of the same three CPRs are present in embryonic axes and cotyledons (Schlereth *et al.*, 2000). The polypeptides of CPR2 and VsPB2 are found in the albumin fraction, that of CPR4 in the globulin fraction of isolated PB of embryonic axes. Papain- and legumain-like CPR are active in extracts from isolated PB of axes and cotyledons. In lysates of isolated PB from both axis and cotyledon endogenous storage globulins are broken down only at acid pH which is characteristic for all CPRs. Furthermore, globulin degradation can be prevented by CPR-specific inhibitors. Storage proteins are degraded in the embryonic axes of imbibed seeds even when translation and *de novo* formation of proteinases is inhibited (Schlereth *et al.*, 2001). All these results lead to the conclusion that stored CPRs which were formed during seed development can initiate globulin mobilization in embryonic axes and cotyledons of germinating vetch seeds.

Proteinases and storage protein mobilization during seed germination

Embryonic axes: So far the proteolytic enzymes and globulin breakdown in embryonic axes during germination have only been analysed in detail in vetch seed (Fig. 1). From the start of germination CPR2, CPR4 and VsPB2 are present in the axes together with their mRNAs (Schlereth *et al.*, 2000). The mRNA of CPR1, and with some temporal delay also the polypeptide of CPR1, are formed during germination. Immunohistochemically CPR1 is found earlier in the radicles than CPR2, a result that contradicts the detection of these two enzymes on immunoblots (Tiedemann *et al.*, 2001). The reason behind this apparent discrepancy is that whereas CPR1 is initially absent from most tissues, local concentration is very high in the radicle. CPR2 on the other hand is present in low concentrations in most axis tissues. Consequently, in extracts the total amount of CPR2 will be higher than that of CPR1, whereas locally the concentration of CPR1 will exceed that of CPR2. Proteolytic activity in vetch embryonic axes needs an

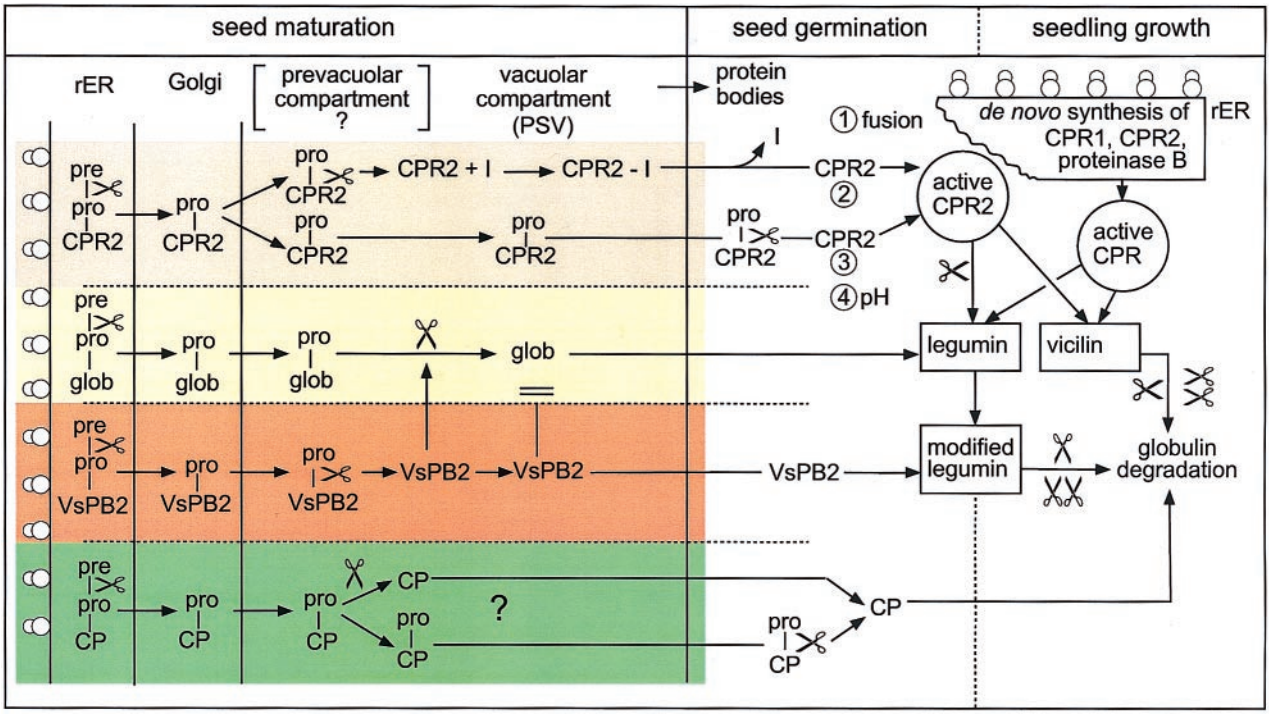

Fig. 2. Cell compartmentalization and processing of protease precursors during seed maturation, germination and seedling growth in relation to storage globulin deposition and mobilization. Single scissor and multiple scissors indicate limited and complete proteolysis, respectively. Hypothetical alternatives are given for protease inactivation/activation. (1) Differential compartmentalization followed by fusion of enzyme- and globulin-containing compartments. (2) Association/dissociation of inhibitor (I) and protease. (3) Delayed proprotease processing. (4) pH shift and changing ion concentrations. CP, carboxypeptidase; CPR, papain-like cysteine proteinase; glob, storage globulin; VsPB2, legumain-like cysteine proteinase (vacuolar processing enzyme from developing seeds, βVPE). It is not shown that VsPB2 is substituted by proteinase B in the post-germinative period.

acidic pH, as does storage protein breakdown in lysates of dry PB, and is prevented by CPR-specific inhibitors (Schlereth *et al.*, 2000).

The histopattern of CPR detection is similar to the pattern obtained after *in situ* hybridization with corresponding cRNAs (Tiedemann *et al.*, 2001) and coincides with the profile of globulin degradation (Tiedemann *et al.*, 2000). This underlines that at least the papain-like CPR1 and CPR2 are responsible for the mobilization of globulin in embryonic axes. Whereas VsPB2 was proven to be present in total axis extracts, the local concentration of this legumain-like CPR presumably was too low to be detected immunohistochemically. After protein mobilization commences in the radicle, it proceeds towards the embryonic shoot (Tiedemann *et al.*, 2000). Globulin breakdown first occurs in epidermis and prevascular strands. In the prevascular strands of the axis differentiation into functional conductive tissue starts during germination (Tiedemann *et al.*, 2000). Here and in the radicle only vicilin, the 7S storage globulin of vetch, was found. In vetch the ratio of vicilin to legumin, the 11S globulin, is much higher in the embryonic axis than in the cotyledons (Schlereth *et al.*, 2000). A predominance of vicilin is characteristic for organs and tissues where globulin mobilization begins. This mobilization is locally initiated at sites where growth and differentiation start.

In garden bean seed (*Phaseolus vulgaris* L.) the 7S storage globulin phaseolin is present in the embryonic axis with the exception of prevascular strands whereas phaseolin is found in all tissues of cotyledons including prevascular strands (Tiedemann *et al.*, 2000). Garden bean seeds contain a set of CPRs similar to that found in vetch. During germination the axis tissues contain CPR1- and CPR2-like polypeptides but lack legumain-like CPR (Senyuk *et al.*, 1998; Tiedemann *et al.*, 2001). Most of the phaseolin in the axis is degraded during germination. Similar observations are made in rape seed (*Brassica napus* L.) where napin and cruciferin, the 2S and 11S storage globulins of rape seed, are predominantly degraded in embryonic axes during germination (Tiedemann *et al.*, 2000).

In vetch a slight initial decrease in the amount of total free amino acids occurs at the beginning of imbibition which is attributed to leakage from the axis. Later during germination the amount of free amino acids remains unchanged as did the amount of total axis protein. No net import or export of amino acids was measured (Schlereth *et al.*, 2000).

Isolated cotton embryos that are incubated without nutrient supply in a humid atmosphere display proteolytic activity and degrade axis proteins similar to embryos that remain attached to the cotyledons (Vigil and Fang,

1995*a*, *b*). The proteinases involved and their substrates were not specified. Cotton seeds store 7S and 11S globulins in their cotyledons (Delseny and Raynal, 1999) but it is unknown whether this also applies for the axis.

Cotyledons: At the beginning of germination vetch cotyledons contain CPR2, CPR4 and VsPB2 and their mRNAs but lack CPR1 and proteinase A and B (Fig. 1), a situation similar to that found in the embryonic axes (Schlereth *et al*., 2000). However, the ratio between CPR2 and storage protein is much smaller in cotyledons than in embryonic axes. The strong predominance of storage globulins in cotyledons in relation to stored proteinases explains why, at the start of germination, protein breakdown by stored proteinases is so small that it was previously undetected (Fig. 1).

Garden bean cotyledons contain CPR1-, CPR2-, CPR4- and proteinase B-like CPRs (Fischer *et al*., 2000; Senyuk *et al*., 1998) as well as a proteinase A-related papain-like CPR, the so-called EP-C1 (Tanaka *et al*., 1993; Rotari *et al*., 1997). A homologue of the latter, found in mung bean, *Vigna mungo* L., is called SH-EP (Yamauchi *et al*., 1992; Okamoto and Minamikawa, 1998). In the three legumes, garden bean, mung bean and vetch, the proenzymes of the proteinase A-like CPRs contain the C-terminal ER-retention signal KDEL. Contrary to the embryonic axes of garden bean where CPR1 and CPR2 are already detected histochemically at day 1 of germination, no CPRs are found by this method in the cotyledons at this time (Tiedemann *et al*., 2001). Germination of garden bean seeds is finished 2–3 dai. In cotyledons CPR1 appears in the second half of the germination period where it is first detected in the epidermis and in prevascular strands. Shortly afterwards CPR2 and the proteinase B-homologue legumain-like proteinase (LLP) appear simultaneously. Since in cotyledons of both vetch and garden bean major storage protein breakdown occurs after germination the histopattern of these processes will be described below.

During germination the amounts of free amino acids and proteins did not change in the vetch cotyledons (Schlereth *et al*., 2000).

Cotyledons of buckwheat (*Fagopyrum esculentum* L.) contain a stored Zn-metalloproteinase which, in contrast to other known MPRs from either prokaryotes and eukaryotes, has a narrow substrate specificity for storage protein from seeds of this species (Belozersky *et al*., 1990). It is localized in PB (Elpidina *et al*., 1991) and has *in vitro* optimal activity at slightly acidic to slightly basic pH (Belozersky *et al*., 1990; Dunaevsky and Belozersky, 1989*a*). Translation inhibition by cycloheximide does not prevent the initiation of globulin breakdown (Dunaevsky and Belozersky, 1989*b*). However, imbibition of buckwheat seeds in the presence of EDTA, which binds bivalent cations, prevents globulin mobilization (Dunaevsky and Belozersky, 1993). This effect can be overcome by adding bivalent cations in excess to EDTA to the incubation medium (Elpidina *et al*., 1991). *In vitro* the MPR mediates a limited proteolysis of the legumin-like 13S storage globulin of buckwheat and generates a fragment pattern similar to that which appears during *in vivo* globulin degradation (Fig. 3). As a result of the initial cleavage of approximately 1.5% of its peptide bonds the holoprotein undergoes a conformational change (Voskobyonikova *et al*., 1989) without being dissociated. These observations are comparable to the initial degradation of globulins in cotyledons of plants like vetch (Shutov and Vaintraub, 1987), field bean, *Vicia faba* L. (Lichtenfeld *et al*., 1979, 1981) and soybean, *Glycine max* (L.) Merr. (Wilson *et al*., 1986). The conformational change leads to an increased mobility of the holoprotein in non-denaturing gel electrophoresis. This mobility shift is independent of whether the degradation of 13S globulin was initiated *in vivo* or *in vitro* (Fig. 3). All evidence thus indicates that the stored MPR is responsible for the initiation of globulin breakdown in buckwheat cotyledons (Fig. 4).

During and after germination the MPR shows an increase in activity which is insensitive to the presence of cycloheximide (Dunaevsky and Belozersky, 1993). The increased activity is attributed to the dissociation of a 12 kDa polypeptide inhibitor (Belozersky *et al*., 1990; Elpidina *et al*., 1991) (Fig. 4) which is localized together with the MPR in PB of buckwheat cotyledons (Elpidina *et al*., 1991). The inhibitor is thought to keep the MPR inactive during seed maturation. Dissociation of inhibitor from the MPR during germination is thought to be triggered by the release of stored Zn, possibly from phytate (Voskobyonikova *et al*., 1990).

Cotyledons of dry buckwheat seeds also contain stored CP. Neither alone nor in combination with the CP can the

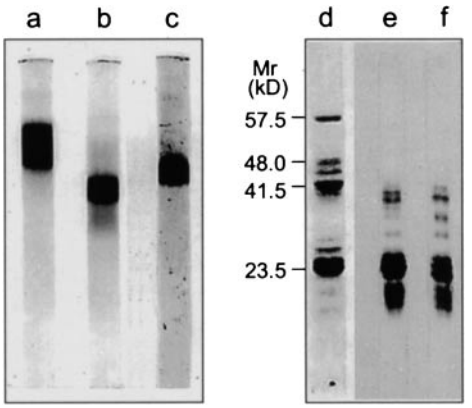

Fig. 3. Start of 13S globulin mobilization in cotyledons of buckwheat. Analysis of 13S globulin on polyacrylamide gels under non-denaturing (a–c) and denaturing (d–f) conditions. Globulin from cotyledons 3 dai but before germination (a, d), globulin from cotyledons modified *in vivo* (b, e), globulin from cotyledons modified *in vitro* by a MPR (c, f).

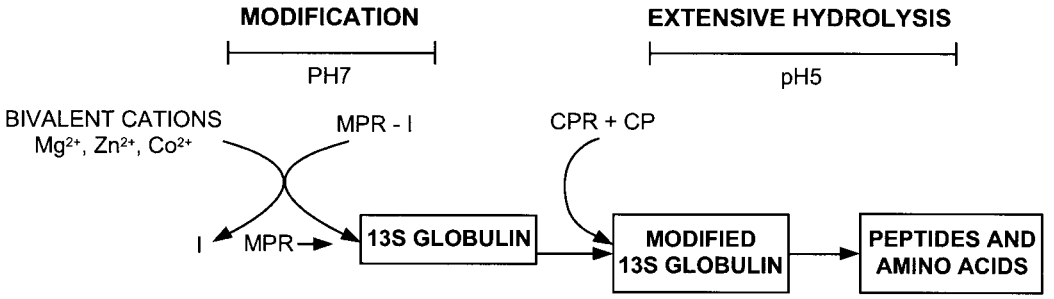

Fig. 4. Functions of different proteases in 13S globulin mobilization in cotyledons during and after germination of buckwheat seeds.

MPR bring about a complete degradation of buckwheat 13S globulin prepared from dry cotyledons. CP from dry vetch seed does not act on unmodified globulin from mature seed (Shutov *et al.*, 1981), but becomes active after globulin fragmentation by endopeptidases has taken place which gives the CP access to an increasing number of free C-termini of peptides. Involvement of CPs in the co-operative breakdown of storage proteins has been demonstrated for seeds of other dicotyledonous plants and cereals too (Dunaevsky and Belozersky, 1989*b*; Dunaevsky *et al.*, 1989). The molecular characteristics of vetch carboxypeptidases have not been described. Partial sequences of seed carboxypeptidases from pea and mung bean have been deduced from their cDNAs (Jones *et al.*, 1996) or directly determined by peptide sequencing (Wilson *et al.*, 1995). Both sequences are homologous to a carboxypeptidase sequence from *Arabidopsis thaliana* (Bradley, 1992). The corresponding gene encodes for a pre-propolypeptide with an N-terminal signal peptide downstream followed by a propeptide and the sequence of the mature enzyme. This agrees well with the fact that the carboxypeptidases of mung bean and vetch cotyledons are located in vacuoles and PB (Fig. 2). Carboxypeptidase families including similar enzymes are also known from cereal grains (Mikola, 1986; Washio and Ishikawa, 1994).

Proteases and storage protein mobilization during post-germinative seedling growth

Embryonic axis: In embryonic axes of vetch and garden bean storage globulin breakdown is completed within the first 1–2 post-germinative days (Schlereth *et al.*, 2000; Tiedemann *et al.*, 2000). Whereas the radicle of vetch stores only vicilin, which is degraded during germination, the embryonic shoot also contains legumin localized there where storage globulins are finally mobilized. Within these first two post-germinative days the content of papain-like CPR1, CPR2 and CPR4 decreases in the axes of vetch and garden bean, and shortly afterwards they can no longer be detected (Fig. 1). Whereas the legumain-like VsPB2 from vetch (Schlereth *et al.*, 2000) and the related βVPE from garden bean (Okamoto *et al.*, 1996) also decline during this time, proteinase B and its mRNA make their first appearance and later increase in vetch embryonic axes (Schlereth *et al.*, 2000). Interestingly, the homologous LLP was not found in the axis of garden bean (Senyuk *et al.*, 1998; Tiedemann *et al.*, 2000). Storage protein breakdown in the axes of vetch is nearly finished now which means that proteinase B must have a function outside this process.

Meanwhile functional vascular bundles have developed (Tiedemann *et al.*, 2000). The amount of protein increases in the axis and an influx of amino acids takes place (Schlereth *et al.*, 2000).

Cotyledons: New insight on protein mobilization and proteinases in cotyledons of various dicotyledonous species has come from the detailed analyses of this process in vetch, garden bean and buckwheat.

At the beginning of the post-germinative period the mRNAs and polypeptides of CPR4 and VsPB2 disappear from the vetch cotyledons. At the same time an increase in the mRNAs and polypeptides of CPR1 and CPR2 takes place (Fig. 1). In addition, the mRNA and polypeptide of proteinase B are now found for the first time and their amounts rapidly increase (Fischer *et al.*, 2000; Schlereth *et al.*, 2000). Whereas mature proteinase A appears 5–6 dai, its mRNA and precursor peptide are already found from the beginning of the post-germinative period onwards (Fig. 1). A stepwise processing of the proteinase A-precursor, including the detachment of the C-terminal KDEL, may be part of the control of enzyme activation (Becker *et al.*, 1997). This was demonstrated for the homologous SH-EP enzyme from mung bean cotyledons (Okamoto and Minamikawa, 1998, 1999; Toyooka *et al.*, 2000; Chrispeels and Herman, 2000). Since proteinase A is not found in PB and the mature enzyme appears late in the post-germinative period its role in storage protein mobilization in vetch cotyledons remains obscure.

The histopattern of storage globulin mobilization in cotyledons of vetch and garden bean is species-specific (Smith, 1981; Tiedemann *et al.*, 2000). In vetch, globulin mobilization proceeds in a small zone from the abaxial epidermis and, with some delay, from the adaxial

epidermis as well towards the central vascular bundle of the cotyledon. Globulin mobilization in garden bean starts in a ring around each of the scattered vascular bundles and proceeds in a small zone towards the vascular bundle in the centre. In both vetch and garden bean the immunohistopatterns of CPR1, CPR2 and proteinase B (vetch) or LLP (garden bean) coincides with the moving zones of globulin breakdown (Tiedemann *et al*., 2001). In this very same zone protein bodies fuse into big vacuoles. Cells behind this zone are devoid of globulin but remain alive in vetch whereas they die away in garden bean cotyledons (Tiedemann *et al*., 2000). The globulin breakdown in prevascular strands precedes that in the other cotyledonary tissues and concurs with the formation of functional vascular tissues. After the vascular bundles have been completed, the amounts of free amino acids as well as the quantity of globulin decrease in vetch cotyledons (Schlereth *et al*., 2000).

In buckwheat cotyledons a CPR is produced in the post-germinative period (Fig. 4). Its synthesis can be prevented by translation inhibition (Dunaevsky and Belozersky, 1989*b*). This particular CPR has an acidic pH-optimum and is localized in PB. Its activity increases as the seedlings grow and the 13S storage globulin is mobilized in the cotyledons. *In vitro* this CPR actively degrades isolated buckwheat 13S globulin, but only when this has been modified previously by the buckweat MPR and only when degradation products are continuously removed from the incubation medium by dialysis. Under these conditions, however, preparations of the buckwheat CPR and CP are capable of degrading the modified globulin into cleavage products that no longer can be detected by immunological and electrophoretic methods (Dunaevsky and Belozersky, 1989*b*).

Conclusions and generalizations

(1) So far, stored proteinases have been found in embryonic axes of dry vetch seeds and in the cotyledons of dry vetch, buckwheat, soybean and cucumber seeds. In vetch and cucumber these stored proteinases are CPRs, in buckwheat and soybean they were identified as MPRs. In vetch and buckwheat the stored proteinases start globulin mobilization or at least contribute to the initiation of this process since it can not be excluded that other proteinase(s) might also be involved (Fig. 5).

(2) Major storage protein mobilization occurs in the embryonic axes of vetch, garden bean and rape during germination. This happens before, in the post-germinative period, the bulk of storage globulin is degraded in the cotyledons. In the axes stored proteinases are more important for storage protein breakdown than they are in the cotyledons. Due to the breakdown of the internal protein reserves, axes are self-sufficient in amino acids for the start of protein biosynthesis during germination

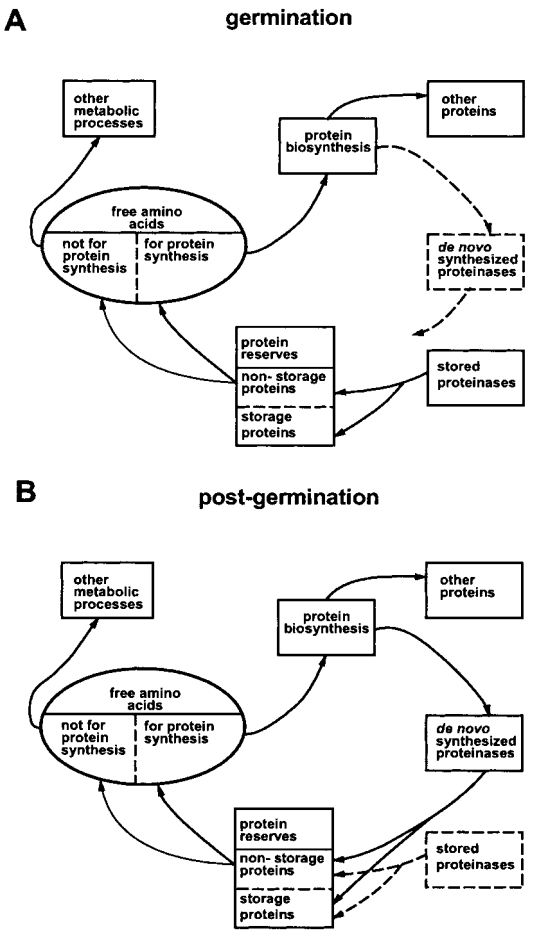

Fig. 5. Role of stored and *de novo* synthesized proteases in globulin mobilization in vetch seed during (A) and after (B) germination (Schlereth *et al*., 2001).

(Fig. 6). Storage proteins are locally degraded where growth and differentiation take place so that long distance transport of degradation products is not needed.

(3) In vetch and garden bean seeds prevascular strands are among the first tissues where protein mobilization is initiated during germination. While protein breakdown proceeds the prevascular strands are transformed into functional vascular bundles. In the post-germinative period these bundles enable a fast transport of amino acids from protein breakdown in the cotyledons into the growing axis (Fig. 6).

(4) Storage proteins in PB are degraded by several members of the same class (vetch) or from different classes (buckwheat) of endopeptidases. CPs are the only exopeptidases which contribute to storage protein breakdown in PB.

(a) In vetch, CPRs mediate the globulin breakdown in embryonic axes and cotyledons. Two papain-like and one legumain-like CPRs together with their mRNAs are stored in the embryonic axes and cotyledons of dry seeds. The mRNAs might be used to produce additional enzyme molecules during germination. New CPRs are formed

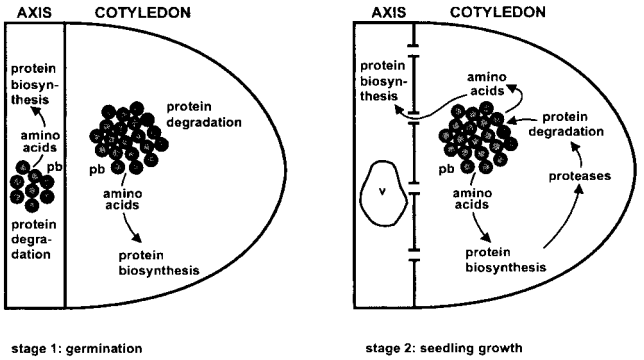

Fig. 6. Storage protein mobilization in embryonic axis and cotyledons during germination (stage 1) and after germination (stage 2). In stage 1, endogenous sources supply amino acids for protein biosynthesis in both organs whereas, in stage 2, the cotyledons are the source of amino acids for the embryonic axis which now acts as a sink (Schlereth *et al*., 2000).

during and after germination. Each CPR with its mRNAs has a typical spatial and temporal pattern of formation, increase and decline during germination and seedling growth, which differs between axes and cotyledons. Members of the papain- and legumain-like CPR families are always present simultaneously (Fig. 1). Shutov and Vaintraub hypothesized that at least the 11S globulins (legumins) have to undergo an initial limited proteolysis by a papain-like CPR before legumain-like CPRs, like proteinase B, can participate in the co-operative breakdown of this storage globulin (Shutov and Vaintraub, 1987). The so-called initiating role was attributed to proteinase A, but in vetch seeds this role presumably is played by CPR1 and CPR2. These two proteinases are found in PB together with the legumain-like VsPB2 and are present in axes and cotyledons during germination when proteinase A is still absent. Since legumains are supposed to be processing enzymes in developing seeds they might have a similar function during and after seed germination. In *Vigna mungo* a legumain-like enzyme, called VmPE-1, contributes to the activation of the papain-like SH-EP by an Asn-specific limited proteolysis (Okamoto *et al*., 1999; Toyooka *et al*., 2000).

(b) Buckwheat cotyledons contain two endopeptidases belonging to different proteinase classes. Both are needed for storage globulin breakdown (Fig. 4). During germination a stored MPR brings about a conformational change in the legumin-like 13S globulin by means of a limited proteolysis. The storage globulin is thereby opened to the post-germination attack by *de novo* formed CPR, which so far has not been assigned to a specific CPR family. A shift from neutral pH which is optimal for the MPR to an acidic pH needed for the CPR is supposed to occur in buckwheat cotyledons when seedling growth starts. Despite the fact that proteinases of two different classes are involved, this model is similar to that proposed by Shutov and Vaintraub for the degradation of 11S globulin (Shutov and Vaintraub, 1987). Complete globulin breakdown in buckwheat cotyledons is mediated by the two endopeptidases in co-operation with a CP.

To what extent can the results from vetch and buckwheat be generalized for other plant species? PB with storage proteins have been found in embryonic axes of many mono- and dicotyledonous seeds (Tiedemann *et al*., 2000). Protein mobilization in the axes precedes storage protein breakdown in cotyledons not only in vetch but also in kidney bean and rape (Tiedemann *et al*., 2000) and in cotton it takes place even in the absence of cotyledons and any external nutrient supply (Vigil and Fang, 1995*a*, *b*). This agrees well with the situation in germinating cereal grains where storage compound mobilization starts in the embryo and scutellum before it proceeds into the living part of the endosperm, the aleurone cell layers. From here *de novo*-synthesized hydrolases, including CPRs are secreted into the non-living starchy endosperm to mobilize storage proteins after the grains have germinated (Fincher *et al*., 1989; Bewley and Black, 1994). Many of the CPRs found in vetch and garden bean are also known from seeds of other dicotyledonous species (Fischer *et al*., 2000). Besides in buckwheat, MPRs are also found in dry seeds of soybean (Bond and Bowles, 1983) and maize (Mitsuhashi and Oaks, 1994) although their presence in PB has not been documented. Whereas the soybean MPR degrades soybean globulin *in vitro*, the MPR from maize is inactive in *in vitro* assays against zein, the major storage protein in corn.

Double labelling with immunogold showed that storage globulin and CPRs are present in the same PB (Fischer *et al*., 2000). This raises the question as to how storage proteins are protected against premature degradation during late embryogenesis and how this protective mechanism is overcome during the seed germination that follows (Fig. 2). In the case of legumin it has been proposed that structural characters and their change are responsible for the protection against and the initiation of proteolytic attack by legumains, respectively (Shutov and Vaintraub, 1987). While association with an inhibitor may inactivate a proteinase, its dissociation during germination may activate the enzyme, as happens in case of the MPR in buckwheat. In seeds like mung bean (Wilson and Tan-Wilson, 1987) and soybean (Wilson *et al*., 1988), proteinase inhibitors are broken down. Those particular inhibitors most probably do not act on endogenous proteinases, in contrast to the cystatins, a class of inhibitory polypeptides known from seeds, which have been demonstrated to act specifically on endogenous CPRs from rice grains (Abe *et al*., 1991). Cystatins have been isolated as a complex together with CPR from maize (Yamada *et al*., 2000). There is no evidence that, during seed maturation, proteinases and substrate are stored in different compartments which at the time of germination fuse to enable protein degradation. The coexistence of

lytic and storage vacuoles was recently shown in barley aleurone (Swanson *et al*., 1998) and pea cotyledons (Robinson *et al*., 1995). A storage of inactive proenzymes that theoretically could be activated by processing during germination has never been observed. Instead, researchers always found mature active enzymes in the PB of dry seed. Though the processing of the proenzyme of a papain-like CPR by a legumain-like CPR has been demonstrated, this happened in post-germination mung beans (Okamoto and Minimikawa, 1998, 1999; Okamoto *et al*., 1999). Finally, changes in the concentration of free metal cations and/or the pH, may contribute to a further control of proteinase activation during germination. In barley aleurone a shift towards an acidic pH also affects the proteolytic activity after germination (Bethke *et al*., 1998).

The new insights into the temporal course of sink–source relations between axis and cotyledons again raise questions concerning the mechanisms by which the axis controls the temporal and quantitative course of storage protein mobilization in cotyledons. The temporal coincidence of depletion of internal protein reserves in the axis with the establishment of functional conductive tissue connections between axis and cotyledons as well as with the beginning of the mobilization of the bulk of stored globulins in cotyledons, suggests that gradients of metabolite concentrations play an important role. This is underlined by the finding that the CPR of buckwheat which seems to be responsible for the mobilization of the bulk of stored 13S globulin, is subject to feedback inhibition by accumulating protein degradation products *in vitro* as well as *in vivo* (Dunaevsky and Belozersky, 1989*b*, 1993). There also is the possibility of hormonal control by the axis (summarized in Bewley and Black, 1994; Bewley, 1997). In cereals, the formation of CPRs in the aleurone layer, is under phytohormonal control of the embryo (Fincher *et al*., 1989; Koehler and Ho, 1990; Bewley and Black, 1994; Bethke *et al*., 1998). New experimental approaches are needed to elucidate the mechanisms by which the embryonic axes in dicotyledonous seeds control protein mobilization in storage tissue and organs. Until now it is still uncertain whether gradients of metabolites and transport compounds directly control sink–source relations between axis and storage organ and whether proteolysis in dicotyledons is controlled by a phytohormonal system as in cereals.

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