RESEARCH ARTICLE

Proteomics reveal tissue-specific features of the cress (*Lepidium sativum* L.) endosperm cap proteome and its hormone-induced changes during seed germination

Kerstin Müller[1], Claudette Job[2], Maya Belghazi[3], Dominique Job[2] and Gerhard Leubner-Metzger[1]

[1] Faculty of Biology, Institute for Biology II, Botany/Plant Physiology, University of Freiburg, Freiburg, Germany
[2] Centre National de la Recherche Scientifique-Université Claude Bernard Lyon-Institut National des Sciences Appliquées-Bayer CropScience Joint Laboratory (UMR 5240), Bayer CropScience, Lyon, France
[3] Centre d'Analyse Protéomique de Marseille, Institut Fédératif de Recherche Jean Roche, Marseille, France

Received: July 29, 2009
Revised: November 3, 2009
Accepted: November 5, 2009

Mature angiosperm seeds consist of an embryo surrounded by the endosperm and the testa. The endosperm cap that covers the radicle plays a regulatory role during germination and is a major target of abscisic acidinduced inhibition of germination. Cress (*Lepidium sativum*) is a close relative of the model plant Arabidopsis thaliana (Arabidopsis). Cress seeds offer the unique possibility of performing tissue-specific proteomics due to their larger size while benefiting the genomic tools available for Arabidopsis. This work provides the first description of endosperm cap proteomics during seed germination. An analysis of the proteome of the cress endosperm cap at key stages during germination and after radicle protrusion in the presence and absence of abscisic acid led to the identification of 144 proteins, which were clustered by the changes in their abundances and categorized by function. Proteins with a function in energy production, protein stability and stress response were overrepresented among the identified endosperm cap proteins. This strongly suggests that the cress endosperm cap is not a storage tissue as the cereal endosperm but a metabolically very active tissue regulating the rate of radicle protrusion.

Keywords:
Cress (*Lepidium sativum*) / Endosperm weakening / Micropylar endosperm cap / Plant proteomics / Seed germination

1 Introduction

Seed germination is defined as the process from imbibition of the dry seed to radicle protrusion [1]. Optimal seed germination is a prerequisite for successful seedling establishment and plant growth, and seeds are a major target for crop improvement in the agricultural sector. Understanding the mechanisms involved in the complex process of seed germination is therefore not only of academic interest, but also relevant to economy and the world food supply. Model plants such as the established model *Arabidopsis thaliana* (Arabidopsis), [2] and garden cress (*Lepidium sativum*, ''cress'') [3] can be used to elucidate processes of general importance for seed germination and seedling establishment.

The mature seeds of most angiospermic species consist of an embryo surrounded by the triploid endosperm and the dead maternal testa. Arabidopsis and cress seeds germinate in two sequential steps [3, 4]. First the testa ruptures, revealing the underlying micropylar endosperm (endosperm cap) covering the radicle. Then the radicle ruptures the endosperm cap, thereby completing germination *sensu*

Correspondence: Dr. Gerhard Leubner-Metzger, Faculty of Biology, Institute for Biology II, Botany/Plant Physiology, University of Freiburg, Schänzlestr. 1, D-79104 Freiburg, Germany
E-mail: gerhard.leubner@biologie.uni-freiburg.de
Fax: +49-761-2032612

Abbreviations: ABA, abscisic acid; **CON,** control; **GA,** gibberellin; **TCA,** tricarboxylic acid

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

stricto. A two-stage germination is common in endospermic species, and is observed, *e.g.* in tobacco (*Nicotiana tabacum*) [5] and *Petunia hybrida* [6], and in white goosefoot (*Chenopodium album*) [7]. The abundance of endosperm in mature seeds differs widely between endospermic species. In the Brassicaceae species Arabidopsis and cress, the endosperm consists of only one cell layer [3, 4]. The endosperm was traditionally considered solely as a nutrient reserve for the growing seedling. However, evidence has accumulated that the endosperm, and particularly the endosperm cap, plays a major role in regulating germination timing and dormancy [3, 7–9]. In accordance with this regulatory role, abscisic acid (ABA) specifically inhibits endosperm rupture, but not testa rupture of Arabidopsis and cress [3] as well as other species with two-step germination [5–7].

Protein translation is an essential process during germination. Seeds will not germinate if translation is pharmacologically inhibited by cycloheximide [10]. However, because seeds can use stored mRNAs as templates for protein synthesis [10, 11] they are able to germinate (although at a slow rate) in the presence of a transcription inhibitor (α-amanitin). These results imply that the potential for germination is largely programmed during seed maturation on the mother plant. Hence, proteomics can reveal major features of seed germination. Based on the progress achieved in sensitive and rapid separation of proteins and in their high-throughput identification by electrophoresis and MS, proteomic approaches have opened up new perspectives to analyze the complex functions of model plants and crop species and allow simultaneous characterization of a large number of proteins [12]. Extensive whole seed proteome data is available for Arabidopsis and is very helpful in the analysis of related Brassicaceae species (www.seed-proteome.com; using data from [10, 13–18]. However, while transcriptomic data have been obtained for separate tissues of Arabidopsis seeds by amplifying small amounts of extracted RNA [8], this procedure is not applicable to proteins for which the small seed size of Arabidopsis precludes separate tissue proteomics by presently available technologies.

Here we chose to work with cress, an emerging model system in seed biology. Cress seed anatomy and germination physiology are highly similar to those of Arabidopsis, but the seeds are about 20 times larger [3] (see Fig. 1A). The size of the seeds makes it possible to dissect the radicle and the endosperm cap, which makes cress an ideal research object for endosperm-related processes in the mature seed. It has been shown in a number of species with endospermic seeds that protein abundance and enzyme activities differ between the micropylar and the non-micropylar endosperm. For example, a peroxidase is expressed and active specifically in the micropylar endosperm of tomato seeds [19]. A micropylar endosperm-specific expansin was found in *Datura ferox* [20] and a β-1,3-glucanase has been shown to be specifically regulated in the micropylar endosperm of germinating tobacco seeds [5]. These proteins are proposed to play a role in cell wall loosening of the micropylar endosperm.

Seeds can complete germination when the growth potential of the radicle overcomes the tissue resistance of the endosperm cap. We have biomechanically quantified the tissue resistance of cress endosperm caps in the course of germination and found that the endosperm weakens prior to radicle emergence and that this process is required for endosperm rupture [3]. The weakening process is initiated by a signal from the embryo, presumably gibberellins (GA) or a GA precursor, but is organ-autonomous once the signal has been perceived. Coordination between different parts of the seed is thus a vital part of the germination process.

Only very few studies have so far analyzed seed covering tissues separately from embryonic tissues during seed germination. Separate tissue proteomics of sugarbeet (*Beta vulgaris*) seeds led to a metabolic map for the different seed parts and their interactions and to the discovery of metabolic compartmentalization in various biochemical pathways [21]. That work demonstrated the great value of separating tissues, as strong differences between protein accumulation patterns in the separate organs were identified. However, the seed anatomy of sugarbeet, which belongs to the Amaranthaceae, differs strongly from that of endospermic species, as sugarbeet fruits have only a remnant of an endosperm layer, a central perisperm as storage tissue and no visible two-step germination [22]. Sheoran *et al.* [23] explored the embryo and the seed coats of germinating tomato (*Lycopersicon esculentum*, Solanaceae) seeds. In agreement with the role of thick endosperms as nutrient reserves for the seed, the authors mainly identified storage proteins. The proteome of separate organs of barley (*Hordeum vulgare*, Poaceae) caryopses was studied by Bønsager *et al.* [24] during and after germination. Barley contains a large amount of dead starchy endosperm surrounded by the living aleurone layer. Both are separated from the embryonic axis by the scutellum. Thus, the proteome of the thin micropylar endosperm cap with its regulatory role in germination still remained unexplored.

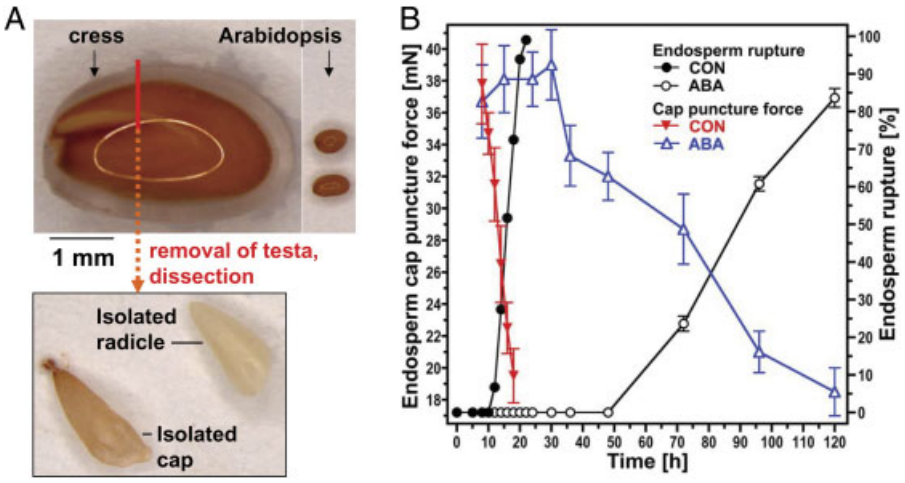
In the work described here, we investigated the tissue-specific proteome of endosperm caps of germinating cress seeds. We compared protein extracts of cress endosperm caps at key points during germination and investigated the effect of ABA inhibition of endosperm weakening and rupture on the endosperm cap proteome.

2 Materials and methods

2.1 Seed germination, tissue dissection and biomechanical measurements

Cress seeds (*L. sativum*, ''Gartenkresse einfache'', Juliwa, Heidelberg, Germany) were germinated in Petri dishes on two layers of filter-paper with 6 mL 1/10 Murashige-Skoog salts at 18°C in continuous white light (101 $\mu M\, m^{-2}\, s^{-1}$).

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim



C

sample	% ER (puncture force [mN])	Collected caps ruptured
8 h	0 % (37.8 +/- 2.5)	No
18 h	70-80 % (19.5 -/- 1.7)	No
18 h ruptured	70-80 % (n.d.)	Yes
18 h ABA	0 % (21 +/- 1.3)	No
96 h ABA	70-80 % (18.5 +/- 1.5)	No

Figure 1. Protein sample collection of cress seed endosperm caps and radicles at physiologically defined time points during germination. (A) A germinating cress seed in the stage of testa rupture (the brownish testa has split, revealing the endosperm cap still covering the radicle) is shown next to two Arabidopsis seeds for size comparison. For dissection, the bit of testa attached to the cap is carefully removed and the radicle with the cap cut off at the place indicated with a red line. The radicle is then carefully removed from the cap with a dissection needle, yielding isolated radicles and caps as shown in the lower panel. (B) The cress endosperm weakens during the germination process. Cress endosperm rupture and endosperm weakening are inhibited by ABA. The tissue resistance of the endosperm was measured biomechanically as puncture force in the absence (CON) and presence of 10 μM ABA. (C) List of the protein samples used for this work. For each sample, average endosperm rupture at the point of collection and tissue resistance of the endosperm (puncture force, from (B)) are listed. n.d. = not determinable, ER = endosperm rupture.

Where indicated, 10 μM *cis*-S(+)-ABA (Duchefa, NL) was added to the germination medium. Puncture force was measured as described previously [3]. Seeds were cut in half, the radicle removed and a metal pin lowered into the empty intact endosperm cap. The force necessary to puncture the endosperm was measured with a custom-made machine.

Dissection of seeds was performed with a scalpel and dissection needle and the tissues collected on ice, frozen in liquid nitrogen and stored at −20°C until protein extraction.

2.2 Protein extraction

For each experimental condition examined, three biological replicates of 1000–2000 endosperm caps were ground with mortar and pestle in liquid nitrogen and thawed in thiourea/urea buffer [25] with the protease inhibitor cocktail "Complete Mini" (Roche Diagnostics, Mannheim, Germany), 60 U/mL DNAse and 6 U/mL RNAse A, as described previously [19]. After shaking for 30 min at 4°C, samples were centrifuged at 20 000 × *g* for 15 min at 4°C until the supernatant was clear. The final supernatant corresponded to the soluble protein extract. Protein concentration was determined with a Bio-Rad assay kit following Bradford's method [26]. Bovine γ globulin was used as a standard.

2.3 2-D gel electrophoresis and data analysis

In 2-D PAGE analyses gels were run as described previously [17, 21]. An amount of 300 μg of protein was used for isoelectric focusing on gel strips with an immobilized pH gradient from 3 to 10 (Immobiline DryStrip pH 3–10 NL, 24 cm, Amersham Biosciences, GE Healthcare, Munich, Germany). The second dimension was carried out as described previously [10] using 10% polyacrylamide-gels. Three gels loaded with biological replicates were run for each treatment. Ten gels were run in parallel overnight. The gels were then stained with silver nitrate. Stained gels were scanned and analyzed with the software ImageMaster 2D Elite version 4.01. Normalization was performed in the total spot volume mode [21]. Only spots in which no more than one protein was identified were used for quantitative

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

comparisons. A *t*-test was calculated to compare the different treatments. Changes with $p < 0.05$ were considered significant.

2.4 Protein identification by MS

Spots were excised from 2-D PAGE gels and treated with trypsin as described previously [21]. Peptide fragments were then analyzed by MS/MS on a nanoelectrospray ionization quadrupole TOF hybrid mass spectrometer (Q-TOF Ultima; Waters Micromass) coupled with a nano-HPLC (Cap-LC; Waters, GE Healthcare, Munich, Germany) as described previously [21]. The following parameters were used for database search: trypsin as digestion enzyme (1 max miss cleavage), peptide mass tolerance 0.1 Da, fragment mass tolerance 0.1 Da, carbamidomethylation of cysteine and oxidation of methionine as variable modifications, Database: NCBInr version 20080704 (6 680 430 sequences; 2 291 697 606 residues), taxonomy: Viridiplantae. Protein scores of more than 41 for NCBInr database were considered as positive identification ($p < 0.05$) with at least two different peptides with individual score > 18. For proteins characterized by only one peptide, this peptide must contain at least five consecutive fragments (b or y ions) and yield a score > 41 for NCBInr. A first pass standard database search was done with only carbamidomethylation on cysteines and oxidation of methionine as variable modifications. Whenever this search gave a positive hit (according to our criteria given above), a second pass search was done only with this positive hit using the error tolerant feature of MASCOT software, in order to assign some more unmatched spectra to the identified protein. The error-tolerant search allows enzyme semi-specificity, testing of all possible modifications and amino acid substitution. Only one of the above was allowed *per* peptide.

3 Results

3.1 Rationale of the proteomics approach

To get information about proteome changes in the endosperm cap during cress seed germination, we dissected cress seeds (Fig. 1A) at key time points during the germination process. We chose these points based on quantification of endosperm cap weakening and endosperm rupture (Fig. 1B). At each point, seeds were dissected and the soluble protein fraction extracted from the separated tissues. Under control conditions (CON), we chose 8 h (beginning of endosperm weakening, no endosperm rupture) and 18 h (endosperm strongly weakened, 60–80% of seeds have ruptured endosperms). At 18 h, we collected ruptured and intact caps separately (18 h = intact endosperm caps; 18 h ruptured = ruptured endosperm caps). A comparison could thus be made between the proteomes at 8 and 18 h (intact caps), corresponding to imbibition and endosperm weakening, while a comparison between the proteomes at 18 h (intact caps) and 18 h ruptured could disclose specific proteins associated with radicle protrusion.

Our choice of samples has the advantage of yielding a physiologically homogenous pool of seeds. As germination is a process that is temporarily spread within a seed population, a pool of seeds will often include a variety of germination stages. By using detailed germination kinetics and our knowledge about endosperm weakening (Figs. 1B and C), we made sure that at the time points we used for sampling, the dissected seeds were physiologically as homogenous as possible. We used 1000–2000 cress endosperm caps *per* biological replicate in order to extract enough protein for 2D-PAGE. This rather large number of tissues had the additional advantage of delivering a well-averaged protein extract.

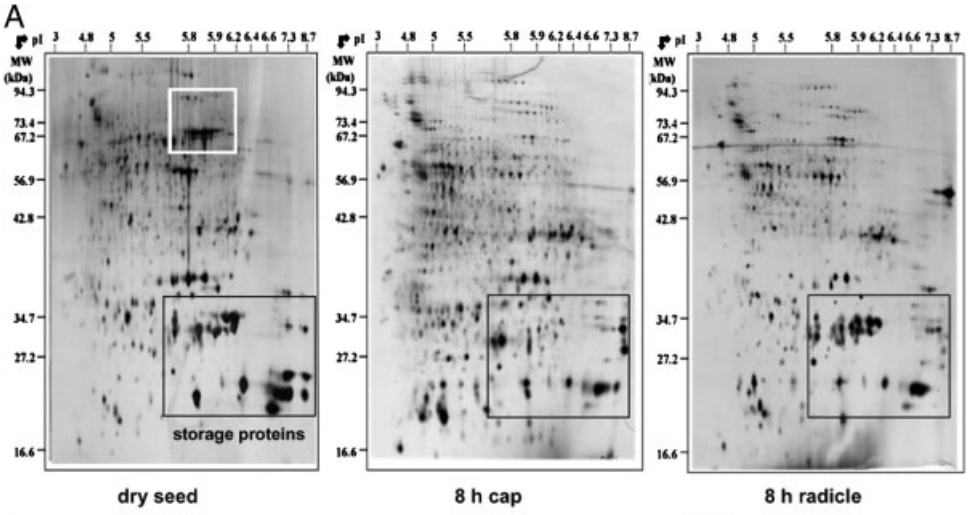
After incubation in medium with 10 μM ABA, we sampled at 18 h (no weakening yet, no endosperm rupture) and 96 h (endosperm strongly weakened, 60–80% of seeds have endosperm rupture). Thus, 8 h CON and 18 h ABA can be considered physiologically equivalent with respect to the stage in the germination process the seeds have reached, as can 18 h CON and 96 h ABA (Fig. 1C). For 96 h ABA samples, only intact endosperm caps were collected, as the primary aim of our study was to investigate germination *sensu stricto*.

The value of separating tissues for protein extraction becomes clear in the comparison between silver-stained 2-D gels with protein extracts of dry cress seeds, radicles and endosperm caps (Fig. 2A). The pattern of stained spots differs visually between all three extracts. In whole seed extracts specific endosperm cap proteins can be hidden by the larger amount of embryo proteins, which in turn would derive mainly from the cotyledons.

Interestingly, the large group of spots in which most storage proteins congregate (marked by black squares in Fig. 2A) was less prevalent in the cap extracts than in the radicle and less in the radicle than in whole dry seeds. While the latter was to be expected as most storage reserves of cress seeds are located in the large storage cotyledons, the low presence of storage proteins in the endosperm cap further supports its role as a germination regulator rather than a nutritional reserve for the growing seedling.

The fact that cress and Arabidopsis are closely related made it possible to predict the identities of some of the spots in cress whole seed extracts by comparing them to the migration behavior and accumulation of corresponding spots on the Arabidopsis proteome maps that are publicly available on the internet (www.seed-proteome.com). For example, two spots containing methionine synthase and β-glucosidase could be identified in extracts of whole cress seeds (Fig. 2B) by the similarity of their migration behavior and changes in protein abundance to the corresponding spots in the Arabidopsis proteome. While the abundance of methionine synthase visibly increased in whole seed extracts during germination, the accumulation level of β-glucosidase

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim



Spot	volume change	Experimental MW [kD]	Experimental pI
Cress whole seeds			
spot a	increase	80	5.85
spot b	constant	67.2	5.89
Arabidopsis whole seeds			
- (from [27])	increase	82.27	5.94
# 96 www.seed-proteome.com	constant	64.6	6.08

a: methionine synthase, b: ß-Glucosidase

Figure 2. The proteomes of radicle, endosperm cap, and whole cress seeds differ. (A) Comparison between representative silver-stained 2-D gels of whole dry cress seeds, 8 h imbibed cress radicles and 8 h imbibed cress endosperm caps. Note that the storage proteins (marked by a black square) are most abundant in the whole seed, followed by the radicle. The cap displays a low abundance of storage proteins. (B) Many cress proteins can already be identified by the similarity of their migration and their volume changes during germination in whole seed extracts to their Arabidopsis orthologous gene products. The section of the dry seed - 2-D gel marked with a white square in (A) is enlarged and compared with the corresponding section of a 2-D gel with extracts of whole cress seeds at 18 h after imbibition (''18 h imbibed''). The experimental MW and p*I* of the two spots marked a and b are given in the table to the right of the gel sections and compared with the corresponding Arabidopsis spots. The values for Arabidopsis are taken from [27] and www.seed-proteome.com, respectively.

remained constant. This led us to assign putative identities, which were later confirmed by MS (endosperm cap spot nos 26 and 53, Supporting Information Table S1, Fig. S4).

Methionine synthase is also an example for a tissue-specific change in protein abundance that would necessarily be overlooked in whole seed extracts in which the embryo is strongly over-represented compared with the endosperm. The role of methionine synthase during Arabidopsis germination has been studied in detail [27]. In this previous study it has been shown that the accumulation level of methionine synthase increases strongly prior to radicle emergence in Arabidopsis, a finding which is in accordance with our observation in whole cress seed extracts (Fig. 2B). In contrast, in the cress endosperm cap samples methionine synthase level remained constant during germination (8–18 h) and increased upon imbibition with ABA (Fig. 3). This applies to both methionine synthase spots (spot nos 26 and 27, Supporting Information Table S1, Fig. S4).

3.2 The endosperm cap proteome exhibits tissue-specific features

We performed a software-based analysis of spot volumes in our 2-D-gels for three biological replicates of the five distinct endosperm cap samples. Spot volumes of a total of 329 spots were normalized by total spot volume. In total, 110 spots whose volume was large enough for MS analysis were digested with trypsin and analyzed by MS. A total of 97 spots led to the successful identification of 144 protein forms as

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

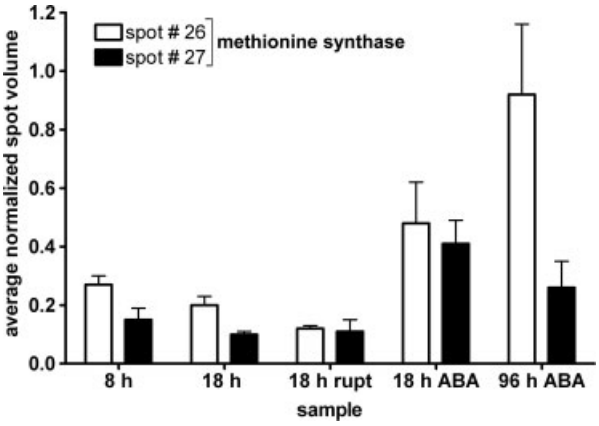


Figure 3. Changes in methionine synthase abundance (methionine synthase spot nos 26 and 27, Supporting Information Table S1) in the endosperm cap. Average normalized volumes of the two unique spots containing methionine synthase in the cap are plotted for all five cap samples. Means of three biological replicates ± SD are presented.

assessed by sequence similarities to proteins represented in the NCBInr protein database. Out of 97, 33 successfully analyzed spots contained more than one and up to four proteins. Supporting Information Table S1 lists all identified proteins with their associated data. The corresponding protein spots can be located on the cress cap proteome map in Supporting Information Figs. S1–S4.

A major part (77%; 111 out of 144 proteins) of the identified proteins showed the highest sequence similarity to Arabidopsis proteins, which underpins the close phylogenetic relationship between cress and Arabidopsis and lends support to the quality of the protein characterizations. As only very few cress protein sequences were presently available in available databases (11 retrievable in an NCBI search), only one protein was identified by its homology to an actual cress protein. It corresponds to the thiocyanate forming protein, which was identified in three spots (spot nos 220, 222 and 224, Supporting Information Table S1). This enzyme plays a role in the antiherbivoral defense of plants by controling glucosinolate hydrolysis, a reaction that is typical for the Brassicaceae [28].

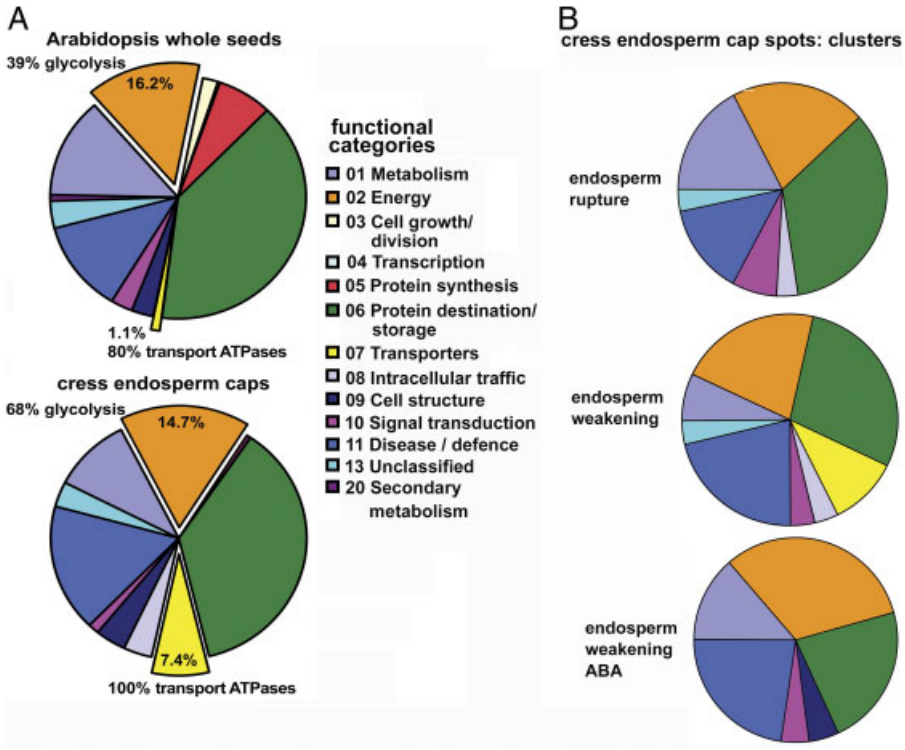


Figure 4. Proteins belonging to the functional categories energy (02), protein destination and storage (06) and disease and defense (11) are overrepresented in the identified cap proteome. (A) Comparison of the distribution of functional categories in the Arabidopsis whole seed proteome and the cress endosperm cap. Numbers in the pie pieces give the fraction of the identified proteome represented by the respective functional categories. Numbers next to the pie pieces are percentages of the functional descriptions ''glycolysis'' and ''transport-ATPases'' in the functional categories 02. energy and 07. transporters, respectively. Functional categories and their color codes are listed. (B) Clustering and distribution of functional categories of the 64 spots representing only one protein. Protein expression signatures are shown for the endosperm cap after endosperm rupture (cluster 2, ''endosperm rupture'', 29 proteins), and during endosperm weakening in the absence (cluster 3, ''endosperm weakening'', 28 proteins) and in the presence of ABA (cluster 4, ''endosperm weakening ABA'', 22 proteins). A signature for cluster 1 (constant abundance) is not shown because it contains only 11 proteins. All clusters can be found in Supporting Information Table S1. Functional categories and their color codes are listed under (A).

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

We have followed Catusse *et al.* [21] and Pawlowski [29] in classifying the identified proteins into the ontological categories for plant proteins introduced by Bevan *et al.* [30] (Fig. 4). We compared the functional categories represented in the cress endosperm cap proteome (this work) with those represented in the Arabidopsis whole seed proteome (Fig. 4A, www.seed-proteome.com; Loïc Rajjou, personal communication). Taking into account the fact that only very strong differences in representation of a category can be considered valid, as about three times the number of spots has been analyzed for Arabidopsis compared with cress, the main difference was an over-representation of category 07 (transporters) in the cress endosperm proteome. The category was composed exclusively of transport ATPase subunits (function 07.22) in cress (Fig. 4A). As mitochondrial ATPases would be expected to be associated with energy production, we also took a closer look at category 02 (energy) in both species. Indeed, in the endosperm cap 65% of the proteins belonging to this category belonged to function 02.01 (glycolysis), while only 27% did so in the Arabidopsis whole seed extracts. If we take the function 02.01 (glycolysis) and category 07 (transporters) together, 32 of 475 proteins in Arabidopsis and 26 of 144 proteins in the cress endosperm cap belong to one of these categories, which is an approximate threefold higher representation in the endosperm cap (Fig. 4, Supporting Information Table S1). This is evidence for the importance of glycolytic metabolism and ATP synthesis in the endosperm of imbibed seeds.

3.3 Endosperm weakening is associated with changes in accumulation levels of endosperm cap proteins

From the quantitative data in Supporting Information Table S1, we clustered the endosperm cap proteins according to their specific accumulation signatures during germination *sensu stricto* in the presence and absence of ABA as well as after endosperm rupture. In this way, the 64 proteins present in unique spots could be grouped by belonging to one or more of the following four clusters (Supporting Information Table S1, Fig. 4B): 11 proteins remained constant over all treatments (cluster 1); 29 proteins changed significantly after endosperm rupture (cluster 2), with only seven proteins being strictly specific to this cluster. Cluster 3 contains 28 proteins that changed between 8 and 18 h in water, thereby corresponding to endosperm weakening and the germination phase in water. Out of them, ten proteins were present in both clusters 2 and 3 (Supporting Information Table S1). Finally, 22 proteins changed between 18 h ABA and 96 h ABA, thereby representing endosperm weakening occurring in the presence of ABA (cluster 4). Out of them, three proteins were present in both clusters 3 and 4 (Supporting Information Table S1).

We compared the distribution of the functional categories after Bevan *et al.* [30] within the four clusters with the profile of the whole protein set (Fig. 4B). Each cluster showed its own specific signature that differed from the protein profile of all 64 proteins present in unique spots (Supporting Information Table S1). The major functional categories that are represented are energy (Bevan *et al.* [30] category 2, Supporting Information Table S1) as well as protein destination and storage (category 6, Supporting Information Table S1) and disease and defense (category 11, Supporting Information Table S1).

A closer look at proteins in functional category 2 (Supporting Information Tables S1 and S2, Fig. S1) showed key enzymes of glycolysis and the tricarboxylic acid (TCA) cycle. Fructose-bisphosphate aldolase showed a strong increase in protein abundance in the endosperm cap during germination and seemed to be a major target of ABA inhibition at 18 h (Supporting Information Table S1). When seeds progressed towards endosperm rupture in the presence of ABA, the abundance of fructose-bisphosphate aldolase increased. A similar, although less pronounced, pattern was observed for glyceraldehyde-3-phosphate dehydrogenase (Supporting Information Table S1). We also identified mitochondrial malate dehydrogenase, which is part of the TCA cycle (Supporting Information Table S1). Furthermore we identified a cytosolic malate dehydrogenase, which is typical for the gluconeogenesis, and, together with its mitochondrial counterpart, for the malate shuttle (*via* oxaloacetate) between the mitochondria and the cytosol. Only one enzyme of the glyoxylate cycle, namely cytosolic aconitase, was found (Supporting Information Table S1). Thus, metabolic activity generally increased during imbibition, and this was most evident for the glycolytic enzymes in the endosperm cap proteome compared with Arabidopsis whole seed (Fig. 4).

We found mainly three groups of proteins with functions in protein destination and storage in cress endosperm caps (Bevan *et al.* [30] category 6, Supporting Information Tables S1 and S3, Fig. S2). These are aspartic proteases that are involved in storage protein degradation, heat shock proteins and other chaperones, and subunits of proteasome complexes.

Stress-related proteins were represented by peroxiredoxin and glutathione-*S*-transferases as well as a thiocyanate-forming protein (Bevan *et al.* category 11 [30], Supporting Information Tables S1 and S4, Fig. S3).

3.4 The proteome of endosperm caps is markedly affected at the time of radicle protrusion

We observed that the cress endosperm cap dries out and dies within a few days after its rupture, while the rest of the seed covering layers, which sometimes remain stuck to the cotyledons, seem to live longer. With the 18 h ruptured samples, we observed that remarkably strong changes in the proteome already take place within less than 6 h after endosperm rupture (Fig. 4C, cluster 2), as the ruptured caps

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

we sampled had been intact until at least 12 h and more often 14 h after imbibition (Fig. 1B). In addition to the six proteins specific to cluster 2, 23 proteins could also be found in Supporting Information Table S1 as showing changes in accumulation at this developmental stage.

Alongside heat shock proteins, the abundance of peroxiredoxin and storage proteins fragments increased (Supporting Information Table S1), whereas the abundance of almost all of the proteins involved in energy metabolism decreased (Supporting Information Table S1), most likely because it is no longer necessary to keep up the full set of metabolic pathways. In accordance with this, an investigation of the cress seedling proteome by Gianazza *et al.* [31] identified mostly storage proteins in the leftovers of the seeds still stuck to the plantlets (encompassing the endosperm and testa). In this context it is also interesting to note the strong drop in the accumulation level of a key enzyme of the "activated methyl cycle", *S*-adenosyl-L-homocysteine hydrolase (spot no. 118), in the endosperm cap at the time of radicle protrusion (Supporting Information Table S1), considering the general importance of this metabolic cycle in plants [32] and seeds [27].

4 Discussion

This work followed the development of the cress endosperm cap proteome during seed germination in the absence and presence of 10 μM ABA, respectively (see Section 3.1 and Fig. 1). Amongst the 144 protein forms we identified, those playing roles in energy production, protein folding and stability and in defense were overrepresented. The same groups were found to be overrepresented in the proteome of germinating barley seeds [24]. We identified proteins in these overrepresented categories whose abundances were associated temporally, hormonally and spatially with endosperm cap weakening and rupture, as shown by the clustering analysis in Fig. 4B. Since the clustering approach provided proteins of the above mentioned groups, we assume that they represent important processes associated with endosperm cap function during Brassicaceae seed germination and after endosperm rupture. In agreement with this, when whole seeds of Arabidopsis and cress endosperm caps were compared using the classification of Bevan *et al.* [30], we found that the functional categories 02 (energy), 07 (transporters) and 11 (disease/defense) were overrepresented in the cap. These findings are discussed in the following sections in the context of the current knowledge in the field.

4.1 Specific features of the endosperm cap proteome

Seeds rely on their storage reserves for energy production. Brassicaceae, *e.g.* the major crop oil seed rape (*Brassica napus*), have oil seeds. A content analysis of cress seeds identified fat as the major storage substance [33, 34]. In addition, our 2-D-gels (Fig. 2A) and protein analyses (Supporting Information Tables S1 and S3) showed significant deposits of storage proteins, mainly in the embryo, and to a much lower extent in the endosperm cap. Lipid mobilization in the endosperm starts prior to its rupture. The early onset is evident in Arabidopsis [35, 36], castor bean [37] and tobacco [38]. ABA does not inhibit lipid mobilization in the Arabidopsis endosperm, while lipid mobilization in the embryo is ABA-inhibitable [8, 39]. We identified a cytosolic aconitase in the cress endosperm. This enzyme plays a role in storage lipid metabolism in the glyoxylate cycle and gluconeogenesis. In accordance with the findings by Penfield *et al.* [39], its protein abundance was not influenced by ABA at 18 h.

We identified enzymes catalyzing the reversible reactions that are part of gluconeogenesis and glycolysis, respectively, such as fructose-bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase whose abundances were associated with the progression of germination (Supporting Information Tables S1 and S2). However, since we did not identify any of the enzymes catalyzing an irreversible step, our proteome analysis does not allow deciphering which pathway is prevalent in the endosperm caps during germination.

Transcript analysis of whole germinating Arabidopsis seeds showed that glycolysis and TCA cycle mRNA expression is upregulated already at 3 h after imbibition in water [39]. This early upregulation of transcript expression was followed by a further increase in mRNA abundance later during germination. It is therefore possible that the inhibiting effect of ABA on the accumulation of enzymes of the glycolytic pathway we observed in the cress endosperm cap at 18 h is already manifested during the earlier phase of imbibition. While the inhibiting effect of ABA on glycolysis during seed germination is a new finding, the inhibition of seed germination by glycolysis inhibitors has been already observed in pine (*Pinus laricio*) seeds [40]. Furthermore, in peanut (*Arachis hypogaea*) seed after-ripening or breakage of dormancy with kinetin leads to glycolytic enzyme activity accumulation in the embryonic axis [41]. After-ripening and loss of dormancy are known to reduce ABA levels in seeds [42]. Our observation that ABA regulates glycolytic enzyme accumulation in the endosperm cap in association with endosperm weakening and rupture suggests that energy production is essential for the developmental function of the cap as a germination-regulating tissue.

Another metabolic enzyme showing specific accumulation behavior in the cress cap endosperm is methionine synthase (Fig. 3). This enzyme plays a vital role as a hub in metabolism [32]. Contrary to previous findings with whole Arabidopsis seeds [27] the accumulation of this enzyme was not triggered in the cress cap endosperm by seed imbibition (Fig. 2). A salient feature was that considerable accumulation of this enzyme occurred in cress ABA-treated imbibed endosperm caps (Fig. 2). Considering that methionine is the precursor of ethylene [32] and that ABA and ethylene act

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

antagonistically in seed germination [35, 43] one possibility would be that the observed accumulation of methionine synthase upon ABA treatment (Fig. 2) represent an attempt of the cress seeds to overcome germination inhibition brought about by ABA.

Function 06.13-proteolysis [30] was represented in our samples by aspartic protease, whose abundance was associated with germination, as well as by 26S proteasome subunits (Supporting Information Tables S1 and S3). Aspartic proteases are known to degrade storage proteins [44]. Mobilization of the bulk of storage reserves is a post-germination event required for seedling growth [1]. However, it has been observed that the start of storage protein degradation in the endosperm cap can occur very early upon imbibition and clearly prior to radicle protrusion in many species [9]. The initiation of protein body degradation in the endosperm cap does not occur in dormant or GA-deficient Arabidopsis seeds, and seems to be an early and general hallmark of endospermic seed germination in many species. Early protein body degradation in the cap of Arabidopsis, *Datura*, tomato and other species occurs only when the seeds are induced to germinate [9, 20, 45]. In accordance with these observations, we found that the abundance of the putative aspartic protease in the endosperm cap increased from 8 to 18 h, *i.e.* during endosperm weakening. ABA inhibits this accumulation at 18 h, but as seeds progressed in endosperm weakening toward endosperm rupture in the presence of ABA, an increase of protein abundance was evident. Especially in the case of Arabidopsis and cress with their single cell layer of endosperm, this early mobilization of protein bodies in the cap is likely to serve a non-nutritional function in the control of germination in addition to delivering amino acids for translation.

A putative 26S proteasome regulatory subunit accumulated in the cress endosperm cap (Supporting Information Table S1). The 26S proteasome is involved in the degradation of unneeded or damaged proteins, which are marked for degradation by ubiquitination, and plays a role in various hormonal pathways known be involved in germination, such as GA. Thus transduction of the GA signal, which promotes germination, involves degradation of negative regulators by the proteasome, *e.g.* RGA in Arabidopsis or Slender1 in barley [46, 47]. In Arabidopsis seeds GA-promoted and ABA-inhibited vacuolation of protein storage vacuoles is first evident in the endosperm cap prior to endosperm rupture and takes place only much later in the non-micropylar endosperm and the embryo [9].

A variety of heat shock proteins were present in our endosperm cap extracts (Supporting Information Tables S1 and S3). Heat shock proteins are expressed when plants are exposed to stress and play an important role in plant stress tolerance [48]. Stress commonly leads to an increase in the production of reactive oxygen species, leading to a more oxidative environment in the cell, which may lead to oxidative protein damage [49] and ultimately to cell death [50]. Heat shock proteins help in the refolding of damaged proteins and stabilize proteins at intermediate stages of folding or degradation. Seed germination, particularly imbibition and the return to full metabolism, are a strong stress for the plant [1, 51]. The importance of the antioxidant machinery for successful rehydration has also been documented in resurrection plants, which, like seeds, progresses from an air-dry to a fully imbibed state in a very short period of time [52].

In accordance with the seeds being subjected to oxidative stress, proteins involved in stress defense (Bevan *et al.* [30] category 11, Supporting Information Tables S1 and S4) were overrepresented amongst identified proteins, and we observed an increase in the abundance of antioxidant enzymes such as glutathione-*S*-transferase and cysteine-peroxiredoxin during germination. Glutathione-*S*-transferase also participates in the defense against other toxic substances by coupling glutathione to molecules that threaten the plant cell. The coupled substances are then removed by depositing them into the vacuole or the apoplast [53]. The identified proteins with a high sequence similarity to the Arabidopsis cysteine-peroxiredoxin PER1 was identified in several spots, which decreased or remained constant, respectively, as seeds progressed to endosperm rupture under control conditions, while its behavior in the presence of ABA varied between the spots in which it was detected. Peroxiredoxins are expressed during late seed germination [54]. 1-Cys peroxiredoxin transcripts are found only in seed parts that can survive desiccation, *i.e.* the embryo and the endosperm [55]. Interestingly, while a reduced transcript abundance of AtPER1 in mutant seeds does not lead to a germination phenotype, seeds over-accumulating AtPER1 show a reduced germination under abiotic stress conditions, *i.e.* under conditions of increased ABA-content [56].

In conclusion, this study provides the first description of tissue-specific endosperm cap proteomics during seed germination. By combining physiological and biomechanical investigations of germination and endosperm weakening with a proteomics approach, we could associate time- and ABA-dependent tissue-specific proteome changes in the endosperm cap with key stages of germination and radicle protrusion. Our work provides a solid basis for future studies using cress to further study the function of the endosperm cap. Our findings are in accordance with a regulatory rather than a nutritional role of the endosperm cap in seed germination and show the suitability of cress as a seed model system.

The authors thank Loïc Rajjou for providing the functional categorization of the Arabidopsis seed proteome. K.M. was supported by Bayer CropScience to perform the proteomics experiments in D. J. and C. J. laboratory. This work was financially supported by grants of the ''Deutsche Forschungsgemeinschaft'' (DFG LE720/6) to G.L.-M.

The authors have declared no conflict of interest.

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

5 References

[1] Bewley, J. D., Seed germination and dormancy. *Plant Cell* 1997, *9*, 1055–1066.

[2] Holdsworth, M., Finch-Savage, W., Grappin, P., Job, D., Post-genomics dissection of seed dormancy and germination. *Trends Plant Sci.* 2008, *13*, 7–13.

[3] Müller, K., Tintelnot, S., Leubner-Metzger, G., Endosperm-limited Brassicaceae seed germination: Abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant Cell Physiol.* 2006, *47*, 864–877.

[4] Liu, P.-P., Koizuka, N., Homrichhausen, T., Hewitt, J. *et al.*, Large-scale screening of Arabidopsis enhancer-traps lines for seed germination-associated genes. *Plant J.* 2005, *41*, 936–944.

[5] Leubner-Metzger, G., Fründt, C., Vögeli-Lange, R., Meins, F., Jr., Class I β-1,3-glucanase in the endosperm of tobacco during germination. *Plant Physiol.* 1995, *109*, 751–759.

[6] Petruzzelli, L., Müller, K., Hermann, K., Leubner-Metzger, G., Distinct expression patterns of β-1,3-glucanases and chitinases during the germination of Solanaceous seeds. *Seed Sci. Res.* 2003, *13*, 139–153.

[7] Karssen, C. M., Two sites of hormonal action during germination of *Chenopodium album* seeds. *Physiol. Plant* 1976, *36*, 264–270.

[8] Penfield, S., Li, Y., Gilday, A., Graham, S. *et al.*, Arabidopsis ABA INSENSITIVE4 regulates lipid mobilization in the embryo and reveals repression of seed germination in the endosperm. *Plant Cell* 2006, *18*, 1887–1899.

[9] Bethke, P. C., Libourel, I. G. L., Aoyama, N., Chung, Y. Y. *et al.*, The Arabidopsis aleurone layer responds to nitric oxide, gibberellin and abscisic acid and is sufficient and necessary for seed dormancy. *Plant Physiol.* 2007, *143*, 1173–1188.

[10] Rajjou, L., Gallardo, K., Debeaujon, I., Vandekerckhove, J. *et al.*, The effect of α-amanitin on the Arabidopsis seed proteome highlights the distinct roles of stored and neosynthesized mRNAs during germination. *Plant Physiol.* 1997, *134*, 1598–1613.

[11] Dure, L., Waters, L., Long-lived messenger RNA: Evidence from cotton seed germination. *Science* 1965, *147*, 410–412.

[12] Bradshaw, R., Burlingame, A., From proteins to proteomics. *IUBMB Life* 2005, *57*, 267–272.

[13] Gallardo, K., Job, C., Groot, S., Puype, M. *et al.*, Proteomic analysis of Arabidopsis seed germination and priming. *Plant Physiol.* 2001, *126*, 835–848.

[14] Gallardo, K., Job, C., Groot, S., Puype, M. *et al.*, Proteomics of Arabidopsis seed germination A comparative study of wild type and gibberellin-deficient seeds. *Plant Physiol.* 2002, *129*, 823–837.

[15] Rajjou, L., Belghazi, M., Huguet, R., Robin, C. *et al.*, Proteomic investigation of the effect of salicylic acid on Arabidopsis seed germination and establishment of early defense mechanisms. *Plant Physiol.* 2006, *141*, 910–923.

[16] Rajjou, L., Lovigny, Y., Groot, S. P. C., Belghazi, M. *et al.*, Proteome-wide characterization of seed aging in Arabidopsis: A comparison between artificial and natural aging protocols. *Plant Physiol.* 2008, *148*, 620–641.

[17] Job, C., Rajjou, L., Lovigny, Y., Belghazi, M. *et al.*, Patterns of protein oxidation in Arabidopsis seeds and during germination. *Plant Physiol.* 2005, *138*, 790–802.

[18] Chibani, K., Ali-Rachedi, S., Job, C., Job, D. *et al.*, Proteomic analysis of seed dormancy in Arabidopsis. *Plant Physiol.* 2006, *142*, 1493–1510.

[19] Morohashi, Y., Peroxidase activity develops in the micropylar endosperm of tomato seeds prior to radicle protrusion. *J. Exp. Bot.* 2002, *53*, 1643–1650.

[20] Mella, R. A., Maldonado, S., Sanchez, R. A., Phytochrome-induced structural changes and protein degradation prior to radicle protrusion in *Datura ferox* seeds. *Can. J. Bot.* 1995, *73*, 1371–1378.

[21] Catusse, J., Strub, J.-M., Job, C., Van Dorsselaer, A. *et al.*, Proteome-wide characterization of sugarbeet seed vigor and its tissue specific expression. *Proc. Natl. Acad. Sci. USA* 2008, *105*, 10262–10267.

[22] Hermann, K., Meinhard, J., Dobrev, P., Linkies, A. *et al.*, 1-Aminocyclopropane-1-carboxylic acid and abscisic acid during the germination of sugar beet (*Beta vulgaris* L): a comparative study of fruits and seeds. *J. Exp. Bot.* 2007, *58*, 3047–3060.

[23] Sheoran, I. S., Olson, D. J. H., Ross, A. R. S., Sawhney, V., Proteome analysis of embryo and endosperm from germinating tomato seeds. *Proteomics* 2005, *5*, 3752–3764.

[24] Bønsager, B., Finnie, C., Roepstorff, P., Svensson, B., Spatio-temporal changes in germination and radicle elongation of barley seeds tracked by proteome analysis of dissected embryo, aleurone layer, and endosperm tissues. *Proteomics* 2007, *7*, 4528–4540.

[25] Harder, A., Wildgruber, R., Nawrocki, A., Fey, S. *et al.*, Comparison of yeast cell protein solubilization procedures for two-dimensional electrophoresis. *Electrophoresis* 1999, *20*, 826–829.

[26] Bradford, M., A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. *Anal. Biochem.* 1976, *72*, 248–254.

[27] Gallardo, K., Job, C., Groot, S., Puype, M. *et al.*, Importance of methionine biosynthesis for Arabidopsis seed germination and seedling growth. *Physiol. Plant.* 2002, *116*, 238–247.

[28] Burow, M., Bergner, A., Gershenzon, J., Wittstock, U., Glucosinolate hydrolysis in *Lepidium sativum* - identification of the thiocyanate-forming protein. *Plant Mol. Biol.* 2007, *63*, 49–61.

[29] Pawlowski, T., Proteomics of European beech (*Fagus sylvatica* L) seed dormancy breaking: Influence of abscisic and gibberellic acids. *Proteomics* 2007, *7*, 2246–2257.

[30] Bevan, M., Bancroft, I., Bent, E., Love, K. *et al.*, Analysis of 19 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature* 1998, *391*, 485–488.

[31] Gianazza, E., Wait, R., Sozzi, A., Regondi, S. *et al.*, Growth and protein profile changes in *Lepidium sativum* L plantlets exposed to cadmium. *Environ. Exp. Bot.* 2007, *59*, 179–187.

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[32] Ravanel, S., Gakière, B., Job, D., Douce, R., The specific features of methionine biosynthesis and metabolism in plants. *Proc. Natl. Acad. Sci. USA* 1998, *95*, 7805–7812.

[33] Andersson, A. M., Merker, A., Nilsson, P., Sorensen, H. *et al.*, Chemical composition of the potential new oilseed crops *Barbareea vulgaris*, *Barbarea verna* and *Lepidium campestre*. *J. Sci. Food Agric.* 1999, *79*, 179–186

[34] Gokavi, S. S., Malleshi, N. G., Guop, M., Chemical composition of garden cress (*Lepidium sativum*) seeds and its fractions and use of bran as a functional ingredient. *Plant Food Hum. Nutr.* 2004, *59*, 105–111.

[35] Penfield, S., Rylott, E. L., Gilday, A. D., Graham, S. *et al.*, Reserve mobilization in the Arabidopsis endosperm fuels hypocotyl elongation in the dark, is independent of abscisic acid, and requires PHOSPHOENOLPYRUVATE CARBOXYKINASE1. *Plant Cell* 2004, *16*, 2705–2718.

[36] Pritchard, S., Charlton, W., Baker, A., Graham, I., Germination and storage reserve mobilization are regulated independently in Arabidopsis. *Plant J.* 2002, *31*, 639–647.

[37] Marriott, K., Northcote, D., The induction of enzyme activity in the endosperm of germinating castor-bean seeds. *Biochem. J.* 1975, *152*, 65–70.

[38] Manz, B., Müller, K., Kucera, B., Volke, F. *et al.*, Water uptake and distribution in germinating tobacco seeds investigated in vivo by nuclear magnetic resonance imaging. *Plant Physiol.* 2005, *138*, 1538–1551.

[39] Penfield, S., Graham, S., Graham, I. A., Storage reserve mobilization in germinating oilseeds: Arabidopsis as a model system. *Biochem. Soc. Trans.*, 2005, *33*, 380–383.

[40] Muscolo, A., Panuccio, M. R., Sidari, M., The effect of phenols on respiratory enzymes in seed germination. *Plant Growth Regul.* 2001, *35*, 31–35.

[41] Swamy, P. M., Sandhyarani, C. K., Contribution of the pentose phosphate pathway and glycolytic pathway to dormancy breakage and germination of peanut (*Arachis hypogaea* L) seeds. *J. Exp. Bot.* 1986, *37*, 80–88.

[42] Cadman, C., Toorop, P., Hilhorst, H., Finch-Savage, W., Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. *Plant. J.* 2006, *46*, 805–822.

[43] Cheng, W., Chiang, M., Hwang, S., Lin, P., Antagonism between abscisic acid and ethylene in Arabidopsis acts in parallel with the reciprocal regulation of their metabolism and signaling pathways. *Plant Mol. Biol.* 2009, *71*, 61–80.

[44] Mutlu, A., Gal, S., Plant aspartic proteinases: enzymes on the way to a function. *Physiol. Plant.* 1993, *105*, 569–576.

[45] Nonogaki, H., Nomaguchi, M., Okumoto, N., Kaneko, Y. *et al.*, Temporal and spatial pattern of the biochemical activation of the endosperm during and following imbibition of tomato seeds. *Physiol. Plant.* 1998, *102*, 236–242.

[46] Dill, A., Jung, H.-S., Sun, T.-p., The DELLA motif is essential for gibberellin-induced degradation of RGA. *Proc. Natl. Acad. Sci. USA* 2001, *98*, 14162–14167.

[47] Fu, X., Richards, D. E., Ait-ali, T., Hynes, L. W. *et al.*, Gibberellin-mediated proteasome-dependent degradation of the barley DELLA Protein SLN1 repressor. *Plant Cell* 2002, *14*, 3191–3200.

[48] Wang, W., Vinocur, B., Shoseyov, O., Altman, A., Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends Plant Sci.* 2004, *9*, 244–252.

[49] Möller, I. M., Jensen, P. E., Hansson, A., Oxidative modifications to cellular components in plants. *Ann. Rev. Plant Biol.* 2007, *58*, 459–481.

[50] Kranner, I., Birtíc, S., Anderson, K., Pritchard, H., Glutathione half-cell reduction potential: A universal stress marker and modulator of programmed cell death? *Free Radical Biol. Med.* 2006, *40*, 2155–2165.

[51] Bailly, C., Active oxygen species and antioxidants in seed biology. *Seed Sci. Res.* 2004, *14*, 93–107.

[52] Kranner, I., Beckett, R., Wornik, S., Zorn, M. *et al.*, Revival of a resurrection plant correlates with its antioxidant status. *Plant J.* 2002, *31*, 13–24.

[53] Marrs, K., The functions and regulation of glutathione *S*-transferases in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 1996, *47*, 127–158.

[54] Dietz, K., Plant peroxiredoxins. *Ann. Rev. Plant Biol.* 2003, *54*, 93–107.

[55] Haslekas, C., Viken, M. K., Grini, P. E., Nygaard, V. *et al.*, Seed-1 cysteine peroxiredoxin antioxidants are not involved in dormancy, but contribute to inhibition of germination during stress. *Plant Physiol.* 2003, *133*, 1148–1157.

[56] Aalen, R. B., Peroxiredoxin antioxidants in seed physiology. *Seed Sci. Res.* 1999, *9*, 285–295.

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim