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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston vanhassa juhlasalissa (S212)
tammikuun 27. päivänä 1996 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Mathematics and Natural Sciences of the University of Jyväskylä,
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UNIVERSITY OF JYVÄSKYLÄ

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URN:ISBN:978-951-39-9023-7
ISBN 978-951-39-9023-7 (PDF)
ISSN 0356-1062

Jyväskylän yliopisto, 2022

ISBN 951-34-0682-2
ISSN 0356-1062

Jyväskylä University Printing House
and Sisäsuomi Oy, Jyväskylä 1996

ABSTRACT

Marttila, Salla
Differential expression of aspartic and cysteine proteinases, glutamine synthetase, and a stress protein, HVA1, in germinating barley
Jyväskylä: University of Jyväskylä, 1996, 54 p.
(Biological Research Reports from the University of Jyväskylä,
ISSN 0356-1062; 49)
ISBN 951-34-0682-2
Yhteenveto: Typen mobilisaatioon osallistuvien entsyymien sekä stressiproteiinin esiintyminen itävässä ohrassa
Diss.

Nitrogen is considered as a rate-limiting factor in plant growth. In barley (*Hordeum vulgare*,L.) seed, most of the nitrogen is deposited in storage proteins, which are remobilized for the growing seedling during germination. Proteolytic enzymes are of central importance in the hydrolysis of the reserve proteins. The main transport form of nitrogen in cereal seeds is glutamine, which is synthesized by glutamine synthetase. An ABA-responsive protein, HVA1, is induced during seed maturation but also due to desiccation stress during germination. In the present study, the expression of two different acid proteases, an aspartic and a cysteine proteinase, together with glutamine synthetase and a stress protein, HVA1, was studied by immuno-microscopy during barley germination.

Aspartic proteinase is already present in the mature barley seed. During germination, it was gradually expressed in all tissues of the seed, excluding the dead starchy endosperm. Subcellulary, aspartic proteinase was localized in the protein storage vacuoles. Tissue distribution and the amount of the two molecular forms of the enzyme varied, suggesting different physiological roles for the two heterodimers. Contrary to the case of aspartic proteinase, the synthesis and secretion of the 30 kD cysteine proteinase was started in the scutellar epithelium after the beginning of germination, and continued in the aleurone layer. The enzyme was found to be secreted via the Golgi complex and the putative secretory vesicles to the cell exterior. Glutamine synthetase (GS) was expressed as a cytosolic isozyme in the scutellum and the aleurone layer of germinating barley seed. At the beginning of germination, GS was likely to be expressed as a homo-octamer, and later as different hetero-octamers.

Under the stress conditions, a barley LEA3 protein, HVA1, was strongly induced, although it was present in the mature seed and declined gradually during germination. Although HVA1 protein has no putative signal peptide, it was localized both in the cytoplasm and protein storage vacuoles, suggesting the presence of a direct uptake mechanism. HVA1 might be degraded by the aspartic proteinase, as they were colocalized in the protein storage vacuoles. The exact function of HVA1 remains an open question.

Key words: Aspartic proteinase; cysteine proteinase; glutamine synthetase; *Hordeum*; HVA1; immunolocalization; LEA; stress response.

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List of original publications

This thesis is based on the following original articles, which will be referred to in the text by their Roman numerals:

I Marttila, S., Jones, B.L. & Mikkonen, A. 1995: Differential localization of two acid proteinases in germinating barley (*Hordeum vulgare*) seed. - Physiol. Plant. 93: 317-327.

II Marttila, S., Porali, I., Ho, T.-H.D. & Mikkonen, A. 1993: Expression of the 30 kD cysteine endoprotease B in germinating barley seeds. - Cell Biol. Int. 17: 205-212.

III Marttila, S., Saarelainen, R., Porali, I. & Mikkonen, A. 1993: Glutamine synthetase isozymes in germinating barley seeds. - Physiol. Plant. 88: 612-618.

IV Marttila, S., Tenhola, T., & Mikkonen, A.: A barley (*Hordeum vulgare*) LEA3 protein, HVA1, is abundant in protein storage vacuoles. (Revised manuscript submitted to Planta)

Abbreviations

ABA	abscisic acid
ABRE	abscisic acid responsive element
ADP	adenosine diphosphate
Asp	aspartic acid
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumine
cDNA	copy/complementary deoxyribonucleic acid
CI-2	chymotrypsin inhibitor 2
CPase	carboxypeptidase
Cys	cysteine
d	day
dhn	dehydration-inducible
E-64	trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane
E.C.	Enzyme Commission
Em	early methionine labelled
EP-A	cysteine endoprotease A
EP-B	cysteine endoprotease B
EP-C1	endopeptidase C1
ER	endoplasmic reticulum
FCS	fetal calf serum
GA	gibberellic acid
GDH	glutamate dehydrogenase
GS	glutamine synthetase
GOGAT	glutamate synthase
GS/GOGAT	glutamate synthase cycle
HVA1	*Hordeum vulgare* A1
HvAP	*Hordeum vulgare* aspartic proteinase
kD	kilodalton
LEA	late embryogenesis abundant
min	minute
mRNA	messenger ribonucleic acid
mw	molecular weight
NBT	Nitro Blue Tetrazolium
P_i	inorganic phosphate
PBS	phosphate-buffered saline
PSV	protein storage vacuole
PVDF	polyvinylenedifluoride
Rab	responsive to ABA
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulphate
Ser	serine
SH-EP	cysteine endopeptidase
TBS	Tris-buffered saline
TGN	*trans*-Golgi network

Contents

1 INTRODUCTION

Cereal seeds are either directly, or indirectly as food for livestock, the main source of carbohydrate and protein for human nutrition. Barley is the fourth most important cereal in the world after wheat, rice and maize, and the most widely cultivated, since it is harvested also in peripheral climatic regions, such as Finland (Briggs 1978). Cultivated barley, *Hordeum vulgare*, L., is systematically a monocot belonging to the tribe Triticeae of the family Poaceae (former Gramineae). The same tribe includes the common cereals wheat (*Triticum aestivum*, L.) and rye (*Secale cereale*, L.), whereas oats (*Avena sativa*, L.) is a member of the nearby tribe, Avenae (Shewry 1993). In Finland, barley is the most common cereal due to its rapid maturation. It is cultivated mainly for stock feed and malting, and to some extent for human food (Sallasmaa 1993).

Barley breeding is directed differently according to the use of the crop; feeding barley should be rich in protein of good quality, whereas malting barley should contain as high concentration of carbohydrates as possible and degrade its protein content efficiently (Aikasalo 1991). Traditional breeding strategies are no longer sufficient to achieve these targets, and consequently gene technology is needed. However, to be able to use modern techniques to improve seed quality, physiological events and their regulation during the life cycle of plants need further study under different environmental conditions. To understand better the physiology of the seed, it is important to remember that cereal seeds do not develop for human consumption but for the needs of the cereal plant itself. A mature grain contains everything, except water, necessary for the growth of the seedling until it is capable of photosynthesizing and uptaking minerals from the ground.

Mature barley seed is composed of the living embryo and the endosperm, which contains the large amount of dead tissue, the starchy endosperm, surrounded by the living tissue of a few layers of cells, known as the aleurone layer (Fig. 1). The embryo, which is deposited in the basal end of the seed, contains the embryonic axis and the scutellum, the

modified cotyledon of monocots, which consists of the parenchymal tissue and the single cell layer of the epithelium facing the starchy endosperm (Briggs 1978). On average, barley seed is composed of about 80 % of carbohydrates, of which starch is the major component, 8-15 % of proteins, 2-4 % of lipids, and 0.2 % of nucleic acids (Shewry 1992).

In the present study, we investigated nitrogen metabolism during germination of barley seed. Proteolytic enzymes play a major role in the remobilization of the nitrogen, which is accumulated in the seed for the use of the growing seedling. The role of different proteinases in germinating seed was studied from the physiological point of view. The results are combined with the studies of the expression of glutamine synthetase to understand further the flow of nitrogen from the endospermal storage proteins to the components of vegetative tissues. When normal development ceases following environmental stress, specific stress response proteins are expressed. Here we have studied the role of a stress protein expressed when the germination of the barley seed is disturbed by desiccation stress.

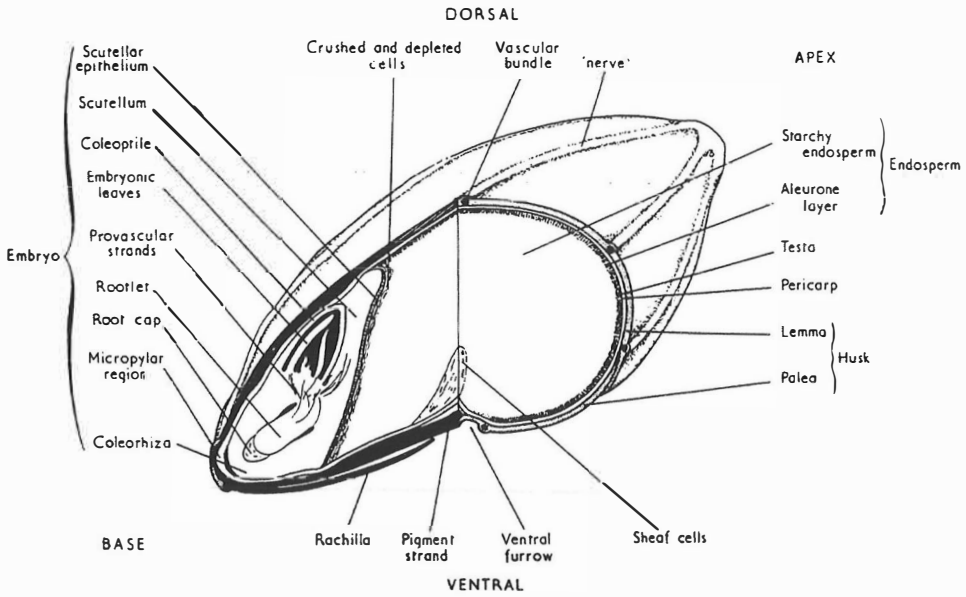

FIGURE 1 A schematic drawing of a mature barley seed (Briggs 1978). The embryo with root and shoot initials is marked on the left. The scutellum is located next to the embryo, its epithelial cell layer facing the huge starchy endosperm. The aleurone layer surrounds the starchy endosperm.

2 REVIEW OF THE LITERATURE

2.1 Nitrogen metabolism in germinating barley seed

Nitrogen is considered as a rate-limiting factor in the growth of plants (e.g. Sechley et al. 1992). Most of the nitrogen content of seed is incorporated in proteins, which form 8-15 % of the dry weight of the cereal seed. Nucleic acids also contain some nitrogen (Briggs 1992). Seed proteins consist mostly of storage proteins of which two thirds are deposited in the starchy endosperm and one third in the adjacent living tissues (Enari & Sopanen 1986). According to the original classification, storage proteins of cereal seeds are divided into four groups based on their different solubility properties: water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins, and acid- or alkali-soluble glutelins (Osborne 1924). Seed proteins can also be divided to storage and non-storage proteins, the former functioning solely as reserves (prolamins and globulins), and the rest as metabolic and structural proteins and, possibly, as secondary storage proteins.

The major group of storage proteins is composed of prolamins, called hordeins in barley, which occur only in cereal and related grass seeds. Prolamins are rich in proline and glutamine, which contain about half of the seed nitrogen, but lack the two essential amino acids, lysine and threonine (Kreis & Shewry 1992). The tissue distribution of proteins varies in the seed; hordeins are localized in the starchy endosperm (Brandt & Ingversen 1976, Matthews & Miflin 1980), and globulins in the scutellum and aleurone layer (Yupsanis et al. 1990).

During germination, reserve proteins are mobilized for the needs of the growing seedling. Mobilization can be classified into three stages (Mikola 1983a): 1) The hydrolysis of reserve proteins in the scutellum and aleurone layer for the production of hydrolytic enzymes. 2) The bulk hydrolysis of the storage proteins in the starchy endosperm. 3) Final breakdown of the remaining proteins in the starchy endosperm and

aleurone layer. Since the starchy endosperm is a non-living tissue, proteolytic enzymes can not be synthesized in situ during germination. They are produced in the adjacent living tissues and secreted into the starchy endosperm unless they are already present in the mature seed.

Germination of the mature grain begins when the grain has imbibed enough water, about 2-3 times the dry weight of the seed (Bewley & Black 1985). In a simplified model, a phytohormone, gibberellic acid (GA), is believed to mediate the germination signal by diffusing from the embryo proper to the scutellum and the aleurone layer. However, different species and cultivars vary significantly in their responsiveness to GA (Fincher 1989). Synthesis and secretion of different hydrolases, for example β-glucanases and α-amylases, begin from the scutellar epithelium and continue gradually along the aleurone layer (Gibbons 1981, McFadden et al. 1988, Pogson et al. 1989). Secretory cysteine proteinases are assumed to play a central role in the initial stages of storage protein hydrolysis in the starchy endosperm (Koehler & Ho 1988, 1990b, Poulle & Jones 1988, Phillips & Wallace 1989). Acid carboxypeptidases continue the degradation of polypeptides, and the resultant small peptides and amino acids are then taken up by active transport into the scutellar epithelium, where they are further degraded by aminopeptidases and dipeptidases (Enari & Sopanen 1986). In cereals, the main form in which the nitrogen is transported to the seedling is glutamine, formed by glutamine synthetase (Saarelainen & Mikola 1987).

2.1.1 Proteolytic enzymes

Proteases are divided into two classes: 1) endopeptidases or proteinases, which hydrolyze peptide bonds inside the polypeptide, and 2) exopeptidases, which hydrolyze amino acid bonds either from the carboxy-terminal end (carboxypeptidases) or from the amino-terminal end (aminopeptidases) of the polypeptide. Proteinases are further divided into four subclasses according to the residues at their active sites: aspartic proteinases, cysteine proteinases, serine proteinases and metalloproteinases (Barret 1986). The corresponding mammalian and microbial proteinases were originally called cathepsins.

In germinating barley grain, there exist at least five different serine carboxypeptidases, CPases I-V (Mikola 1983b), which account for most of the exopeptidase activity in the starchy endosperm. CPase I is synthesized in the scutellum, and CPase III in the aleurone layer; both enzymes are secreted into the starchy endosperm. CPase II mRNA is present already in the developing seed, where the activity is localized in the starchy endosperm; thus the enzyme is localized at the site of action (Ranki et al. 1994). Recently, three additional cDNAs for Ser-CPases were cloned from GA-induced barley aleurone layers (Degan et al. 1994).

Barley aminopeptidases are less well-characterized and their function

is less clear than that of carboxypeptidases. Neutral aminopeptidases have been detected only in the living tissues of the barley grain (Mikola & Kolehmainen 1972). Two alkaline aminopeptidases, a leucine aminopeptidase and a dipeptidase, have been partially purified from barley seeds (Sopanen & Mikola 1975, Sopanen 1976). Their activities increase in the course of germination in the scutellum, but stay at the same level in the aleurone layer (Mikola & Kolehmainen 1972). This supports the hypothesis that they participate in the degradation of the small peptides in the scutellum. Aleurain, which was originally predicted to be a cathepsin H - like cysteine proteinase, is now better described as an aminopeptidase with a mildly acidic pH optimum (Holwerda & Rogers 1992). The function of aleurain in barley aleurone layer is not clear.

Wrobel & Jones (1993) have shown by activity gel electrophoresis that five serine proteinases and five metalloproteinases are present in the germinating barley seed. These enzymes were most active either in neutral or slightly alkaline pH. A metalloproteinase is already present during the first day of germination. However, the location and function of these proteinases during germination are not known.

Aspartic and cysteine proteinases are described more detailed in the next two sections.

2.1.1.1 Aspartic proteinases

Aspartic proteinase (E.C. 3.4.23) is a widely distributed enzyme; animal, microbial and retroviral aspartic proteinases are well-known, whereas the corresponding plant enzymes have been less-well studied. Aspartic proteinases have two Asp residues in their active center, an acidic pH optimum and they are inhibited by pepstatin. Both extracellular and intracellular forms have been found (Davies 1990). The molecular weight of plant aspartic proteinases vary from 20 to 89 kD, since monomers, dimers and larger complexes exist. Typical examples of well-known extracellular aspartic proteinases are the gastric enzymes pepsin, gastricsin and chymosin, the last-mentioned being commercially important in the cheese-making industry. The best known intracellular aspartic proteinases are lysosomal cathepsin D and yeast proteinase A (Davies 1990, Szecsi 1992).

Although aspartic proteinases have been purified from many plants, their function is not clear, or at least it seems to be involved in various degradation processes as can be seen from the following examples. The pitchers of the insectivorous plants contain aspartic proteinases analogous to animal gastric enzymes (Amagase 1972, Takahashi et al. 1974, Tökés et al. 1974). Some aspartic proteinases present in species of the family Compositae have been traditionally used as coagulating agents, such as chymosin in cheese-making (Heimgartner et al. 1990, Faro et al. 1992, Tamer 1993). Tomato and tobacco leaves have an extracellular aspartic proteinase, which probably regulates the pathogenesis-related proteins (Rodrigo et al. 1989,

1991). However, plant aspartic proteinases have been purified mostly from dry seeds: for example, from monocots such as wheat (Belozersky et al. 1989, Galleschi & Felicioli 1994), rice (Doi et al. 1980), and barley (Sarkkinen et al. 1992), and from dicots such as hemp (St. Angelo et al. 1969), buckwheat (Belozerskii et al. 1984), rape (D'Hondt et al. 1993) and cocoa (Biehl et al. 1993), and from gymnosperms such as Scots pine (Salmia et al. 1978) and Jack pine (Bourgeois & Malek 1991). Since aspartic proteinase is already present in resting seeds, it has been thought that it could start the hydrolysis of storage proteins before the de novo synthesis of cysteine proteinases (Doi et al. 1980, Mikola 1983a). In some cases, aspartic proteinase is capable of degradating storage proteins; for example, edestinase degrades edestin of hemp seed in vitro, and barley aspartic proteinase degrades 2S albumin of *Arabidopsis* seed in vitro (D'Hondt et al. 1993). Also in wheat flour and cocoa seeds aspartic proteinase enhances the degradation of storage proteins together with carboxypeptidases (Belozersky et al. 1989, Voigt et al. 1994). However, barley aspartic proteinase is not able to hydrolyze globulins or hordeins (Sarkkinen et al. 1992).

Aspartic proteinase is purified to homogeneity from dry barley seeds, and the activity has also been detected in senescing barley leaves (Sarkkinen et al. 1992, Kervinen et al. 1990). Barley aspartic proteinase is composed of two heterodimeric isozymes with molecular weights of 32 and 16 kD (48 kD), and 29 and 11 kD (40 kD). Sequencing data suggests that the larger form is a precursor for the smaller form, which was confirmed by the presence of only one form of mRNA. Barley enzyme (HvAP) is closely related to lysosomal cathepsin D and yeast proteinase A with the exception that HvAP includes a 104 amino acid sequence (Runeberg-Roos et al. 1991), which seems to be typical of plant aspartic proteinases. Besides barley, it is also found in rice and cardoon aspartic proteinases (Hashimoto et al. 1992, Cordeiro et al. 1994). The three-dimensional structure of HvAP closely resembles that of human cathepsin D (Guruprasad et al. 1994). The hydrolytic specificity of HvAP is narrow, as is typical of aspartic proteinases, preferentially cleaving peptide bonds between amino acids with large hydrophobic side chains (Kervinen et al. 1993). Recently, it has been shown that HvAP is capable of cleaving barley prolectin to lectin in vitro (Runeberg-Roos et al. 1994).

2.1.1.2 Cysteine proteinases

Cysteine proteinases (E.C. 3.4.22), which are found in microbes, animals, plants and viruses, are characterized by a Cys residue at their active center and inhibition by thiol-blocking reagents, like E-64 and leupeptin. Cysteine proteinases are divided into three clans or superfamilies, which include about 20 families; almost half of the families are found only in viruses. The best known family is that of papain, named after a cysteine proteinase found in the latex of *Carica papaya* (Rawlings & Barrett 1994). Well-known

lysosomal cysteine proteinases cathepsins B, L and H, which are important in intracellular protein turnover, have a high sequence homology with papain (Agarwal 1990). Generally, papain-like enzymes are synthesized with a signal peptide, which leads them either to the lysosome/vacuole or to the secretory pathway (Rawlings & Barrett 1994). Most plant cysteine proteinases belong to the papain family. Typically, these enzymes have pH optima of 5-7 and their molecular weight is about 25-30 kD, although bigger forms also exist; they exist mostly as monomers (Ryan & Walker-Simmons 1981).

In plants, cysteine proteinases are mostly used for protein degradation in contrast to animals and microbes, which primarily use serine proteinases. It is hypothesized that reason for this is the high level of serine proteinase inhibitors, such as chymotrypsin and trypsin, found in many plants, although serine proteinases also occur in plants (Ryan & Walker-Simmons 1981). However, cysteine proteinase inhibitors have been found in plants as well; for example rice oryzacystatin, which inhibits the major proteinases present in the midguts of the certain herbivorous insects (Liang et al. 1991). Physiological function of proteinase inhibitors in plants is unclear.

Cysteine proteinases have important roles throughout the life cycle of plants, as the following examples show. Some of them are expressed during stress response; for example, a low temperature induced cysteine proteinase gene is found in tomato, and two drought-inducible genes in *Arabidopsis thaliana* (Schaffer & Fischer 1988, Koizumi et al. 1993). A senescence-specific cysteine proteinase, which is purified from unpollinated pea ovaries, is suggested to degrade Rubisco (Cercós & Carbonell 1993). The most extensively studied cysteine proteinases participate in the mobilization of nitrogen. Many of them have roles in different developmental stages of plants, e.g. during seed development and germination; for example, oryzain ß in rice (Watanabe et al. 1991), SH-EP in *Vigna mungo* (Yamauchi et al. 1992), EP-C1 in *Phaseolus vulgaris* (Tanaka et al. 1993), and a cysteine proteinase in *Ricinus communis* (Cornel & Plaxton 1994). Thiol proteases are also involved in the post-translational modification of proteins, for example in the maturation of a storage protein, glycinin, in *Glycine max* (Muramatsu & Fukazawa 1993). Cysteine proteinases take part in the hydrolysis of seed storage proteins, being the major proteases synthesized de novo during germination. They have been characterized in a number of germinating seeds: in legumes, such as *G. max* (Wilson et al. 1988),*V. radiata* (Baumgartner & Chrispeels 1977, Yamaoka et al. 1990), *V. mungo* (Mitsuhashi et al. 1986, Mitsuhashi & Minamikawa 1989), *V. faba* (Yu & Geenwood 1994), *V. sativa* (Becker et al. 1994); in monocots, such as rice (Abe et al. 1987, Watanabe et al. 1991, Shintani et al. 1995), wheat (Cejudo et al. 1992, Capocchi et al. 1994), maize (Barros & Larkins 1990), and barley (see references in the next section).

Several cysteine proteinases have been characterized from germinating barley seed or green malt. Three of them probably represent the same enzyme or they are isozymes, having acid pH optima, essentially same molecular weight (29-30 kD) and the capacity to hydrolyze hordeins (Poulle

& Jones 1988, Phillips & Wallace 1989, Koehler & Ho 1990b). However, only one of them, 30 kD EP-B, is sequenced (Koehler & Ho 1990a). A cysteine proteinase SH-EP of *V. mungo* has 94 % homology with the EP-C1 of *P. vulgaris* and 60 % with barley EP-B (Yamauchi et al. 1992). The 37 kD cysteine proteinase EP-A also digests hordein, but it is expressed later than 30 kD EP-B, suggesting it continues the hydrolysis of soluble polypeptides (Koehler & Ho 1988, 1990b). These barley cysteine proteinases are secretory enzymes, which hydrolyze hordein in the starchy endosperm. However, intracellular transport of these proteases have not been intensively studied. Another barley cysteine proteinase, 32 kD aleurain, is not a secretory enzyme but it is localized in special protein storage vacuoles in the aleurone layer (Holwerda et al. 1990). Aleurain also exhibits aminopeptidase activity, resembling mammalian cathepsin H (Holwerda & Rogers 1992).

2.1.2 Glutamine synthetase

Glutamine synthetase (GS; E.C. 6.3.1.2.) is a universal enzyme, which is present in all prokaryotes and eukaryotes. The evolutionary history of the GS genes shows that they have an old common ancestor, which existed before the divergence of prokaryotes and eukaryotes over one thousand million years ago (Kumada et al. 1993). GS plays a central role in nitrogen assimilation: free ammonia, which is highly toxic to cells, is rapidly assimilated to organic form by GS in the following reaction:

$$\text{L-Glutamate} + \text{ATP} + NH_3 \rightarrow \text{L-Glutamine} + \text{ADP} + P_i + H_2O.$$

This reaction belongs to the GS/GOGAT or glutamate synthase cycle, where glutamate synthase (GOGAT) converts glutamine and 2-oxoglutarate to two molecules of glutamate, and GS assimilates ammonia to glutamate to form glutamine (Tempest et al. 1970, Lea & Miflin 1974).

In higher plants, GS generally occurs as an octamer with a molecular weight of about 360 000 - 450 000 daltons (McNally & Hirel 1983), although a tetrameric isoform is active in sugarbeet leaves (Mäck & Tischner 1990). Although GS is distributed throughout the plant, its activity differs from one tissue to another (Stewart & Rhodes 1978). Subunit composition of GS also varies between tissues and is dependent on developmental stage (Bennet & Cullimore 1989, Edwards et al. 1990). In higher plants, GS occurs in two basic forms: cytosolic GS_1 and plastidic GS_2. Plants can be classified into four groups according to GS pattern: 1) cytosolic GS only, 2) chloroplastic GS only, 3) cytosolic GS as a minor component, and chloroplastic GS as a dominant component, and 4) equal amounts of cytosolic and chloroplastic GS (McNally et al. 1983). In nitrogen-fixing root nodules GS can form up to 2 % of the total soluble protein (McParland et al. 1976).

GS is not the only nitrogen assimilating enzyme; asparagine

synthetase assimilates ammonia to form asparagine, which has a better N:C ratio than glutamine. Asparagine synthesis is preferred in dark-grown plants, where photosynthetic carbon is a limiting factor (McGrath & Coruzzi 1991). Glutamate dehydrogenase (GDH) was earlier thought to be the major nitrogen assimilating enzyme in higher plants until the GS/GOGAT cycle was found. The role of GDH in plants is still unclear, since it is not capable of replacing GS, when GS is inhibited (Lacuesta et al. 1990, Sechley et al. 1992). Remobilization and reassimilation of nitrogen take place at certain times during the plant life cycle. Glutamine and asparagine synthesis are strongly induced, when nitrogen transport is needed from one organ to another, particularly in germinating seeds and senescing leaves. In addition to primary sources of ammonia, e.g. nitrate reduction or direct uptake from the soil, and nitrogen fixation from the air, ammonia is continuosly formed during various metabolic processes. Nitrogen atoms are probably recycled several times inside the plant (Lea et al. 1990).

In barley, light-grown shoots contain both cytosolic and chloroplastic GS activity, whereas roots, seeds and etiolated shoots have only cytosolic GS activity (Mann et al. 1979). During germination of barley, GS activity of the scutellum is high. Glutamine acts as a transport form of nitrogen, whereas asparagine is used for short-term storage (Saarelainen & Mikola 1987). Recently, it was shown that the expression of barley GS isoforms is induced rather by developmental events than the availability of different nitrogen sources. However, the activity of some, but not all, isoforms was affected by changes in ammonia levels (Mäck 1995).

2.2 Stress-induced gene expression

2.2.1 Abscisic acid

In higher plants, intracellular communication is mediated by hormones, which can be roughly classified into two groups: growth stimulators including auxins, gibberellins (GA) and cytokinins, and growth inhibitors suc as abscisic acid (ABA) and ethylene (Taiz & Zeiger 1991). During the plant life cycle, ABA is involved in many physiological events such as seed maturation, seed and bud dormancy, abscission and senescence, but it also takes part in the conversion of environmental signals to a stress response. Stomatal closure is the best characterized ABA-mediated response to dehydration (Chandler & Robertson 1994). The concentration of ABA increases when cellular dehydration occurs as a consequence of a natural desiccation during seed maturation or as a stress response caused by drought, high salt, cold or heat. ABA also mediates local and systemic wound responses (Skriver & Mundy 1990). It has been suggested that ABA is a general stress hormone in plants. However, the existence of ABA-independent pathways mediating stress response has also been

demonstrated (Giraudat et al. 1994). Recently, it has been found that among lower and higher plants ABA and stress-inducible responses are conserved (Knight et al. 1995).

ABA is a small organic acid which is synthesized in the cytosol from mevalonic acid via the carotenoid pathway (Parry & Horgan 1991). Compartmental changes in pH values cause rapid redistribution of ABA in plant tissues (Slovik & Hartung 1992). Although no receptor protein for ABA has yet been found, there is strong evidence that the reception site of both ABA and GA is situated at the external face of the plasma membrane (Anderson et al. 1994, Gilroy & Jones 1994). The signal transduction route involving ABA remains rather unclear, but it is apparent that ABA mediates environmental signals by regulating intracellular calcium levels (Gilroy & Jones 1992).

2.2.2 ABA- and stress-inducible LEA proteins

A special group of proteins was first found to be expressed during the late state of maturation in cotton embryos (Dure et al. 1981). These proteins were called "late embryogenesis abundant" (LEA), according to the stage of their expression. Typical of LEA proteins is a rapid disappearance after the beginning of germination, although LEA mRNAs are the principal mRNAs left in the mature seed. LEA proteins are ABA-inducible; they could be induced earlier during development or later during germination by exogenous ABA (Galau et al. 1986). LEA proteins are probably universal in higher plants, since LEA homologs have been found in the mature seeds of a number of monocots and dicots (Dure et al. 1989). Interestingly, LEA-like proteins have also been detected in vegetative tissues subjected to desiccation or treated with ABA, analogous to desiccation during seed maturation (Skriver & Mundy 1990).

Sequence homologs have been found for four of the six different LEA protein groups of cotton called LEA1 (cotton D-19), LEA2 (D-11), LEA3 (D-7) and LEA4 (D-113; Dure 1993b). The nomenclature is variable: in addition to LEA, there are also rab (responsive to ABA), dhn (dehydration-inducible) and Em (early methionine labelled) proteins, all of which can be classified into LEA families. The precise physiological function of these proteins is still unclear, although it is known that they are involved in desiccation response.

Typically, LEA proteins are heat-stable and highly hydrophilic. It is common that regions of high homology are surrounded by non-homological sequences. However, the **LEA1** group is an exception, since members of this family are highly homologous throughout the sequences. LEA1 proteins have an amorphous structure, which is predicted to enable the water-binding capacity of these molecules (Dure 1993b). The best known LEA1 protein is wheat Em, which is the most abundant cytosolic polypeptide in the mature wheat embryo and also ABA- and drought-

inducible (Morris et al. 1990). The **LEA2** group, also known as dehydrins, contains at least 30 members from 13 species with deduced molecular weights of 14 to 85 kD. Common to all of them is a 15-mer motif that occurs once or twice in the C-terminal end (Close et al. 1993). Many, but not all dehydrins, contain a contiguous sequence of serine residues, which form a putative phosphorylation site (Vilardell et al. 1990). An example of this group could be maize dehydrin, RAB17, which may have a role in regulating the transport of nuclear proteins (Close et al. 1989, Goday et al. 1994).

The **LEA3** group is characterized by a tandemly repeated 11-mer comprising about 50 % of the molecule, whereas the rest of the polypeptide, mostly at the C-terminal end, is non-homologous (Dure 1993b). Variation in the number of repeats causes the wide range of molecular weights, from 15 to 60 kD. The properties of the 11-mer repeats suggest that they could form an amphiphatic α-helical structure, which probably is important for the function of the proteins (Dure et al. 1989). It is suggested that this special structure enables the sequestration of ions during desiccation to prevent precipitation (Dure 1993a). The **LEA4** group proteins are predicted to have a long α-helical structure of high homology in their N-terminal end, the rest of the molecules being non-homologous. This structure could possibly replace water molecules, and thus protect membrane structures (Dure 1993b). In developing cotton, a LEA4 protein, D-113, is evenly distributed in the cytosol of the embryonic tissues (Roberts et al. 1993).

2.2.3 HVA1, a barley LEA3 protein

Lin and Ho (1986) found a dozen ABA-inducible polypeptides in isolated barley aleurone layers. One of them, HVA1, appeared to be encoded by a single copy gene, which is located on barley chromosome 5, and belongs to the *lea3* group (Straub et al. 1994). As is typical of LEA3 proteins, about 50 % of HVA1 protein is composed of tandemly repeated 11-mers. The repetative sequence is a palindrome T-E-A-A-K-Q-K-A-A-E-T, which is repeated nine times (Hong et al. 1988). Based on computer modelling, this structure is predicted to form an amphiphatic α-helix (Dure et al. 1989). However, all the tandem repeats are not perfect; interruptions may represent so-called hinge regions in the potential three-dimensional structure (Dure 1993a).

Barley HVA1 is 95 % similar to the wheat pMA2005 protein at the amino acid level (Curry et al. 1991). They are closely related to carrot Dc3 (Seffens et al. 1990) and cotton D-7 proteins (Baker et al. 1988). Genes for these LEA3 proteins have characteristically one short exon, an intron and a long exon. In the promoter region of the *HVA1* gene, four ABA-responsive elements have been found. However, mutations in two of them, the C-box and pABRE1 region, had no influence on the ABA induction of the gene. Mutations to pABRE2 or pABRE3 affected the absolute level of the gene expression, but ABA inducibility nonetheless remained at a high level

(Straub et al. 1994). On the other hand, the two elements, pABRE2 and pABRE3, are necessary for the ABA induction of another stress-regulated barley gene, *HVA22* (Shen et al. 1993).

The expression of HVA1 is started in the developing barley seed around day 25 post-anthesis. Its level remains high in the mature seed constituting about 1 % of the total soluble protein in the embryo. The HVA1 mRNA disappears rapidly during imbibition but the protein is still detected after 2 days of imbibition. HVA1 is also induced in vegetative organs by ABA treatment as well as drought, salinity, cold and heat stress, but not by anaerobic treatment. However, HVA1-induction is stronger in 3-day-old than in 7-day-old seedlings in terms of developmental regulation (Hong et al. 1992).

Curry et al. (1993) suggested a subgroup (II) for the LEA3 group on the basis of sequence differences; carrot Dc8, soybean pGmPM2 and wheat pMA1949 are different from a group containing HVA1, although they belong clearly to the LEA3 group (Table 1). Cotton D-29, which is also classified as a member of the LEA3 group, has a remote homology to dsp21, a desiccation related protein from a resurrection plant, *Craterostigma plantagineum* (Piatkowski et al. 1990).

TABLE 1 Members of the LEA 3 group.

Name	Source	Repeats	Mw	Reference
LEA3 (I)				
HVA1	barley	9	22	Hong et al. 1988
pMA2005	wheat	10	23	Curry et al. 1991
MLG3	maize	11	23	White & Rivin 1995
Dc3	carrot		17	Seffens et al. 1990
D-7	cotton	5	15	Baker et al. 1988
pLEA76	*B. napus*	13	30	Harada et al. 1989
LEA3 (II)				
Dc8	carrot	17	60	Franz et al. 1989
pGmPM2	soybean		50	Hsing et al. 1992
pMA1949	wheat	11	33	Curry et al. 1993
LEA3 -like				
D-29	cotton			Baker et al. 1988
dsp21/pcC3-06	*C. plantagineum*		22	Piatkowski et al. 1990

2.3 Protein transport in plant cells

2.3.1 Secretory pathway

Metabolic processes in eukaryotic cells are compartmentalized into different organelles isolated by membranes. Except for the minor part of the proteins synthesized in chloroplasts and mitochondria, protein synthesis takes place in the cytoplasm or on the membrane-bound ribosomes (Bednarek & Raikhel 1992). The endomembrane system of plant cells, which is also called the secretory pathway, includes the rough and smooth endoplasmic reticulum (ER), Golgi complex, *trans*-Golgi network (TGN), vacuole, tonoplast and the plasma membrane (Fig. 2). These organelles are connected to each other by numerous vesicles that mediate intracellular transportation between them. The nuclear membrane is also considered part of the system. Chloroplasts and mitochondria as well as peroxisomes/glyoxysomes are excluded from the endomembrane system, since proteins targeted to these organelles have positive sorting information to direct the newly-synthesized protein to its destination straight from the cytosol (Harris 1986, Chrispeels 1991, Bednarek & Raikhel 1992, Vitale et al. 1993). The endomembrane system was first identified during a study of the targeting of secretory proteins to the cell surface, which explains the name secretory pathway (Palade 1975). In fact, the secretory pathway also delivers vacuolar and tonoplast proteins to their targets (Vitale et al. 1993).

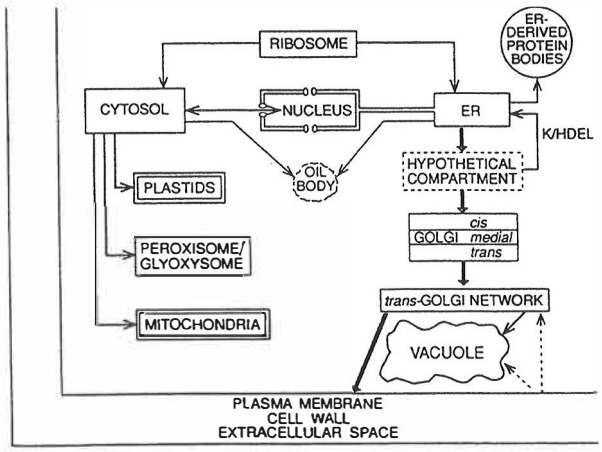

FIGURE 2 The endomembrane system/secretory pathway of a plant cell (Bednarek & Raikhel 1992). The arrows show the route of the newly-synthesized proteins to different organelles inside the cell. The thick black arrows depict bulk flow from the ER to the exterior of the cell.

The sorting and targeting of nuclear-encoded proteins in plant cells to the right destination out of about 30 different possibilities, e.g. various organelles, the membranes surrounding them and the extracellular space, is currently under intensive research. Each protein has to contain the necessary targeting information in its primary amino acid sequence. Positive sorting signals direct proteins along the branched pathway. Targeting signals can initiate or stop translocation of a protein through the membrane, or retain a protein in a certain compartment (Chrispeels 1991, Bednarek & Raikhel 1992). The so-called default pathway is thought to be a bulk flow along which a protein is transported non-selectively for secretion unless it is retained somewhere (Denecke et al. 1990).

To enter the secretory pathway a polypeptide is targeted co-translationally into the ER by an amino-terminal signal peptide, which can be from 13 to 50 amino acids long and is characterized by a strech of hydrophobic amino acids. The ER is the site for co-translational and post-translational modifications such as cleavage of the signal peptide, disulfide bond formation, N-linked glycosylation, oligomerization and proper conformation of the nascent protein. Proteins leave the ER by bulk flow unless they are misfolded or retained by a specific ER-retention sorting signal (Vitale et al. 1993). After the ER, the proteins are directed to the forming or *cis*-face of the Golgi complex proceeding through the medial and *trans*-Golgi towards the *trans*-Golgi network (TGN), also called the partially coated reticulum. So far, the retention signals for the plant Golgi complex are not known. Possibly, some proteins leave the Golgi before the TGN. However, the bulk flow proteins are presumed to continue their journey from the TGN to the plasma membrane, where they are retained, or secreted either into the cell wall or extracellular space. The rest of the proteins leaving the TGN are probably targeted to the vacuole or the vacuolar membrane, tonoplast (Chrispeels 1991, Bednarek & Raikhel 1992).

2.3.2 Vacuolar targeting

The plant vacuole is a multi-purpose organelle, the role of which depends on the developmental stage, organ or cell type of the plant (Marty et al. 1980). Vacuoles are often compared to mammalian lysosomes, since they have an acidic content and contain a set of hydrolytic enzymes like lysosomes. In addition to degradative purposes, vacuoles can store inorganic ions, organic acids, sugars and storage as well defense proteins. Turgor regulation by space filling is also an important function of the vacuole (Wink 1993).

Although the animal, yeast and plant secretory systems show high similarity in many details, the major difference is in lysosomal/vacuolar targeting (Chrispeels 1991). In mammalian cells, newly-synthesized lysosomal proteins are recognized by the mannose-6-phosphate receptor (Sahagian et al. 1981). In plant cells, glycans do not participate in the

targeting of vacuolar proteins, but the sorting information is included in the amino acid sequence. Three differently positioned signals for vacuolar targeting have been found, but no common sorting sequence exists (Gal & Raikhel 1993).

A carboxy-terminal propeptide directs a barley prolectin and a chitinase to vacuoles in transgenic tobacco (Wilkins et al. 1990, Neuhaus et al. 1991). Barley aleurain and sweet potato sporamin have an amino-terminal targeting sequence like that found in yeast vacuolar proteins. Aleurain and sporamin have been correctly targeted to the vacuole in transgenic tobacco, which shows that different targeting systems can exist in the same cell (Holwerda et al. 1992, Matsuoka & Nakamura 1992). A third type of vacuolar targeting signal belongs to the mature protein, i.e. there is no cleavable propeptide other than the signal peptide. Several proteins have this type of signal, for example potato patatin and bean phytohemagglutinin (Sonnewald et al. 1991, von Shaewen & Chrispeels 1992).

In developing cereal seeds, storage proteins accumulate in the protein bodies, also called protein storage vacuoles, which represent a special type of plant vacuoles. Protein bodies can form by several different ways, which are not yet completely understood. First, rice glutelins (globulins) follow the normal secretory pathway to reach the protein storage vacuole (Krishnan et al. 1992). Second, within the lumen of the ER, prolamins of maize and rice aggregate into protein bodies, which remain attached to the ER (Krishnan et al. 1986, Shotwell & Larkins 1988). Third, in wheat, the ER-derived protein bodies containing gliadins (prolamins) are translocated into the vacuole by an autophagy-like process (Levanony et al. 1992). Fourth, maize zeins (prolamins) do not have the ER-retention signal, although they are retained in the ER. It has been shown that the structural properties of zeins contain the targeting information for ER retention and protein body formation (Geli et al. 1994). Generally, storage proteins have the targeting signal for the ER, the signal peptide. However, some proteins have no signal peptide but they are localized in vacuoles, like a barley chymotrypsin inhibitor, CI-2 (Rasmussen et al. 1990).

3 AIM OF THE STUDY

Cereals are a valuable source of proteins for the nutrition of human beings and domestic animals. In combination with legumes, the nutritional value can reach a level where all essential amino acids are present. However, in undeveloped countries, both sources are not always available. If nutritional quality is to be improved, then more information is needed about the seed proteins, their accumulation and remobilization processes. Insufficient knowledge hinders otherwise successful breeding processes, which could be facilitated by modern transformation techniques.

The aim of the present thesis was to compare the expression and intracellular location of two acid endoproteases, aspartic and cysteine proteinases, during barley seed germination. The occurrence of glutamine synthetase was investigated to understand better the flow of nitrogen to the growing seedling after the hydrolysis of storage proteins. Tissue distribution and intracellular localization of a barley stress protein, HVA1, was compared during germination and desiccation stress.

The specific aims of this study are described below.

1. Tissue distribution and subcellular localization of aspartic and cysteine proteinases in the intact seed, and the role of the scutellum and the aleurone layer as secretory tissues during progressive germination.

2. The occurrence and intracellular localization of glutamine synthetase during germination of barley seed.

3. The expression and intracellular localization of a stress-induced protein, HVA1, during germination of barley seed and under stress conditions.

4 SUMMARY OF MATERIALS AND METHODS

Materials and methods are described in detail in the original articles (I-IV).

4.1 Plant material

Barley (*Hordeum vulgare*, cv. Himalaya) seeds from the harvests of 1985 and 1988 were obtained from the Agronomy Club, Washington State University, Pullman, WA, USA. The seeds were surface-sterilized in 1% $NaOCl_4$ for 20 min, washed in sterile dH_2O, incubated in 10 mM HCl for 10 min, and washed with dH_2O several times. The seeds were then germinated aseptically on 0.75% water agar up to 7 days at 20°C in the dark.

Stress and ABA treatments of germinated seeds were performed as in Hong et al. (1992). For cold stress, 1-d and 3-d germinated seeds were kept on ice at +4°C for 4 days. For drought stress, 3-d germinated seeds were dehydrated to 85 % of original fresh weight, and thereafter kept for 24 h in a moist chamber. For ABA treatment, 3-d germinated seeds were incubated with 100 µM ABA.

4.2 Antibodies

Aspartic proteinase antiserum and a pre-immune serum (I) were kindly provided by Dr. Leena Mikola (University of Jyväskylä, Finland) and Dr. Jukka Kervinen (University of Helsinki, Finland). An antiserum against a 30 kDa cysteine proteinase (I) was the generous gift of Prof. Berne L. Jones (University of Wisconsin, Madison, WI). Polyclonal antibodies against 30 kD cysteine endoprotease B (II) and a ß-galactosidase-HVA1 fusion protein (IV) were kindly provided by Prof. David Ho (Washington University, St. Louis,

MO). Polyclonal antiserum against glutamine synthetase (III) was the kind gift of Dr. Brian G. Forde (Rothamsted Research Station, Harpenden, UK). Non-immune rabbit serum was obtained from Zymed, San Francisco, CA, USA (I-IV). All the polyclonal antibodies were raised in rabbit, and the IgG fractions were separated using protein G-Sepharose (MabTrap G, Pharmacia, Uppsala, Sweden).

4.3 Biochemical methods

4.3.1 Protein extraction

For immunoblotting, proteins were extracted from germinated and/or stressed whole seeds or separated seed parts as earlier described (I-IV).

4.3.2 Immunoblotting

Proteins were separated (I-IV) by standard SDS-polyacrylamide gel electrophoresis in reducing conditions (Laemmli 1970), and the subsequent immunoblotting performed according to Towbin et al. (1979). The blotting membrane was either nitrocellulose (II, III) or PVDF (I, IV). After primary antibody incubation antigens were detected by alkaline phosphatase secondary conjugate by NBT/BCIP method (Sambrook et al. 1989).

4.4 Microscopical methods

4.4.1 Immunohistochemistry

Immunolocalization was first performed at tissue level in all studies. Imbibed, germinated and/or stressed barley seeds were fixed with 4 % PFA in PBS overnight at +4°C, dehydrated in ethanol and infiltrated in paraffin. Deparaffinized sections of 8-15 µm were incubated with primary antibodies and the immunolabelling was detected either by silver enhanced gold labelling (I-III) or fuchsin staining after alkaline phosphatase secondary conjugate (IV).

4.4.2 *In situ* hybridization

Deparaffinized sections (II) were treated with proteinase K solution to enhance the probe penetration, prehybridized for one hour at 40°C in

hybridization buffer, and hybridized with ^{35}S-labelled cDNA probe for EP-B mRNA for 16 h at 42°C, washed, dehydrated, air dried and laid on X-ray film for 3 days. Slides were coated with Kodak NBT2 emulsion, exposed for 2 weeks at 4°C, and finally developed and fixed.

4.4.3 Immuno-electron microscopy

Subcellular localization studies were performed either on ultrathin cryosections (I, III) or LR White resin sections (IV). For cryosectioning, pieces of scutellar tissue from germinated seeds were fixed in a mixture of 4 % paraformaldehyde and 1 % glutaraldehyde in PBS for 1 h, incubated in 20 % polyvinylpyrrolidone containing 1.84 M sucrose in slow rotation at 4°C overnight, and then frozen in liquid nitrogen according to Tokuyasu (1989). Ultrathin sections were cut at -90°C with a Reichert Ultracut FC4 ultramicrotome equipped with cryo-attachment.

For LR White sectioning, pieces of tissue from stressed and unstressed barley seeds were fixed with 4 % paraformaldehyde and 0.25% glutaraldehyde in PBS for 2 h at room temperature by continuous mixing. The samples were then dehydrated, gradually infiltrated in LR White resin (EMS, Fort Washington, PA, USA) and polymerized at 58°C for 48 h. Subsequent immunolabelling of both LR White and cryosections was performed using the protein A-gold method.

4.4.4 Quantitation of gold label

Density of immunogold labelling (IV) was determined on the surface area by means of the point-counting method (Griffiths 1993). Gold particles of 10 nm were counted on the cytoplasm and PSVs of the micrographs taken from the scutellum of the imbibed seed and cold stressed aleurone layer of the 1-d germinated seed treated either with HVA1 antiserum or non-immune serum. Sections were cut from three LR White blocks per sample. Gold particles were calculated from 14 to 23 micrographs per treatment printed at the final magnification of 30,000. Statistical analyses were performed by t-test, or when the assumptions of the t-test were not fulfilled, by the Mann-Whitney U-test.

5 REVIEW OF THE RESULTS

5.1 Expression sites of aspartic proteinase (I)

Aspartic proteinase content is high in the mature seed, and increases only a little during germination (Törmäkangas et al. 1994). Barley aspartic proteinase is translated as a proenzyme which is processed into two forms of the active enzyme, first into the 48 kD and then into the 40 kD enzyme. The 48 kD form is composed of 32 and 16 kD subunits, which are processed into 29 and 11 kD forms, respectively (Runeberg-Roos et al. 1991). In the present study, the aspartic proteinase was found to be present in all the living tissues of the grain, including shoots and roots, throughout germination (I: Fig. 1a, b, e). In addition to the subunits, the antiserum also detected a 48 kD unprocessed form, and a 43 kD polypeptide that was probably the enzyme after modification but before hydrolysis into two subunits (Sarkkinen et al. 1992). The high molecular mass form (32 + 16 kD) was detected in all the living tissues, while the low molecular mass form was found only in the aleurone layer and embryo extracts. The low molecular mass form of aspartic proteinase did not show notable change, whereas the high molecular mass form increased about two-fold during germination.

Aspartic proteinase was present in the parenchymal cells of the scutellum and the proximal aleurone layer of ungerminated seed (I: Fig. 2a, b). During germination, the enzyme also became present in the epithelial cell layer and gradually appeared throughout the whole aleurone layer. No secretion of the aspartic proteinase into the starchy endosperm was observed at any stage (I: Fig. 2c, d, f, h). Consistent with the immunoblotting results, aspartic proteinase was found in all the living tissues of the grain, including the shoots and roots (I: Fig. 4a, b). Subcellulary, aspartic proteinase first occurred in the protein bodies and, later, in the large vacuoles of the scutellum. No label was seen in either the cytoplasm or in the other organelles (I: Fig. 5).

5.2 Expression sites of 30 kD cysteine proteinases (I, II)

The strong synthesis of sulfhydryl enzymes is started after the beginning of germination. In three different papers, a 29-30 kD cysteine proteinase is reported to be one of the main proteases synthesized (Poulle & Jones 1988, Phillips & Wallace 1989, Kohler & Ho 1990b). It is very likely that the 30 kD cysteine proteinase from green malt and the 30 kD cysteine endoprotease B (EP-B) from isolated aleurone layers either represent the same enzyme or are at least isoenzymes, since they behaved in a similar manner in the localization studies. However, sequence data is only available for EP-B (Koehler & Ho 1990a), so that final confirmation is not possible.

Both 30 kD cysteine proteinases were not expressed until after the beginning of germination (I, II). A small amount of the enzyme was detected after the first day, which increased rapidly with progressive germination, indicating a high rate of de novo synthesis (I: Fig. 1c; II: Fig. 1). The 30 kD cysteine proteinase was found in all tissues of the grain throughout germination except in shoots or roots (I: Fig. 1d, e). Bands (42.5, 38, 32 kD) migrating more slowly than the 30 kD enzyme were likely to correspond to the putative preproenzyme or the proenzyme and processing intermediates formed during processing, if compared to the processing of EP-B (Koehler and Ho 1990a). The putative preproenzyme of 42.5 kD was found only in the aleurone layer, but the possible intermediate forms (32, 38 kD) were secreted into the starchy endosperm, as Koehler and Ho (1990a) also discovered with EP-B.

The 30 kD cysteine proteinase as well as EP-B first appeared in the scutellum during the first day of germination. Both were strongly localized in the scutellar epithelium, from where secretion into the starchy endosperm began during the first day of germination (I: Fig. 3a, c; II: Fig. 3a, b, c). Later during germination, labelling in the scutellum decreased (I: Fig. 3e; II: Fig. 3e). In the course of germination, the localization of the enzyme proceeded along the aleurone layer towards the distal end of the grain and, at the same time, enzyme secretion into the starchy endosperm was observed (I: Fig. 3b, d, f; II: Fig. 3d, h, i, j). The 30 kD cysteine proteinase did not occur in shoots or roots, consistent with immunoblotting (I: Fig. 4c, d). At the subcellular level, the 30 kD cysteine proteinase was observed in the Golgi complex and inside the membrane-coated vesicles, which were considered as putative secretory vesicles that occurred near the plasma membrane (I: Fig. 6).

5.3 Occurrence of glutamine synthetase (III)

The activity of glutamine synthetase (GS) increases about 10-fold during germination of barley seed (Saarelainen & Mikola 1987). GS corresponds to the synthesis of glutamine, the main form in which nitrogen is transported to the growing seedling. In the present study, the occurrence of GS during germination of barley grain was studied using an antiserum that recognizes both cytosolic and chloroplastic GS isozymes in a number of plants (Cullimore & Miflin 1984). At the beginning of germination, one GS polypeptide of 42 kD (named A) was present in the embryo (III: Fig. 1a). Later, another polypeptide of 40 kD (B) appeared, the amount of which increased 5-fold during germination, whereas the amount of polypeptide A remained unchanged. In the aleurone layer, roots and etiolated shoots, polypeptide A was also detected first, and B appeared later during germination, although weakly (III: Fig. 1b, c, d). No more than two polypeptides were revealed in the scutellum by immunoblotting a two-dimensional electrophoresis gel (III: Fig. 1e).

Immunolight microscopy showed GS to be present in the scutellum and the aleurone layer but not in the starchy endosperm of the germinating grain (III: Fig. 2a, b, c). The intensity of labelling correlated with the GS activity (Saarelainen & Mikola 1987), peaking at the fourth day (III: Fig. 2c). Subcellulary, GS was detected only in the cytoplasm of scutellar cells (III: Fig. 3a, b).

5.4 Occurrence of HVA1 protein (IV)

Barley HVA1 protein belongs to the LEA3 proteins, which are expressed during the late stage of seed development, and also as a response to various stresses (Hong et al. 1988, 1992). In the present study, we compared the tissue distribution and subcellular localization of the HVA1 protein in germinated and stressed barley seed.

By immunoblotting it was shown that the amount of HVA1 protein declined in the course of germination, but did not disappear completely for at least 7 days (IV: Fig. 1a). HVA1 protein was present in shoots from 3 to 7 days but disappeared from roots when germination continued over 5 days (IV: Fig. 1b). The expression of HVA1 protein was clearly induced by ABA or cold/drought stress treatment after three days of seed germination (IV: Fig. 1a, c). HVA1 is normally seen as a 27 kD band in SDS-PAGE, probably due to its high lysine content, since the cDNA sequence predicts it to be 22 kD (Hong et al. 1988). In the course of germination, a second band of 23 kD, which was also detected in the ABA treated and stressed samples, appeared in the aleurone layer (IV: Fig. 1a, b). We showed by in vitro hydrolysis studies that the 23 kD band was not a degradation product (IV: Fig. 1a).

By immunolight microscopy, HVA1 was detected in all the living tissues of the mature barley seed (IV: Fig. 2a-e). After 3 days of germination, HVA1 labelling was hardly detectable in the aleurone layer. When the 3-day germinated seed was subjected to cold stress, a higher level of HVA1 expression was observed in the aleurone tissue (IV: Fig. 2f-h). Subcellulary, HVA1 was found both in the protein storage vacuoles (PSV) and the cytoplasm in the scutellum of the mature seed, as shown by protein A-gold labelling (IV: Fig. 3a, b). The density of labelling was three times higher in the PSVs than in the cytoplasm (IV: Table 1). In the aleurone cells of the 1-day germinated and cold-stressed barley seed, HVA1 was also found in the PSVs and cytoplasm, but the density of labelling was nine times higher in the PSVs than in the cytoplasm (IV: Fig. 3c, d). Although labelling was quite weak in the cytoplasm, it differed significantly from the controls treated with non-immune serum (IV: Fig. 4, Table 1). In the aleurone cells of 3-day germinated and cold stressed seeds, HVA1 was found in vacuoles, and again, in small amounts in the cytoplasm (IV: Fig. e, f).

To confirm the electron microscopy studies, PSVs were isolated from mature aleurone layers by centrifugation in non-aqueous conditions. As expected, HVA1 protein was mainly localized in the PSV fraction and to a lesser extent in the supernatant, which represented the cytoplasmic compartment (IV: Fig. 5). Aspartic proteinase antiserum was used as a control showing that the supernatant was no contaminated with PSVs.

6 DISCUSSION

The metabolism of eukaryotic cells is compartmentalized into different organelles, which are isolated by membranes. Protein trafficking between these organelles is based on various targeting signals, which are included in the primary amino acid sequence, and their interaction with specific receptors. A lot of research has been focused on intracellular protein traffic, but the details of targeting and sorting are still poorly understood. The basic mechanism seems to be similar in yeast, animal and plant cells, although there are also many differences. In the present study, the function of various proteins expressed during germination was clarified by investigating their tissue distribution and subcellular localization.

6.1 The function of aspartic and cysteine proteinases in germinating barley seed

Variation in the tissue distribution and the timing of expression confirmed that barley aspartic proteinase and 30 kD cysteine proteinase play different roles in germinating barley seed. The broad expression of aspartic proteinase in all the living tissues of the seed during germination suggests a role of the house-keeping enzyme. The expression of 30 kD cysteine proteinase started after the beginning of germination and it was first limited in the scutellar epithelium and later, in the aleurone layer. Both tissues secreted cysteine proteinase into the non-living starchy endosperm, suggesting a specific role in the hydrolysis of the main storage proteins.

Barley aspartic proteinase is translated with a putative N-terminal signal sequence (Runeberg-Roos et al. 1991), indicating that the protein is targeted cotranslationally to the ER, the initial point of the secretory route. Since barley aspartic proteinase resembles mammalian cathepsin D and yeast proteinase A, which are intracellular proteinases located in the

lysosome or vacuole, vacuolar localization has been expected. The proenzyme actually has a putative N-terminal vacuolar targeting sequence similar to barley aleurain and sweet potato sporamin, and protein has been localized in the vacuoles of barley leaves and root tips (Runeberg-Roos et al. 1994). Its acidic pH optimum also supports the hypothesis of vacuolar localization. However, the subcellular localization of the enzyme in the seed had not been confirmed. In the present study, barley aspartic proteinase was localized in the scutellar protein bodies of the mature seed, and later, during progressive germination, in the vacuole, as expected on the basis of the sequence data.

According to the immunoblotting, the proforms were present throughout germination, which suggests that the enzyme is activated only when needed. The 29 + 11 kD form of the enzyme did not change notably during germination, but the 32 + 16 kD form increased about two-fold. The 29 + 11 kD form was found only in the embryo and aleurone layer, whereas the 32 + 16 kD form was found also in shoot and root extracts. These results suggest that the two forms of the enzyme could have different roles in the germinating seed, since they differ in amount and tissue distribution.

The function of aspartic proteinase in plant cells is unclear. Localization in protein bodies/vacuoles of the living tissues of the seed indicates the possibility of hydrolyzing storage proteins. However, barley aspartic proteinase is not able to hydrolyze globulins or hordothionins in vitro (Sarkkinen et al. 1992), or hordeins in activity gels (Wrobel & Jones 1992). On the other hand, barley aspartic proteinase is able to hydrolyze 2S albumin, the main storage protein of *Arabidopsis* seed, in vitro (D'Hondt et al. 1993). The involvement of aspartic proteinase in the hydrolysis of storage proteins has not been studied in vivo. On the basis of its narrow hydrolytic specificity, it is suggested that barley aspartic proteinase acts more like a regulatory enzyme (Kervinen et al. 1993). This enzyme is capable of hydrolyzing barley prolectin into lectin in vitro. Aspartic proteinase and barley prolectin are colocalized in the vacuoles of barley leaves and root tips (Runeberg-Roos et al. 1994). Barley lectin is activated as part of the defense reaction induced as a result of pathogen attack. Similarly, the putative hydrolysis of a barley stress-inducible protein, HVA1, in protein storage vacuoles, could also be involved in the stress response reaction as discussed later.

The primary translation product of many proteases is an inactive precursor form (Neurath 1984), where a large pro-region prevents the activation of protease (Baker et al. 1993). In vivo processing of the rat hepatocyte preprocathepsin L involves the cotranslational cleavage of the signal peptide, glycosylation, and transportation via the Golgi complex to the lysosome, where the prosequence is cleaved and the enzyme maturation completed (Nishimura et al. 1988). Papain is synthesized as a 40 kD preproprotein, which maturates due to the change in the electrostatic charge in the conserved motif of the pro-region (Vernet et al. 1995). Barley 30 kD EP-B is synthesized as a preproEP-B with a molecular mass of 42.5 kD. Like

barley aspartic proteinase, EP-B is synthesized with a putative signal peptide (Mikkonen et al., unpublished results), which was highly expected, since EP-B as well the 29 kD and 30 kD cysteine proteinases from green malt are secretory proteinases (Koehler & Ho 1990b, Poulle & Jones 1988, Phillips & Wallace 1989). The maturation site of the EP-B or 30 kD cysteine proteinase is unclear, since processing intermediates of 38 and 32 kD and the mature form of the enzyme are found both in the synthesizing tissues and in the starchy endosperm (Koehler & Ho 1990a). It is suggested that barley CPase I maturates after secretion from the scutellum into the starchy endosperm (Doan & Fincher 1988). On the other hand, another barley cysteine proteinase, the 37 kD EP-A, is secreted in a mature form (Koehler & Ho 1990a). Maturation of α-amylases also occurs in the aleurone layer before secretion via the cleavage of the C-terminal propeptide by a carboxypeptidase (Søgaard et al. 1991).

The C-terminal KDEL or HDEL sequence is known as an ER retention signal in animal, yeast and plant cells. Thus, it is surprising that some secretory proteins contain the ER retention signal. A cysteine endopeptidase in *Vigna mungo*, 33 kD SH-EP, has a C-terminal KDEL (Yamauchi et al. 1992) in the same way as barley 37 kD EP-A has a C-terminal TDEL (Porali et al., unpublished). Okamoto et al. (1994) have shown that the C-terminal propeptide of ten amino acids containing KDEL is cleaved post-translationally to form the mature SH-EP. The auxin-binding receptor protein, which is localized both in the ER and the plasma membrane, also contains the KDEL motif. On the contrary to the SH-EP, the C-terminal KDEL is retained in the secreted protein (Jones & Herman 1993). The purpose and function of the ER retention signal in secretory enzymes is not known.

The actual secretion mechanism is unclear, although it has been intensively studied with barley α-amylases. By immunomicroscopy, it has been shown that the ER and the Golgi complex are involved in the secretion of both high and low pI α-amylases in barley aleurone cells (Gubler et al. 1986, 1987, Zingen-Sell et al. 1990). In the present study, the 30 kD secretory cysteine proteinase was also localized in the *cis*- and *trans*-Golgi area showing that the secretion route of cysteine proteases includes the Golgi complex. Identifying the putative secretory vesicles has been difficult, since in the cereals secretion goes via the constitutive pathway, i.e. secretory products are not accumulated inside the cell but are secreted continuously (Jones & Robinson 1989). In the present study, the existence of the putative secretory vesicles was shown for the first time at electron microscopy level. The vesicles had left the TGN and were localized near the plasma membrane. Some of the vesicles were relatively large in diameter, which may be due to the aggregation of the secretory vesicles. Annexin-mediated aggregation of the secretory vesicles might be a prerequisite for fusion with the plasma membrane (Blackbourn & Battey 1993). Recently, a putative vacuolar targeting receptor has been purified based on the recognition of clathrin in the clathrin-coated transport vesicles (Kirsch et al. 1994). The

nature of the membranes in secretory vesicles demands further studies.

One major difference in secretion between animal and plant cells is the cell wall surrounding plant cells, which thus forms a barrier to the secretory proteins. According to Gubler et al. (1987), α-amylase is delivered through the aleurone cell wall via channels digested presumably by endoxylanases. Tubes around plasmodesmata may facilitate enzyme release.

The role of the scutellum as a secretory tissue has been a matter of controversy (e.g. Palmer & Duffus 1986). So far, it has been shown that the synthesis and secretion of β-glucanases is started from the scutellar epithelium at the beginning of germination, and it continues from the aleurone layer with progressive germination (McFadden et al. 1988). The scutellum is also able to secrete an α-amylase in vivo (Ranki & Sopanen 1984, Pogson et al. 1989). In the present study, the role of the scutellum in the synthesis and secretion of proteolytic enzymes was demonstrated in the intact seeds.

6.2 The role of cytosolic glutamine synthetase in the mobilization of nitrogen

In many higher plants, cytosolic and chloroplastic glutamine synthetase enzymes, called GS_1 and GS_2, respectively, are encoded by distinct nuclear genes (Forde & Cullimore 1989). In general, there is greater similarity in GS_1 in different plants, e.g. monocots and dicots, than GS_1 and GS_2 in the same species. Recently, cDNAs for barley GS isozymes have been isolated and characterized (Baima et al. 1989, Strøman et al. 1990, Freeman et al. 1990, Marigo et al. 1993). The chloroplastic GS is translated as a precursor form containing an N-terminal prosequence, which includes the putative transit peptide. The newly-synthesized protein is directed from cytosol to chloroplast by the transit peptide, which is cleaved off during transfer across the chloroplast membrane (Strøman et al. 1990). The transit peptide is also found in the chloroplastic GS of other plant species, e.g. rice, pea and bean (Sakamoto et al. 1989, Tingey et al. 1988, Lightfoot et al. 1988). A cDNA coding for barley cytosolic GS is highly similar to the other known GS_1 sequences (Marigo et al. 1993).

According to Mann et al. (1979), barley seeds, roots and etiolated shoots exhibit only GS_1 activity, whereas light grown shoots contain both GS_1 and GS_2 activity. In the present study, it was shown that GS is present both in the scutellum and the aleurone layer of the barley seed, and it was confirmed that only cytosolic GS is expressed in germinating barley seed. It was also shown that the subunit composition of GS_1 octamer changes during germination: first, a single polypeptide was expressed and later, another smaller polypeptide appeared. In mature *Phaseolus vulgaris*,

sunflower and mustard seeds, there exists a single GS isoform, which is gradually replaced by different isoforms during germination (Swarup et al. 1990, de la Haba et al. 1992, Sakamoto et al. 1990). In *P. vulgaris*, as in barley seed, cytosolic GS is initially composed of similar subunits; however, during germination the subunit composition changes, and it is possible that different heteromeric isoforms exist at the same time. In barley, the composition of cytosolic GS is affected by the source of nitrogen available (Mäck 1995). Edwards et al. (1990) have shown that GS_1 and GS_2 have non-overlapping roles in the pea, where cytosolic GS is expressed at a high level in the tissues where the mobilization of nitrogen takes place, and the chloroplastic form is dominant in the photosynthetic tissues. Similar situation is very likely to occur also in barley.

6.3 HVA1 protein is abundant in protein storage vacuoles

Although the expression of a LEA3 protein, HVA1, was strongly induced by desiccation stress or ABA treatment in all the living tissues of the seed during germination, HVA1 protein was not totally degraded during normal germination either. Similarly, some dehydrins are present in well-hydrated barley seedling but their amount and size change when desiccation occurs (Close et al. 1993). A 27 kD band represented HVA1 protein in barley tissues, but another band of 23 kD appeared in the aleurone layer during germination and under stress. The role of the smaller band remained unclear, since it wasDual not a degradation product.

Like aspartic proteinase, HVA1 was localized in the protein storage vacuoles (PSV; also called protein bodies) of the mature barley seed. On the contrary to aspartic proteinase, HVA1 does not have a signal peptide and the predicted structure of the protein is highly hydrophilic, suggesting a cytoplasmic location (Dure et al. 1989). In the present study, HVA1 was, however, found both in the cytoplasm and PSVs. Thus, we suggest that the sorting of HVA1 to the PSVs is likely to happen by direct uptake from the cytoplasm. There are examples of ER-independent, direct vacuolar targeting in mammalian, yeast and plant cells. In animal cells, selective lysosomal uptake of cytosolic proteins is mediated through a targeting signal, KFERQ, via an ATP-consuming receptor system (Dice 1990). In yeast cells a cytosolic enzyme, fructose-1,6-bisphosphatase, is imported into vacuoles for degradation (Chiang & Schekman 1991). Yeast fructose-1,6-bisphosphatase contains two remote KFERQ-like motifs. However, HVA1 contains no such consensus sequences. Direct lysosomal uptake in animal cells as well as the regulated vacuolar targeting of fructose-1,6-bisphosphatase in yeast is considered as a stress response, where the purpose is to degrade the selectively imported proteins (Dice 1990, Chiang & Schekman 1991). Another example from yeast is the localization of a mannosidase in the vacuolar membrane even when the secretory route is blocked, which is very

reliable evidence of an alternative pathway (Yoshihisa & Anraku 1990). However, the mechanism of the direct uptake into vacuoles is unknown.

In plant cells, some putatively cytoplasmic proteins are located in vacuoles (Nakamura & Matsuoka 1993). A carrot LEA3 protein, DC 8, is localized primarily in the protein bodies and vacuoles of zygotic embryos, and to a lesser extent in cytoplasm. DC 8 is also secreted into the cell walls of endosperm tissue, although it has no putative signal sequence (Franz et al. 1989). Chymotrypsin inhibitor 2 (CI-2), even though it has a stop codon in the middle of the putative signal peptide sequence, is localized in the protein storage vacuoles of barley seed (Rasmussen et al. 1990). In vegetative tissues, a soybean lipoxygenase and a ß-amylase of *Arabidopsis thaliana* are located in the vacuole, although they do not contain a signal peptide (Tranbarger et al. 1991, Monroe et al. 1991). Since ABA influences Ca^{2+} transport both in the ER and vacuole (Bush et al. 1993), regulated vacuolar import of HVA1 could be due to an increased level of Ca^{2+} induced by water deficit.

Previously, sequestration of ions has been suggested for the role of LEA3 proteins to prevent precipitation during water deficit (Dure 1993a, b). HVA1 protein probably occurs as a dimer at neutral pH, where ion binding is likely to happen. However, vacuolar pH is acidic, and may not favour dimerization. Thus, it is possible that HVA1 has different roles in the cytoplasm and protein storage vacuoles. The C-terminal sequence of HVA1 protein contains at least three putative cleavage sites for barley aspartic proteinase, suggesting that the aspartic proteinase could hydrolyze HVA1 protein in PSVs. However, the function of HVA1 protein in PSVs remains an open question.

7 CONCLUSIONS

The main focus of the present study was on the expression of the aspartic and cysteine proteinases as well as glutamine synthetase during germination of barley, and on the occurrence of a LEA3 protein, HVA1, during stress induction in germinated barley seed. The main conclusions are the following:

1. In the mature barley seed, aspartic proteinase is present in the embryo and the proximal aleurone layer. The expression of the enzyme expands to all the living tissues of the grain during germination, suggesting a role of the house-keeping enzyme. Subcellulary, aspartic proteinase is localized in the protein bodies of the mature seed and later, during germination, in the vacuoles. Tissue distribution and the amount of the two molecular forms of the enzyme varies, suggesting different physiological roles for the two forms of the enzyme.

2. Synthesis and secretion of the 30 kD cysteine proteinase is started from the scutellar epithelium during the first day of germination, thus confirming the central role of the scutellum as a secretory tissue. With progressive germination, the aleurone layer becomes the major source of the 30 kD cysteine proteinase. This cysteine proteinase is transported via the Golgi complex and the putative secretory vesicles to the exterior of the secretory cell. Processing of the enzyme occurs in a similar way to 30 kD cysteine endoprotease EP-B.

3. Glutamine synthetase is expressed as a cytosolic enzyme in the living tissues of the barley seed, showing that GS_1 has a central role in the remobilization of nitrogen for the growing seedling. At the beginning of germination, a single GS subunit is expressed in the seed. The subunit composition changes later during germination, when another, slightly

smaller polypeptide, is synthesized. Since the amount of polypeptides differs, it is probable that GS_1 occurs as heteromeric forms in the seed.

4. A LEA3 protein, HVA1, is present in the living tissues of the mature and germinating seed, although it declines gradually in the course of germination. The expression of HVA1 is induced by the ABA and stress treatments. The HVA1 protein is localized mainly in the protein storage vacuoles, and to a lesser extent in the cytoplasm. A direct uptake mechanism from the cytoplasm to the protein storage vacuole is suggested for the HVA1 protein. Although the function of HVA1 in the protein storage vacuoles is unclear, it might be degraded there by the barley aspartic proteinase.

Acknowledgements

This study was carried out at the Department of Biology, University of Jyväskylä during the years 1989-95. The study was supported by the Foundation for Biotechnical and Industrial Fermentation Research, the Ministry of Agriculture and Forestry and the University of Jyväskylä.

I wish to express my warmest thanks to my supervisor Anita Mikkonen, who's everlasting optimism has encouraged me to go on. I also want to thank Ritva Saarelainen for her valuable advice at the beginning of this study. Leena Mikola and the late Professor Juhani Mikola are greatly acknowledged for the excellent guidance to basic reasearch in the field of plant physiology.

My sincere thanks belong to Antero Salminen for helpful reading of the manuscripts as well as Hilkka Reunanen, Markku Kulomaa and Matti Vuento for the support and valuable advice in many practical arrengements.

I am indebted to Kaarina Pihakaski-Maunsbach and Alan Schulman for the useful and constructive criticism of the thesis manuscript. I also want to thank Michael Freeman for the revision of the English language.

I especially wish to thank Berne Jones and David Ho for the pleasant co-operation and encouragement.

Special thanks are due to Ilkka Porali and Paula Sarkkinen, my nearest coworkers, for fruitful discussions and understanding when my thoughts were wandering somewhere inside the barley grain. Special thanks belong also to Teija Tenhola, who's contribution in the last part of this study was remarkable. I also wish to thank all the students who have been working in our research group for creating a pleasant atmosphere.

I wish to express my warmest thanks to Raija Vassinen and Paavo Niutanen as well as Pirjo Kauppinen, Anne-Riitta Luoma-aho, Marjatta Suhonen, Leena Kaihlavirta, Marianne Salo, Maija-Leena Kiiveri and Anna-Liisa Kotiranta for making my life a little bit easier. Generally, all the staff in the Department has been very kind and helpful.

I also want to thank Eeva-Liisa Punnonen and Varpu Marjomäki for useful advice concerning methodology in the course of this work.

I greatly acknowledge the support of my family, although they did not always know what I was doing. Last, but not least, I want to thank my friends Jyrki and Raisa for reminding me that the most important thing is the life itself.

Yhteenveto (Résumé in Finnish)

Typen mobilisaatioon osallistuvien entsyymien sekä stressiproteiinin esiintyminen itävässä ohrassa

Ohran (*Hordeum vulgare*, L.) jyvässä valtaosa typestä, jota pidetään kasvien kasvua rajoittavana tekijänä, on varastoitu proteiineihin. Jyvän itäessä typpi siirretään kasvavan taimen käyttöön pilkkomalla varastoproteiinit proteolyyttisten entsyymien avulla. Pääasiallinen typen kuljetusmuoto viljakasveilla on aminohappo glutamiini, jota syntetisoi glutamiini-syntetaasi (GS). Kuivumisen häiritessä jyvän itämistä ns. stressiproteiinien synteesi käynnistyy nopeasti. Samoja proteiineja ilmenee luontaisesti jyvän kypsymisen aikana. Tässä tutkimuksessa selvitettiin kahden proteolyyttisen entsyymin, aspartyyli- ja kysteiiniproteinaasin, sekä GS:n ja tietyn stressiproteiinin ilmenemistä itävässä ohran jyvässä.

Aspartyyliproteinaasi esiintyy jo lepäävässä jyvässä ja myöhemmin itävän jyvän kaikissa elävissä solukoissa, mutta sitä ei eritetä kuolleeseen tärkkelysendospermiin, jossa suurin osa varastoproteiineista sijaitsee. Entsyymillä on kaksi aktiivista muotoa, joiden esiintyminen ja määrä vaihtelee jyvän eri solukoissa, mikä viittaa siihen, että entsyymin eri muodoilla saattaa olla erilainen tehtävä. Solunsisäisesti aspartyyli-proteinaasi paikantuu kalvon ympäröimiin proteiinijyväsiin. Entsyymi ei osallistu varsinaisten varastoproteiinien pilkkomiseen vaan todennäköisesti se on muita proteiineja säätelevä entsyymi.

Kysteiiniproteinaasin synteesi alkaa vasta itämisen aikana. Entsyymi paikantuu vain tärkkelysendospermiä ympäröiviin solukoihin, joista se eritetään varastoproteiinien hajotusta varten. Eritysreitti kulkee ER:ltä Golgin laitteen kautta eritysvesikkeleiden avulla ulos solusta. Päinvastoin kuin aspartyyliproteinaasi, kysteiiniproteinaasi on keskeinen entsyymi varastoproteiinien pilkkomisessa.

Glutamiinisyntetaasi esiintyy jyvän elävissä solukoissa sytosolisena muotona. Sen alayksikkökoostumus vaihtelee itämisen aikana liittyen ehkä eri lähteistä tulevan typen assimilaatioon. Sytosolinen muoto ilmenee voimakkasti silloin, kun tarvitaan typen kuljetusta solukosta toiseen.

Jyvä alkaa tuottaa erityisiä stressiproteiineja joutuessaan alttiiksi esimerkiksi kuivumiselle. Ohran LEA3-proteiini, HVA1, esiintyy kuivassa jyvässä, ja sen taso laskee itämisen alettua. Jos jyvä kärsii kuivumisesta, HVA1 ilmenee voimakkaasti. Solun sisällä HVA1-proteiini lokalisoituu sekä sytoplasmaan että proteiinijyväsiin. Vaikka HVA1:n ilmeneminen liittyy selvästi kuivumisstressiin, sen varsinainen tehtävä on vielä epäselvä. On mahdollista, että aspartyyliproteinaasi hajottaa HVA1-proteiinia proteiinijyväsissä.

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ORIGINAL PAPERS

I

Differential localization of two acid proteinases in germinating barley (*Hordeum vulgare*) seed

by

Salla Marttila, Berne L. Jones & Anita Mikkonen

Physiologia Plantarum 93: 317-327, 1995

https://doi.org/10.1111/j.1399-3054.1995.tb02235.x

II

Expression of the 30 kD cysteine endoprotease B in germinating barley seeds

by

Salla Marttila, Ilkka Porali, Tuan-Ho David Ho & Anita Mikkonen

Cell Biology International 17: 205-212, 1993

http://dx.doi.org/10.1006/cbir.1993.1056

III

Glutamine synthetase isozymes in germinating barley seeds

by

Salla Marttila, Ritva Saarelainen, Ilkka Porali & Anita Mikkonen

Physiologia Plantarum 88: 612-618, 1993

https://doi.org/10.1111/j.1399-3054.1993.tb01379.x

IV

A barley (*Hordeum vulgare*) LEA3 protein, HVA1, is abundant in protein storage vacuoles

by

Salla Marttila, Teija Tenhola & Anita Mikkonen

(Revised manuscript submitted to Planta)

https://www.jstor.org/stable/23384375

https://af.booksc.eu/book/5840878/4e9399