Regulation of seed germination in *Lepidium sativum*

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Regulation of seed germination in the close Arabidopsis relative Lepidium sativum: A global tissue specific transcript analysis

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The completion of germination in *Lepidium sativum* and other endospermic seeds (e.g. Arabidopsis) is regulated by two opposing forces, the growth potential of the radicle (RAD) and the resistance to this growth from the micropylar endosperm cap (CAP) surrounding it. We show by puncture force measurement that the CAP progressively weakens during germination and we have conducted a timecourse transcript analysis of RAD and CAP tissues throughout this process. We have also used specific inhibitors to investigate the importance of transcription, translation and posttranslation levels of regulation of endosperm weakening in isolated CAPs. Although the impact of inhibiting translation is greater, both transcription and translation are required for completion of endosperm weakening in the whole seed population. The majority of genes expressed during this process occur in both tissues, but where they are uniquely expressed, or significantly differentially expressed between tissues, this relates to the functions of the RAD as growing tissue and the CAP as a regulator of germination through weakening. More detailed analysis showed that putative orthologs of cell-wall remodelling genes are expressed in a complex manner during CAP weakening suggesting distinct roles in the RAD and CAP. Expression patterns are also consistent with the CAP being a receptor for environmental signals influencing germination. Inhibitors of the aspartic, serine, and cysteine proteases reduced the number of isolated CAPs in which weakening developed, and inhibition of the 26S proteasome resulted in its complete cessation. This indicates that targeted protein degradation is a major control point for endosperm weakening.
The seed germination process begins with imbibition of the dry seed and is completed when the radicle has emerged through all the layers enveloping the embryo (Finch-Savage and Leubner-Metzger, 2006). In both *Arabidopsis thaliana* (Arabidopsis) and *Lepidium sativum* (Lepidium) there are two such layers, an outer dead testa (seed coat) and beneath that a layer of living endosperm cells (aleurone layer). Germination in these species has two separate visible stages: firstly the testa ruptures and then the lower hypocotyl/radicle (RAD) extends to complete germination by rupturing the micropylar endosperm layer (CAP) that covers it. A recent publication by Sliwinska et al. (2009) describes how embryo elongation during Arabidopsis seed germination is due to cell expansion growth in a specific zone in the lower hypocotyl/radicle transition region. During the latter process the CAP weakens through autolysis to reduce the mechanical resistance to radicle protrusion. Biomechanical measurements have been used to record such weakening in species from a variety of different families (e.g. Bewley, 1997; Toorop et al., 2000; da Silva et al., 2004; Finch-Savage and Leubner-Metzger, 2006). However, Arabidopsis seeds are too small for such measurements, with the techniques used to date, and this has limited progress in linking biomechanical and molecular studies. To overcome this obstacle we have demonstrated that the larger seeds of Lepidium can be used as a model system for studying both the molecular and biomechanical mechanisms of endosperm cap weakening (Müller et al., 2006; Müller et al., 2009; Linkies et al. 2009). In this work, direct biomechanical measurement has shown that endosperm cap weakening is promoted by gibberellins (GA) and inhibited by abscisic acid (ABA). This endosperm weakening is induced by an early signal from the embryo, after which, weakening and lysis proceed as an organ-autonomous process. Further experimentation has shown that in isolated endosperm caps GA can replace the embryo signal, that *de novo* GA biosynthesis occurs in the endosperm, and that the weakening is regulated, at least in part, by the GA/ABA ratio.

The genera *Lepidium* and *Arabidopsis* both belong to the lineage I clade of the *Brassicaceae* family and are therefore closely related (Franzke et al., 2009). As may be expected from this close relationship, the above findings in Lepidium are consistent with the known spatial, temporal and GA-mediated regulation of genes during Arabidopsis seed germination (Yamaguchi et al., 2001; Ogawa et al., 2003; Yamauchi et al., 2004). Separate global expression profiles of the whole embryo and endosperm shortly after radicle emergence in Arabidopsis are also consistent with this pattern of regulation (Penfield et al. 2006). Comparison of the transcriptomes of endosperm and embryo tissues at a single time point of 24h also showed large differences in expression between the tissues (Okamoto et al, 2010). However, to date there has
been no similar analysis of the changes in these tissues leading to the completion of germination.

To take advantage of their close relationship we carried out a global transcript analysis of the interaction between individual seed tissues in a time course during germination of Lepidium by cross species hybridization to a full genome Arabidopsis array (Linkies et al., 2009). The larger size of Lepidium enabled us to use RNA samples collected specifically from the CAP and RAD to avoid confounding the results with other tissues in the embryo and other regions of the endosperm. This work demonstrated that the CATMA 25K microarrays (Hilson et al., 2004; Allemeersch et al., 2005), which are spotted with PCR-amplified Arabidopsis gene-specific tags (GST; 150-500 bp), were effective for comparative genomics by cross-species microarray hybridization with Lepidium. Such cross-species hybridizations for closely related species, using several array platforms, have become an accepted approach where no species specific arrays are available (reviewed by Van de Mortel and Aarts, 2006; Bar-Or et al., 2007; Broadley et al., 2008). CATMA microarrays have also been shown to be effective for cross-species microarray hybridization in work by Slotte et al. (2007), in which Capsella bursa-pastoris ('Capsella') accessions differing in flowering time were compared at the transcriptome level. Capsella, like Lepidium and Arabidopsis is from the lineage-I clade of the Brassicaceae (Franzke et al., 2009).

In Linkies et al. (2009) a preliminary analysis of these cross-species Lepidium arrays indicated that ethylene-related transcripts were over-represented in the lists of regulated genes. The array data was therefore used to complement an investigation of the interaction of ethylene with ABA, which resulted in a model for the hormonal regulation of endosperm cap weakening and rupture. In the present work we investigate the importance of the transcription, translation and posttranslation stages in the regulation of germination through endosperm weakening. We also carry out a full global transcript analysis of the RAD and CAP tissues during the germination process.

RESULTS AND DISCUSSION

The progression of germination is clearly linked to endosperm weakening that requires both transcription and translation for completion in the whole seed population.
The completion of germination (radicle emergence) in Lepidium is regulated by two opposing forces, the growth potential of the RAD and the resistance to this growth from the seed covering layers (testa and CAP). After testa rupture the latter is determined by the strength of the endosperm, which can be determined by puncture force measurement, and this progressively decreases towards germination completion (Fig. 1). Onset of endosperm weakening occurs after 8 h of imbibition, on medium without hormonal addition (-ABA in Fig. 1), and its progression results in the occurrence of endosperm rupture and germination completion in an increasing proportion of the seed population up to 18 h (Fig. 1A). Both the onset and the completion of endosperm weakening are delayed by the addition of ABA, shifting its onset to more than 30 h imbibition and its completion to 96/120 h. At the onset of endosperm weakening there is a high variance in the force required to puncture the endosperm (Fig. 1B). This variance declines as the endosperm weakens in an identical fashion, with and without ABA (Fig. 1C), indicating that ABA has an effect only on the timing of this process. ABA therefore provides a means of spreading out the process of endosperm weakening enabling samples to be taken at several stages, both before and during the process. Overall, these results show that endosperm weakening is not just an imbibition-effect but clearly related to the progression of the germination process. Single-tissue analyses of the RAD and CAP should therefore provide a means to identify mechanisms underlying the germination process.

Dry seeds store mRNAs, which are assumed to contain transcripts for genes that are important for both late embryogenesis and for early seed germination (Comai, 1989; Hughes and Galau, 1989, 1991). Upon imbibition transcriptional changes take place, and after the first 3 h huge changes in transcript abundance are already evident in seeds of Arabidopsis (Nakabayashi et al. 2005; Preston et al. 2009; Kimura andNambara 2010). Rajjou et al. (2004) have shown that inhibiting this transcription with α-amanitin delays the germination process of whole seeds and inhibits seedling development in Arabidopsis. Transcription inhibitors have also delayed germination in wheat embryos (Jendrisak, 1980), and endosperm rupture in tobacco seeds (Arcila and Mohapatra, 1992). In contrast, inhibition of translation by cycloheximide entirely blocks the completion of germination in whole seeds (Rajjou et al. 2004). We have utilized the bigger seeds of Lepidium to investigate, in a similar way, the necessity of transcription and translation during endosperm weakening in individual seed tissues. When Lepidium CAPs were dissected from -ABA-imbibed seeds (Fig. 2A, B), and incubated individually, the initial autolysis caused either hole formation close to where the radicle in an intact seed would penetrate through the endosperm, and/or abscission of the CAP tip (Fig. 2C). Subsequent progressive
autolysis later disrupts the whole CAP (Fig. 2D). We exploited this situation to observe the influence of the inhibitors α-amanitin and cycloheximide on the progression of endosperm weakening. Unlike when whole seeds are used, there are no problems with uptake of inhibitors into the tissues using this system.

Incubation on α-amanitin following dissection slowed the progress of autolysis and prevents the completion of the process in a proportion of the CAPs (Fig. 2E). In contrast, cycloheximide completely blocks autolysis of more than 90% of CAPs (Fig. 2E) even during the very late stages of germination, i.e. following dissection at 18 h when some seeds in the population have already begun autolysis in situ. Comparison of the progression of autolysis in control CAPs dissected at 10 and 18 h suggests that the process occurs more quickly in the presence of the RAD in whole seeds than it does after dissection, i.e. CAPs dissected at 18 h are further progressed than CAPs dissected at 10 h plus 8 h further incubation following dissection (Fig. 2F). If the same treatments are applied to the RAD dissected after 18 h no inhibition of growth was observed on α-amanitin, but cycloheximide significantly inhibited radicle growth (Supplemental Table S1). These findings show that, in addition to translation, transcription is very important in the CAP, and comparative global transcriptome analysis of both tissues will be very informative.

**Arabidopis CATMA microarrays can be used effectively to investigate patterns in Lepidium transcript expression**

To investigate how Lepidium gene transcripts in specific seed-tissues are regulated temporally and spatially, we hybridized Lepidium RNA samples to Arabidopsis CATMA 25k microarrays (Complete Arabidopsis Transcriptome Microarray, www.catma.org, Hilson et al., 2004; Allemeersch et al., 2005). The RNA was extracted from specific Lepidium seed tissues (RAD, CAP, and non-micropylar endosperm (NME)) at defined time points during germination. These tissues were collected after testa rupture, before and during endosperm weakening, but prior to endosperm rupture, i.e. only seeds with intact endosperm were used.

The principal experiment (+ABA-arrays) produced samples from seeds imbibed on medium with ABA (10 µM) as this slows the germination process allowing the dissection at earlier developmental stages, i.e. dissection is not possible before 8 h of imbibition, but without ABA (-ABA-arrays) changes that lead to endosperm weakening have already occurred (Linkies et al., 2009). We therefore compared RAD and CAP from seeds incubated in medium containing 10
μM ABA, at 8, 18 and 30 h leading up to the onset of endosperm weakening, and later at 96h, just prior to endosperm rupture (Fig. 1). In this experiment 10 μM ABA slows the germination process, but importantly does not prevent the completion of germination (radicle emergence). Indeed, the relationship between decreasing endosperm cap puncture force and increasing percentage of seeds showing endosperm rupture was almost identical with and without ABA despite the very different rates of this process on these solutions (Linkies et al., 2009; Fig. 1). In a further smaller experiment (-ABA-arrays) seeds were imbibed on medium without ABA and samples were prepared at 8 and 18 h from RAD, CAP and NME. These data are used to confirm results collected in the first experiment and to help aid the identification of CAP specific gene expression.

Normalized expression values for Lepidium were obtained in the +ABA-arrays for 19,794 CATMA probes to which there was significant transcript hybridisation (Supplemental Table S2) and in the -ABA-arrays for 22,025 probes (Supplemental Table S3). Lepidium gene transcripts that hybridised to these probes were assigned as putative Arabidopsis orthologs defined by having an Arabidopsis Genome Initiative (AGI) identifier such as At1g62380 and a gene ontology (GO) annotation associated with this AGI (www.arabidopsis.org). Henceforth, to avoid repetitive use of the term, putative orthologs in Lepidium will be referred to using AGI annotation.

All microarray data including the normalised intensity values for each microarray were deposited in ArrayExpress (www.ebi.ac.uk/microarray/; +ABA-arrays accession no. E-TABM-743; -ABA-arrays accession no. E-TABM-745). To support the use of these cross species hybridizations, Linkies et al. (2009) verified the transcript expression pattern of the arrays by comparing them to corresponding qRT-PCR results obtained with independent biological RNA samples from a separate experiment. They concluded that cross-species microarray hybridization with the CATMA platform is a useful and effective tool for heterologous transcriptomics with Lepidium.

There are differences in the pattern of transcription between the radicle tip and the endosperm cap, but much of the temporal change is common to both tissues

Principal component analysis (PCA) was used to look for global patterns in the Lepidium expression data across all the gene transcripts (Fig. 3). The two components PC1 and PC2 accounted for more than 60% of the variance in gene expression. PC1 clearly separated RAD and CAP (Fig. 3A). PC2 then separated the times in a continuous temporal order. These clear patterns indicate that the data behave in an expected fashion with greatest differences occurring
between the tissues. The comparison indicates that the majority of change in transcript numbers occurs before endosperm weakening (i.e. 8-18 h). The very similar ordering of the time course suggests that much of this change in the earlier stages of germination is common to the two tissues. PC3 confirms the step change between 8 h and 18 h with a subsequent smaller progressive change 18-96 h (Fig. 3B). Distances between RAD and CAP are similar at 8 h and 18 h, least at 30 h and then greatest at 96 h, coinciding with the period of endosperm weakening from 30 to 96 h (Fig. 1) and preparation for radicle expansion and emergence.

The majority of the genes expressed (“present”) during germination occur in both tissues, but where they are uniquely expressed this relates to their specific functions

To determine whether individual genes were expressed or not, the normalised values for each probe were compared to those for the 912 empty spots on the arrays with a one-sided t-test. Probes for which the normalised values were significantly greater than the empty spots (p<0.05) were considered to be “expressed”. The data shown in Table I are the number of probes on the array that indicate expression based on this criterion. In agreement with the PCA the majority of genes expressed at any time point are expressed in both the CAP and RAD (common, Table I). Similarly the majority of genes expressed in successive time points in the same tissue are common. The number of commonly expressed genes range from 8045 to 10493. This is a very similar number to that found by Penfield et al. (2006) shortly after radicle emergence in Arabidopsis seeds. They found 9650 genes in common with approximately 4000 that were expressed differentially in the embryo or the endosperm. They concluded that patterns of gene expression are broadly similar between the two organs suggesting similar post-germinative metabolism occurring in these tissues. We show here that this similarity extends to the germination process.

As Penfield et al. (2006) found post-germination in Arabidopsis, there are a number of genes that are expressed in one tissue, but not the other at all time points leading to germination completion. At 8 h the number of genes uniquely expressed in the RAD (4087) is much greater than in the CAP (917). This suggests much greater early transcriptional activity in the RAD than the CAP. In barley, Barrero et al. (2009) suggest that the coleorhiza is functionally related to the endosperm cap in Arabidopsis and Lepidium since it appears to regulate germination by restricting radicle elongation. In this species they show that 23% of genes are differentially expressed during first 8 h in the coleorhiza, but only 16% in the radicle, suggesting a more active
role for the former. This is opposite to the results shown here, however, the coleorhiza initially elongates (it grows) with the root before the root penetrates it. This may explain the different pattern of gene expression in the two species since the CAP of Lepidium shows no such growth.

By 18 and 30 h this ratio has changed so that the numbers of genes uniquely expressed is 27 and 22% greater respectively in the CAP than the RAD, by 96 h the number of genes expressed in the two tissues is more similar. To investigate whether these differences were linked to functional specialisation of the CAP and RAD tissues we applied the GO-based seed-specific TAGGIT workflow (Carrera et al., 2007) to identify proportional representations of genes into functional categories (Supplemental Table S4). There are also a number of genes whose expression is unique to each tissue/time combination (Table I in parenthesis), these range from 145 (30 h RAD) to 513 (8 h RAD). Again the GO-based seed-specific TAGGIT workflow (Carrera et al., 2007) was used to categorise these genes (Supplemental Table S5).

At 8 h, the numbers of genes represented in TAGGIT categories is greater in the RAD than CAP (Supplemental Table S4), which in general reflects the pattern in the total numbers expressed (Table I). There are also tissue-specific differences in the numbers of genes uniquely expressed at each time point. In general, for the RAD the numbers of genes are greater than in the CAP in the following categories: dormancy related; brassinosteroid; ethylene, cell cycle related; cytoskeleton and translation associated. This reflects the radicle as growing tissue. Whereas, for the CAP after 8 h the numbers of genes are greater than for the RAD in the following categories: gibberellins, jasmonic acid, glycolysis and gluconeogenesis; Krebs cycle; beta oxidation; and stress. This pattern may reflect the function of the endosperm to regulate germination through its autolysis and subsequent death. These differences in the two tissues are broadly similar to those found by Penfield et al. (2006) in post-germinative Arabidopsis tissues. In contrast, there is little difference in the numbers of common genes that are expressed by both tissues in any TAGGIT category between time points and little pattern is shown in the data (Supplemental Table S4). Similarly there is little difference and pattern in other genes expressed in common between time points and tissues (data not shown).

There are differences in the numbers of gene transcripts that are differentially regulated in the RAD and CAP

The transcript abundance of individual genes in the +ABA-array data were compared between
the RAD and the CAP at each time point using t-Tests to identify which genes showed
differential expression relative to each other. P-values were adjusted for false discovery rate
(Benjamini and Hochberg, 1995) and the resulting gene lists (p-values ≤ 0.10) are given in
Supplemental Table S6. These genes were considered to be up- or down-regulated between
tissues at the time point specified. The total number of genes that are differentially regulated
between the RAD and the CAP increases as the seeds progress towards germination and
presumably the functional specialisation of the tissues develop (Table II). The numbers of genes
that are up-regulated in the RAD and CAP are similar at 8, 18 and 96 h, but at 30 h the number
is higher in RAD (1000) compared to Cap (783). If higher stringency is applied to the analysis
the overall pattern shown in Table II remains the same, but with fewer genes (e.g. for 96 h at
P<0.10, 2464; at P<0.05, 1600; data not shown).

To further investigate the functional specialisation of the tissue we applied the seed-specific
TAGGIT workflow (Carrera et al., 2007) to the genes up-regulated in either the RAD or the CAP
relative to the other (Table II). Although the number of up-regulated genes in both tissues is
similar there are differences in the categories of genes that are over-represented at all time
points. In general, the categories with gene numbers over-represented in the CAP are related to
hormones, aspects of metabolism and reserve mobilisation, and stress. Whereas, the categories
with gene numbers over-represented in the RAD are related to dormancy, late embryogenesis
abundant proteins, aspects of growth, and DNA repair. The most over-represented category in
RAD is translation associated proteins with a 26 fold higher number of genes across the four
time points than the CAP (397 and 15 respectively). However, when viewing these data it should
be remembered that the actual number of different genes is less than this since genes can be
represented at more than one time point. Protein synthesis and ribosomal protein genes were
also highly expressed in the embryo relative to the endosperm in Arabidopsis shortly after
germination, and this was linked to a fivefold higher number of ribosomes in the embryo than the
endosperm (Penfield et al., 2006).

There are similar numbers of genes up-regulated in both tissues in the following categories: cell-
wall modification and protein degradation. This superficial similarity obscures important
differences in the detail that are explored below. TAGGIT effectively summarises the
proportional representation of seed-specific genes into functional categories, however, the gene
lists used are no longer entirely current. In the following sections we investigate categories
identified with TAGGIT, but use comprehensive gene lists that extend beyond those known to be
seed-specific.

**Genes involved in gibberellin (GA) and abscisic acid (ABA) signalling networks have different temporal and spatial patterns consistent with regulation through subtle changes in hormone sensitivity**

In Linkies et al. (2009) a model for the hormonal regulation of endosperm cap weakening and rupture was constructed for Lepidium. In this model, GA is an embryo signal that releases coat dormancy (if present) and induces the CAP weakening process. Thereafter weakening is a cap-autonomous process, with the rate regulated by GA-ABA and ethylene-ABA antagonisms that result in the completion of germination. There are many proteins involved in the regulatory networks controlling this process and current understanding can be found in several recent comprehensive reviews (Holdsworth et al., 2008a; Finkelstein et al., 2008; Penfield and King, 2009). Hormone signalling, especially that resulting from the dynamic balance of GA and ABA, is a key component of these networks, which are thought to have significant interactions (Kucera et al., 2005, Holdworth et al., 2008a). Understanding of ABA signal transduction is developing rapidly and a model has recently emerged in which PYR/PYL/RCAR receptors bind to ABA to remove the repression by PP2Cs of downstream signalling via SnRK2s to ABRE-regulated gene expression by the transcription factors ABI3, ABI4, and ABI5 (Cutler et al., 2010; Fig. 5A). On the other side of this balance, DELLA proteins repress GA responses and therefore germination (Sun and Gubler, 2004). DELLA proteins are degraded to remove this repression when they form a complex with GA and GID1 receptors (Hartweck, 2008; Fig. 5A). These signalling networks are influenced by a diverse range of environmental signals during germination, principally temperature and light. Key components of the interaction between these two environmental signals and GA are the two phytochrome-interacting bHLH transcription factors, PIL5 and SPT5; these both repress germination in the dark (Penfield et al., 2005).

In the Lepidium after-ripened seed used in this work the kinetics of endosperm rupture is strongly dependant on temperature, but not light and there is no obvious evidence of residual dormancy. This situation contrasts with Arabidopsis seeds that are light sensitive and so expression patterns may differ from those anticipated from work on Arabidopsis. Transcript levels of genes encoding the components of the hormone signalling networks in the CAP and RAD during Lepidium germination are shown in Fig. 5B. Although germination of our Lepidium seed batch was not responsive to light, phytochrome genes are surprisingly shown to be some of the most highly
expressed in both tissues. These genes are up-regulated in the CAP relative to the RAD, in particular *PHYA*. –ABA-array results indicate that expression of *PHYA* is higher in the CAP than the NME and is therefore CAP specific. In Arabidopsis, *SOM* is thought to encode a component of the phytochrome signal transduction pathway that regulates genes in hormone metabolism, and acts as negative regulator in PHYA-mediated promotion of germination (Kim et al., 2008). However, in Lepidium *SOM* is expressed very highly in both tissues, which from its function in Arabidopsis appears counter intuitive in these actively germinating seeds. The reason for these very clear expression patterns with *PHYA* and *SOM* in these light insensitive Lepidium seeds is not clear. *PIL5* expression is low as expected for a negative regulator of germination in this situation. *SPT* tends to be more highly expressed in the CAP, but transcript levels are low. These results are consistent with the CAP being the principal receptor for environmental signals influencing germination.

In general, genes relating to GA signalling are more highly expressed than those relating to ABA signalling (Fig. 5B) and this is consistent with expectations for non-dormant seeds progressing towards the completion of germination. Nevertheless, it is interesting to note that *ABI4* expression is significantly up-regulated in the RAD, whereas, *ABI5* expression tends to be higher in the CAP and *ABI3* is similarly expressed in both tissues at a low level. This is entirely consistent with the results of Penfield et al. (2006) who showed, using GUS fusions, that in Arabidopsis *ABI3* is expressed in embryo and endosperm, *ABI4* expression was specific to the embryo, and although *ABI5* was expressed in the embryo and endosperm expression in the latter was CAP specific. *ABI4* is thought to repress lipid breakdown in the seed (Penfield et al., 2006). Another note of interest with ABA signalling is that genes encoding for ABA receptors each exhibit distinct patterns, but where there is a significant differential expression between tissues, for example *PYL4, PYL5, PYL 6*, and *PYR1* they are up-regulated in the CAP.

Seeds with an absence of GA receptors fail to germinate (Griffiths et al., 2006; Willige et al., 2007) and by binding to GA and DELLAs the latter are degraded to de-repress germination (Fig. 5A). Genes encoding these receptors are up-regulated in the CAP, in particular *GID1A* and *GID1C*, the latter late in the germination process. Interestingly, the reverse is true with DELLA repressor genes, which are expressed more highly in the radicle, in particular *RGL3* early in the germination process. In Arabidopsis, RGL3 represses testa rupture in response to changes in GA and ABA levels (Piskurewicz and Lopez-Molina, 2009). As with the ABA receptors each of these genes displays a different temporal pattern suggesting regulation occurs through a
complex mix of subtle controls with the potential to be highly responsive to the prevailing conditions. Regulation clearly does not result solely from a simple hormone balance, but additionally through differing spatial and temporal sensitivity to these hormones generated in the hormone signalling networks.

**There is a complex pattern of gene expression linked to proteins associated with cell-wall modification that underlies CAP weakening**

Much of the differences in gene expression between the CAP and RAD are likely to result from the functional specialisation of the whole endosperm as an embryo nutritional tissue during seed development and the RAD as a growing tissue in the germinating seed. However, the CAP in Lepidium is specifically associated with the regulation of germination through endosperm weakening (above and Müller et al., 2006; Linkies et al., 2009) which requires cell-wall modification by cell-wall remodelling proteins (CWRPs). Table II indicated that a similar number of genes in TAGGIT cell-wall modification category are up-regulated in the two tissues at different time points. The total number of genes at all time points is 76 and 71 for CAP and RAD respectively. However, on closer inspection there are very different patterns to the expression of CWRP genes in the two tissues (Fig 5; Supplemental Fig. S1).

Plant cell expansion growth is driven by water uptake and restricted by the cell wall. The mechanical strength of the plant cell wall determines the shape and the rate and direction of growth of individual cells as well as the mechanical resistance of whole tissues (Fry, 2004; Cosgrove, 2005; Schopfer, 2006; Knox, 2008). The primary cell wall has a fibreglass-like structure with crystalline cellulose microfibrills that are embedded in a matrix of complex polysaccharides, which are divided into two classes: hemicelluloses and pectins. Hemicelluloses are cellulose-binding polysaccharides, which together with cellulose form a network that is strong yet resilient. Pectins form hydrated gels that push microfibrils apart, easing their sideways slippage during cell growth, while also locking them in place when growth ceases. They are important determinants of wall porosity and wall thickness and they glue cells together in an adhesive layer called the middle lamella. Known wall-remodelling mechanisms include reactive oxygen species (ROS)-mediated polysaccharide scission and CWRP actions include enzymatic hydrolysis, transglycosylation and expansin action. Cell wall loosening is an important developmental process in all stages of plant development requiring elongation growth or tissue weakening. Examples include seed germination (Bewley, 1997; Nonogaki et al., 2007; Müller et
Ikuma and Thimann (1963) in their "hatching hypothesis" of seed biology suggested that "... the final step in the germination control process is the production of an enzyme whose action enables the tip of the radicle to penetrate through the coat". In searching for this "hatching enzyme", evidence has been uncovered for the contribution of various CWRPs, including endo-β-1,4-mannanases (e.g. Bewley, 1997; Nonogaki et al., 2000; Iglesias-Fernandez et al., 2011) and endo-β-1,3-glucanases (e.g. Leubner-Metzger, 2002; Leubner-Metzger, 2003; Petruzzelli et al., 2003), as well as for ROS (Müller et al., 2009), but most of this work was in Solanaceous seeds. However, endosperm weakening is also evident in Brassicaceae seeds where it is promoted by GA and ethylene, and inhibited by ABA (Debeaujon and Koornneef, 2000; Debeaujon et al., 2000; Müller et al., 2006; Bethke et al., 2007; Linkies et al., 2009; Iglesias-Fernandez et al., 2011). Based on the timing of GA-inducible transcripts in whole seeds of Arabidopsis many CWRP genes that remodel hemicellulose are expressed during the early germination phase (Ogawa et al., 2003; Nonogaki et al., 2007). Our tissue-specific transcriptome analysis with Lepidium (this work and Linkies et al., 2009) shows that many of the bigger CWRP families exhibit complex temporal and spatial expression patterns that are presented in the Supplemental Figures/Tables. We therefore restrict our subsequent discussion to a selection of early-expressed hemicellulose-related genes that are abundant during CAP weakening.

Expansins are plant cell-wall loosening proteins which disrupt noncovalent bonds that tether matrix polysaccharides to the surface of cellulose microfibrils, or to each other (Sampedro and Cosgrove, 2005; Choi et al., 2006). Whatever their biochemical mechanism of action, expansins act in catalytic amounts to stimulate wall polymer creep without causing major covalent alterations of the cell wall. The α-expansins (EXPA) act with a pH optimum around 4. They have possible roles in developmental processes like organ size and elongation growth, fruit tissue softening and seed germination (Sampedro and Cosgrove, 2005; Choi et al., 2006; Gaete-Eastman et al., 2009; Lizana et al., 2010). Transcripts of the tomato α-expansin LeEXPA4 and its putative ortholog in Datura ferox were specifically expressed in the micropylar endosperm in association with endosperm weakening (Chen and Bradford, 2000; Mella et al., 2004). This transcript expression was promoted by GA, but not inhibited by ABA. Where there is a significant difference in the level of expression between the tissues the majority of Lepidium expansin genes (Fig. 5A, Supplemental Fig. S1A) are expressed more highly in the CAP than the RAD.
This is particularly true for the α-expansins EXPA1, 2, 7, 8, 9 for which in the +ABA-arrays the transcript levels are higher in the CAP compared to the RAD at all time points (Fig. 5A); only EXPA3 was RAD-specific. The –ABA-arrays show that EXPA1, 2, 9 are specifically expressed in the CAP, but not in the NME. ABA does not down-regulate any of these CAP-specific α-expansin genes. During the early phase of Arabidopsis seed germination, transcripts of AtEXPA1, 2, 8, 9 accumulate 100- to 500-fold (from 0 h to 12 h in whole unstratified seeds) and this induction is promoted by GA, not inhibited by ABA, and mainly localized in the endosperm (Nakabayashi et al., 2005; Penfield et al., 2006; Carrera et al., 2008; Holdsworth et al., 2008a; Preston et al., 2009). In summary, transcript expression analysis during germination of both Lepidium and Arabidopis shows that α-expansin genes, in particular EXPA2, are induced early in the endosperm cap prior to the onset of weakening and are involved in ABA-insensitive processes that lead to testa rupture and cap weakening (this work and Linkies et al., 2009). Based on their temporal, spatial and hormonal expression pattern α-expansins are likely to contribute to processes in the CAP prior to and during endosperm weakening and rupture, but they do not confer the ABA-regulation of these processes.

Xyloglucan endotransglycosylase/hydrolase (XTHs) modify xyloglucans, which are part of the hemicellulose network believed to crosslink cellulose microfibrils (Fry, 2004; Cosgrove, 2005). Xyloglucan is the primary XTH substrate, and most XTHs exhibit XET (transferase) activity that breaks and remakes glycosidic bonds in the backbone of xyloglucan. Some XHTs also exhibit XEH (hydrolase) activity and mediate xyloglucan strand breaks, and a few exhibit only XEH activity. Direct unambiguous proof of XTHs inducing wall stress relaxation and extension is still lacking. However, XTHs are implicated in cell-wall hemicellulose remodelling leading to loosening (Van Sandt et al., 2007) or stiffening (Maris et al., 2009). XTHs are proposed to have roles in many developmental processes including cell growth, fruit ripening and reserve mobilisation following germination of xyloglucan storing seeds (Tine et al., 2000; Fry, 2004; Nonogaki et al., 2007; Van Sandt et al., 2007). During tomato seed germination transcript accumulation of LeXET4 was induced in the micropylar endosperm, promoted by GA, but not inhibited by ABA (Chen et al., 2002). The phylogenetic relationship between the 33 members of the Arabidopsis XTH gene family reveal 3 groups (http://labs.plantbio.cornell.edu/XTH/arabidopsis.html, Becnel et al., 2006). Several XTH genes of Lepidium showed tissue-specific expression during seed germination (Fig. 5B, 5C, 5D, Supplemental Fig. S1B). The group 2 XTHs 15, 16, 20, 22, 23, 24, and group 3 XTHs 27, 28 and 31 exhibit stronger expression in the CAP compared to the RAD. In contrast, the group 1 XTHs 5
and 8, and the group 3 XTH32 exhibit stronger expression in the RAD compared to the CAP. During the early phase of Arabidopsis seed germination, of the above mentioned genes only transcripts of AtXTH5, 16 and 27 accumulated significantly at 6 h, and AtXTH15, 22, 28 and 31 at 12 h, while AtXTH20, 23 and 24 were not induced. Only XTH15, 16, 5, 31 were induced by GA, and only XTH24 was down-regulated by ABA (Nakabayashi et al., 2005; Preston et al., 2009). In summary, transcript expression analysis during seed germination of both Lepidium and Arabidopsis shows that XTH genes are expressed in a complex manner suggesting distinct roles in the RAD and CAP.

**Mannans** are rigidity and mechanical strength conferring hemicellulosic polysaccharides present in the endosperm of many seeds (Bewley, 1997; Reid et al., 2003; Nonogaki et al., 2007). The endosperm cell walls of Solanaceous seeds contain ca. 60% mannose and ca. 10% galactose as galactomannans. Coffee galactomannan contains only ca. 2% galactose which results in hard and brittle endosperm properties. Mannan polysaccharides could be masked and this may have prevented the detection of mannan epitopes in Arabidopsis seeds, but genetic evidence has strongly indicated a functional role for mannan in seed development and germination of this species (Marcus et al. 2010; Iglesias-Fernandez et al. 2011). Galactomannan biosynthesis in seed endosperms involves β-1,4-mannan synthase and galactomannan galactosyltransferase (Reid et al., 2003; Edwards et al., 2004). The β-1,4-mannan synthases are encoded by the cellulose synthase-like A (CslA) gene family (Dhugga et al., 2004; Liepman et al., 2005). AtCslA2 transcripts accumulated in germinating Arabidopsis seeds in a GA-promoted and ABA-unaffected manner (Nakabayashi et al., 2005; Preston et al., 2009). In Lepidium CSL2 and CSL9 showed a radicle-specific expression during seed germination (Fig. 5E, Supplemental Fig. S1C).

Degradation of mannan and galactomannan polymers involves endo-β-1,4-mannanase, α-galactosidase, and β-mannosidase, all of which have been identified in germinating seeds; several endo-β-1,4-mannanases have hydrolase and endotransglycosylase activity (Schroder et al., 2009). Among the many endo-β-1,4-mannanase isoforms of tomato, the LeMAN2 gene is expressed specifically in the micropylar endosperm prior to radicle emergence in association with enzyme activity accumulation (Nonogaki et al., 2000; Toorop et al., 2000; Gong and Bewley, 2007). This induction is promoted by GA, but not inhibited by ABA. Endo-β-1,4-mannanase also accumulates in the micropylar endosperm of Solanum lycocarpum, Datura ferox, and coffee and is thought to contribute to endosperm weakening (Bewley, 1997; Nonogaki et al., 2000; Toorop et al., 2000; da Silva et al., 2004; Arana et al., 2006; Pinto et al., 2007).
Endo-\(\beta\)-1,4-mannanase enzyme activities of individual tomato micropylar endosperm caps varies at least 100-fold (Still and Bradford, 1997). Although the presence of endo-\(\beta\)-1,4-mannanase enzyme activity in the tomato endosperm cap is consistently associated with radicle emergence, it is not the sole or limiting factor under all conditions. Seed germination of tomato lines over-expressing an endo-\(\beta\)-1,4-mannanase was not promoted (Belotserkovsky et al., 2007). Seed-specific regulation of several endo-\(\beta\)-1,4-mannanases are also known from rice (Yuan et al., 2007; Ren et al., 2008). Seven endo-\(\beta\)-1,4-mannanase genes are known in Arabidopsis (Yuan et al., 2007), but of these only \(AtMan7\) (At5g66460) transcripts accumulated in whole unstratified seeds and this induction is promoted by GA, but not inhibited by ABA (Nakabayashi et al., 2005; Preston et al., 2009). In agreement with this, transcripts of the Lepidium \(MAN7\) accumulated in the CAP and to a lesser extent in the RAD during seed germination (Fig. 5E; Supplemental Fig. S6C). This induction was not inhibited by ABA, and at 8 h was stronger in CAP than the RAD and the NME (Fig. 5E). Figure 6A and 6B show that endo-\(\beta\)-1,4-mannanase enzyme activity accumulated in the CAP and the RAD, but less in the NME prior to endosperm rupture. This increasing activity is, at least in part, due to \(LesaMAN7\) as the transcript expression pattern is regulated in a similar manner (Fig. 6C). Late during germination and after endosperm rupture, endo-\(\beta\)-1,4-mannanase enzyme activity and \(LesaMAN7\) transcripts also accumulate in the cotyledons (Fig. 6). In agreement with a role for \(LesaMAN7\) in germination, a recent study shows that Arabidopsis knockout mutants for \(AtMAN7\), 6 and 5 had slower germination than the wild-type (Iglesias-Fernandez et al., 2011). In seeds of \(Sisymbrium officinale\), which is also a Brassicaceae, endo-\(\beta\)-1,4-mannanase enzyme activity accumulated in an ethylene- and GA-promoted manner (Iglesias-Fernandez and Matilla, 2009).

\(\alpha\)-Galactosidases and \(\beta\)-mannosidases contribute to seed galactomannan degradation (Bewley, 1997; Feurtado et al., 2001; Nonogaki et al., 2007), and \(\beta\)-mannosidase enzyme activity has been detected in the micropylar endosperm of tomato seeds, \(Datura\), and coffee (de Miguel et al., 2000; Mo and Bewley, 2002; da Silva et al., 2005). In Arabidopsis seeds, \(At3g18080\) \(\beta\)-mannosidase transcripts accumulate mainly in embryo and \(At3g57520\) \(\alpha\)-galactosidase transcripts were abundant between 3 h and 24 h in whole seeds (Nakabayashi et al., 2005; Penfield et al., 2006; Preston et al., 2009). Transcripts of Lepidium \(\alpha\)-galactosidases and \(\beta\)-mannosidases had complex tissue-specific patterns (Fig. 5F). Taken together, these results support a role for endo-\(\beta\)-1,4-mannanase during the germination of endospermic Brassicaceae seeds.
Cellulase (endo-β-1,4-glucanase) activity was detected in tomato, *Datura* and coffee seeds (Sanchez et al., 1986; da Silva et al., 2004; Nonogaki et al., 2007). In *Datura* and coffee, but not in tomato, this was in association with endosperm weakening and germination. Several putative orthologs of Arabidopsis showed a CAP-specific expression pattern during seed germination of Lepidium, while orthologs of At1g70710 and At1g64390 showed a RAD-specific expression (Supplemental Fig. 6D). During the early phase of Arabidopsis seed germination, transcripts of At1g70710 and At1g64390 accumulate ca. 100-fold (from 0 h to 24 h of whole unstratified seeds) and this induction is promoted by GA, but not appreciably inhibited by ABA (Nakabayashi et al., 2005; Preston et al., 2009). Tomato endosperm cell walls contain up to 10% arabinoce but little xylose. Transcripts of a β-D-xylosidase accumulated in the embryo of germinating tomato seeds (Itai et al., 2003; Nonogaki et al., 2007). In Lepidium β-D-xylosidases (At1g02640, At5g64570, At1g78060, At5g10560) and α-D-xylosidases showed RAD-specific expression during seed germination, while the β-D-xylosidase At5g49360 was higher in the CAP (Supplemental Fig. 1D). In Arabidopsis transcripts of β-D-xylosidases accumulated >100-fold (from 0 h to 24 h of whole unstratified seeds), while the β-D-xylosidase At5g49360 was >20-fold induced in the endosperm, and these inductions were promoted by GA, but not inhibited by ABA (Nakabayashi et al., 2005; Preston et al., 2009). The transcript expression pattern of pectin-related enzymes in Lepidium has already been discussed in Linkies et al. (2009).

**Genes relating to protein degradation and post-translational modification are important in the regulation of cell wall modification**

From a review of recent post-genomic data, Holdsworth et al. (2008b) concluded that RNA translation and posttranslational modification are major levels of control for germination completion. However, there are similar numbers of genes up-regulated in both tissues in the TAGGIT category protein degradation (Table III), but as discussed above this similarity obscures important differences in the detail. There are 620 genes tagged in this category, of which 76 are significantly differentially expressed between the two tissues, with 34 and 42 expressed more highly in the CAP and RAD respectively (Supplemental Figs. S2A and B). Closer inspection of this cohort of genes reveals a prominent role for the aspartic acid and subtilase families of plant proteases. We therefore looked at genes from all members of these two families of plant proteases and included the cysteine protease family of enzymes. Members of these classes of proteases are reported in Beers et al. (2004). Fig. 7 summarises those members that are
significantly (P<0.10) differentially expressed between the two tissues. The SBT protease members are predominantly over-represented in the RAD, whilst the significant aspartic proteases are mainly overrepresented in the CAP. We have also investigated the expression of genes encoding key enzymes in protein modification involving the ubiquitin/26S proteasome E3 ligases, specifically the F box and RING finger proteins (Supplemental Figs. 2C and D).

**SBT proteases:** Subtilases are a diverse family of serine proteases, which number 54 in Arabidopsis and have a high degree of gene duplication and associated redundancy (Rautengarten et al., 2005). This functional redundancy has made it difficult to associate biological function to individual genes with only two knock out mutants, *stomatal density and development 1* (*sdd1*) and *Abnormal leaf shape* (*ale1*), having recognisable phenotypes. It has been postulated that these encode proteins (*SDD1* and *ALE1*) that act as proprotein convertases yielding bioactive peptides (Berger and Altmann 2000; Tanaka et al., 2001). In Lepidium Fig. 7 shows predominantly greater expression of the subtilase gene family members in the RAD than in the CAP, one such transcript in Arabidopsis *AIR3* (*At2g04160*) has been linked to lateral root emergence (Neuteboom et al., 1999). In Arabidopsis, more than 20 of the family members have been shown to be transcriptionally regulated by light, with the expression of *At2g39850* and *At5g59130* demonstrating sole dependence on PHYA under far red light for induction (Zhou, 2009). In Lepidium both these subtilases are expressed significantly higher in the RAD, whereas *PhyA* is expressed significantly more highly in the CAP (Fig. 5B) suggesting the possibility of signalling between the two tissues regulated by light.

**Aspartate proteases:** There are 59 aspartic proteases identified amongst the annotated Arabidopsis genes and little is known about their biological role (Beers et al., 2004). Sub-cellular localisation may help to elucidate their physiological function and a number have been located in the intracellular fluid of the apoplast with a role in disease resistance signalling (Xia et al., 2004). There is also evidence for a role in seeds. Mutlu et al. (1999) characterised an aspartic protease from dry seeds of Arabidopsis and collocated it with the seed storage protein 2S albumin and the vacuolar marker enzyme α-mannosidase. Molecular studies of osmoprimed seeds of cauliflower (Fujikura and Karssen 1995) identified two aspartic proteases with enhanced expression upon priming. A proteomic analysis of Lepidium CAP tissue (Müller et al., 2010) identified aspartic proteases as a main class of proteins involved in storage protein degradation. The abundance of one aspartic protease was shown to increase from 8h to 18h during the period of CAP weakening. The authors concluded that this early mobilization of protein bodies in the cap is
likely to serve a non-nutritional function in the control of germination (Müller et al., 2010). These observations are consistent with the CAP specific expression of a number of putative aspartic protease transcripts within our data set (Fig. 7).

**Cysteine proteases:** A number of cysteine proteases have been described in the literature that are involved in seed germination (Helm et al., 2008; Cervantes et al., 1994). Ethylene was shown to induce the expression of a cysteine protease responsible for the catabolism of major reserve proteins (Cervantes et al., 1994). Helm et al. (2008) have reported a number of KDEL-cysteine proteases involved in programmed cell death (PCD) and the dismantling of extension scaffolds. This led the authors to the hypothesis that the KDEL-tailed cysteine proteases they identified participate in the final cell collapse during PCD by attacking the structural hydroxyproline-rich glycoproteins of the cell wall. In Lepidium, transcript numbers of one of these KDEL-tailed cysteine proteases, At3g48340, was significantly up-regulated in the CAP (Fig. 7).

**RING finger E3 ligases and F box proteins:** E3 ligases are the components of the 26S proteasome that confer substrate specificity to the system. The ubiquitin/26S proteasome pathway is important to most aspects of plant biology (Vierstra 2009) including hormonal signalling (Frugis and Chua 2002). There are 697 F box proteins (Gagne et al., 2002) and 469 RING finger proteins (Stone et al., 2005) in the Arabidopsis genome of which we have transcriptional data for 333 and 327 putative orthologs respectively in our Lepidium data set. Transcript abundances from this set that are significantly differentially expressed between the two tissues is shown in Supplemental Figure 2C and D. There was a greater proportion of both F box and E3 ligase encoding genes up-regulated in the CAP compared to the RAD (2.3 and 2.7 fold respectively). This suggests that posttranslational modification, in the form of selected proteolysis, performs a more significant role in CAP weakening than in the developing RAD. The role of the 26S proteasome pathway in light and hormonal signalling is well characterized in plants (Vierstra 2009) and this apparent enrichment of E3 ligase mRNAs may strengthen the argument for these environmental cues playing a substantial role in endosperm weakening and signalling to the developing seedling.

**The effect of protease inhibitors:** To investigate the role of these proteins in protein degradation we have monitored the progression of CAP hole formation, as described above, when incubated upon specific protease inhibitors. The CAPs isolated after 12 h imbibition were
then incubated upon 1 µM Pepsatin, 4 mM Pefabloc, 28 µM E-64 and 60 µM MG132 which inhibit aspartic, serine, and cysteine proteases, and the 26S proteasome respectively (Fig. 8). It is clear from these data that all 4 classes of proteases investigated have a pronounced affect on endosperm weakening, with the most dramatic affect being the complete cessation of autolysis by the proteasomal inhibitor MG132. The other three inhibitors reduced the number of CAPs exhibiting autolysis to approximately 50% of that shown in the control (84%) over the same time period. This suggests that each class of protease has a specific protein target, and numerous protein targets may be required for complete lysis.

The complete inhibition of hole formation and tissue autolysis in those CAPs treated with MG132 suggests that targeted protein degradation is a major control point for endosperm weakening. It is now well established that the ubiquitination pathway plays a role in various hormonal signalling pathways and is involved in the regulation of germination through the degradation of DELLA proteins (Dill et al., 2001), it could therefore be hypothesised that inhibition of the degradation of an important transcription repressor prevents the cascade of transcriptional activity that we show in our array data. It was recently demonstrated that ABA inhibits CAP hole formation (Linkies et al. 2009) and we show here that the proteasome inhibitor MG132 completely blocks cap hole formation and autolysis. This suggests the involvement of a key transcriptional repressor implicated in several signalling pathways as well as in ABA signalling. Several ubiquitin ligases have been linked to ABA signalling and have the transcription factors ABI3 and ABI5, both important regulators of seed germination, as targets for proteolysis (Santner and Estelle, 2010; Lopez-Molina et al. 2003; Holdsworth et al. 2008). ABI5 and ABI5 BINDING PROTEIN (AFP) mRNA and protein levels increase when seeds are treated with ABA and mutants for ABI5 and ABF exhibit seed phenotypes. NINJA and AFP are related proteins and it has been proposed in recent work that the Groucho/Tup1-type family co-repressors, including TOPLESS (TLP), are part of a general repressor machinery implicated in several signaling pathways (Liu and Karmarkar, 2008; Pauwels et al. 2010). For jasmonic acid and ABA signalling, NINJA and AFP are proposed to mediate the interaction between transcription factors, ABI5 for ABA, and TLP. The TPL-type co-repressors have general functions in plant hormone signalling that are related to transcription factor proteolysis. Supplemental Figure S2C emphasises the influence of the proteasomal degradation pathway on hormonal signalling. There was significant differential expression between CAP and RAD of genes related to auxin (AFB3, TIR1 and MAX2), jasmonic acid (COI1), and ethylene (EBF1). All these except TIR1, showed elevated levels of expression in the CAP.
CONCLUDING DISCUSSION

We have shown that following rupture of the testa, germination in Lepidium is regulated by the opposing forces of RAD (radicle plus lower hypocotyl) extension and the resistance to this by the surrounding CAP (micropylar endosperm), which progressively declines through autolysis. By 18 h some seeds have completed autolysis (germination has occurred), but even at this late stage, progress in some seeds can be stopped by inhibiting transcription and the remainder can be stopped by blocking translation and posttranslational changes. Taken together these results suggest that this is a control point for germination completion that is very late on in the germination process. This late control point therefore acts as a gateway to seedling development, but the rate of germination must be determined earlier in the process as seeds reach this control point at different times, and thus weakening does not determine vigour. Late control is a necessary feature that prevents inappropriate germination when environmental conditions change. Expression of genes involved in hormone signalling networks was shown to have different temporal and spatial patterns consistent with establishing a complex responsive regulation through subtle changes in hormone sensitivity, rather than through a crude hormone balance. Genes encoding cell-wall remodelling proteins were also expressed in a complex, tissue specific manner during endosperm weakening that would allow subtle regulation of weakening and therefore germination completion in response to hormone signals driven by the current ambient environment.

MATERIALS AND METHODS

Plant material, germination and puncture-force measurements

After-ripened *Lepidium sativum* L. FR1 (‘Gartenkresse, einfache’) and FR14 (‘Keimsprossen’) seeds (Juliwa, Heidelberg, Germany) were incubated in petri dishes on two layers of filter paper with 6 ml 1/10 Murashige-Skoog salts as medium in continuous white light (ca. 100 µmol s⁻¹ m⁻²) as described by Müller et al. (2006) at the temperatures indicated. Testa rupture and endosperm rupture were scored using a binocular microscope. Puncture-force measurements were performed as described by Müller et al. (2006).

Inhibitor studies on endosperm hole formation and autolysis

Afterripened seeds of *Lepidium sativum* (Lepidium) `FR1´ were incubated in Petri dishes on two layers of filter paper with 6 ml 1/10 Murashige and Skoog (MS)-salts as medium in continuous
white light (approx. 100 µmol·s⁻¹·m⁻²) at 18 °C. After 10, 12, and 18 h the micropylar endosperm was dissected from the seeds for further incubation on 500 µM cycloheximide (Sigma) or 1 µg/ml α-amanitin (Sigma). Cycloheximide was dissolved in 50% acetone. Following dilution 0.1% acetone remained and so this same amount was added to all treatments and the control. Preliminary work determined that this concentration had no influence on germination, hole formation or radicle growth. In a second experiment dissection at 12 h was followed by incubation on 1 µM pepstatin (Roche), 4 mM Pefabloc (Roche), 28 µM E64 (Roche), and 60 µM MG132 (Merck). The concentrations used were those recommended by the manufacturer. In preliminary work, ten-fold lower concentrations were also used to test for a lower dose response. The inhibitors were dissolved in methanol, water, water/ethanol and DMSO respectively. Controls for each inhibitor differed and contained the appropriate chemical at <0.05%. For every inhibitor and control at least 3 replicates of 20 micropylar endosperm caps each were incubated in small Petri dishes on two layers of filter paper with 2.5 ml 1/10 MS-salts as medium with the indicated inhibitor in continuous white light (approx. 100 µmol·s⁻¹·m⁻²) at 18 °C. Experiments were repeated to confirm results. Analysis of endosperm autolysis was determined at the times indicated by 2 categories, beginning of autolysis (initial autolysis) was recorded as soon as 1 hole was visible, in nearly all cases that happened just below the tip, progression of autolysis was recorded when more than 1 hole was visible which later led to autolysis resulting in digestion of whole parts of the endosperm.

Endo-β-1,4-mannanase enzyme activity assay

Seed tissues (RAD, CAP, NME and cotyledons) were ground in 0.1 M Hepes-0.5 M NaCl buffer (pH 7.5) using an ice-cold mortar. The volume of the Hepes buffer was added at the ratio of fresh weight of tissues (mg)/buffer volume (mL) = 1/3. The extract was centrifuged at 4 °C for 10 min at 10,000 rpm, and the supernatant was used to assay the activity of endo-β-mannanase as described by Bourgault and Bewley (2002).

Semiquantitative RT-PCR

One microgram of RNA was reverse transcribed using oligo(dT) primer according to the PrimerScript™ Reverse Transcriptase Kit instructions (TaKaRa). Aliquots of these first-strand
cDNAs as templates were used in subsequent PCR reactions. For the semi-quantitative PCR analysis; template volumes were determined that result in equal amplification for the actin reference gene for each sample. For actin, optimal conditions were 27 amplification cycles with 52 °C as annealing temperature; forward primer 5’- CTAAAGCCAACAGGGAGA-3’, reverse primer 5’-TTGGTGCGAGTGCGGTGA-3’. The template volumes determined for actin were used for the semi-quantitative PCR analysis of the endo-1,4-mannanase (52 °C annealing temperature, 35 amplification cycles); forward primer 5’-ACCGATTTCATTGCCAATAACCG-3’, reverse primer 5’-TGTCGACTTTGTGCGATGAGA-3’.

**RNA isolation from Lepidium seed tissues**

For each sample ca. 1000 Lepidium endosperm caps (CAP), ca. 1000 non-micropylar endosperms (NME), or ca. 100 radicles (RAD) were collected at the times indicated, frozen in liquid nitrogen and stored at -80 °C. Total RNA extraction was carried out by the CTAB-method followed by quantity and quality control analyses as described (Chang et al., 1993). Four biological replica RNA samples were used for downstream applications.

**Microarray experimental design**

We carried out two separate microarray experiments. The first compared CAP and RAD at 8, 18, 30 and 96 h of imbibition on 10 µM ABA and were termed +ABA-arrays. The second compared CAP, NME and RAD at 8 and 18 h of imbibition on germination medium without ABA and were termed -ABA-arrays. Each experiment used 4 biological replicates. Hybridisations were carried out according to the description below and Linkies et al. (2009). For the -ABA-array experiment the two time points for each tissue were directly compared on four microarrays, balanced for colour. For each tissue in the +ABA-array experiment, all time points were directly compared to each other on one microarray each, and for each time point the two tissues were compared on one microarray. Each treatment was balanced for colour. This design can be thought of as four interlinked loops.

**Cross-species CATMA microarrays and Lepidium RNA hybridisation**

RNA was prepared in the following way for microarray hybridization. The Ambion MessageAmp™ II aRNA Amplification Kit (AM1751, Applied Biosystems, Darmstadt, Germany) was used according to the manual with 2 µg of Lepidium FR1 total RNA as template to generate antisense amplified RNA, called aRNA (Van Gelder et al., 1990). The quality and quantity of the
aRNA was checked by running an aliquot on a 2100 Bioanalyzer (Agilent, UK). The microarrays used carried genome sequence tag (GST) fragments generated using gene-specific primers identified by the CATMA Consortium (http://www.catma.org, Hilson et al., 2004; Allemeersch et al., 2005). CATMA version 2 arrays with 24576 GST were used for the –ABA-array experiment, whilst CATMA version 3 arrays with 30343 GST were used for the +ABA-array experiment. The aRNA was labelled and the CATMA microarrays were hybridized according to the method described in Lim et al. (2007) and Linkies et al. (2009). The microarrays were scanned using an Affymetrix 428 array scanner at 532 nm (Cy3) and 635 nm (Cy5). Scanned data were quantified using Imagene version 4.2 software (BioDiscovery, http://www.biodiscovery.com/). Microarray data were deposited in ArrayExpress (http://www.ebi.ac.uk/microarray/) under accession number E-TABM-745 (-ABA-arrays) and E-TABM-743 (+ABA-arrays).

RNA microarray data handling and analysis

Data from the two experiments (+ABA-arrays and -ABA-arrays) were analysed separately using a similar approach, but differing in line with the different experimental designs used, and the availability of genomic DNA hybridisation data for CATMA v3 arrays used in the ABA experiment (Linkies et al., 2009). In both cases, spot intensity data from Imagene were analysed using the limma package in Bioconductor (Smyth, 2005). There was an initial screen to the data which removed all probes that could not be assigned to an Arabidopsis gene defined by having an Arabidopsis Genome Initiative (AGI) identifier and associated with this a TAIR 7 gene ontology (GO, http://www.arabidopsis.org). Background correction was performed using the 'normexp' method, which is analogous to RMA. Within array normalisation (Smyth and Speed, 2003) was performed using print tip loess. In the -ABA-array experiment between array normalisation was performed using quantile normalisation on the 'A' values. For the +ABA-array experiment, probes which had shown no significant response in the genomic DNA microarrays (Linkies et al., 2009) were weighted out of the normalisation and analysis. The two filtering steps resulted in lists (Supplemental Tables S1 and S2) containing 19794 and 22025 genes for the +ABA-arrays and -ABA-arrays respectively. These gene lists were then used in all downstream analyses. For both experiments the data were analysed as a linear model (Smyth, 2004), for the -ABA-arrays experiment the analysis was adjusted for the intraspot correlation. The variance estimates were adjusted using empirical Bayes estimates of the per-spot variability for use in differential expression analyses. Estimates of the transcript numbers (intensities) for individual spots (genes) on the +ABA- and -ABA-arrays (Tables S1 and S2) were compared across sample time points and tissues using F-Tests to identify those whose intensity had significantly changed.
Statistical significance of differences was assessed using the approach of Benjamini and Hochberg (1995) to control the false discovery rate at the level of 10%. The genes identified were considered to be up- or down-regulated between tissues at the same time or different time points. PCA was performed in R for each experiment to compare the tissues and time points across the probes.

**Functional categorisation of genes**
Gene lists were created for genes that were significantly expressed (“present”) on the arrays and significantly up- or down-regulated between tissues using procedures described at appropriate points in the text. In order to investigate whether these genes were linked to functional specialisation categories the lists were subjected to the GO-based established seed-specific TAGGIT workflow (Carrera et al., 2007) to identify proportional representations of genes in functional categories.

**Supplemental Data**
The following materials are available in the online version of this article.

**Supplemental Table S1.** Effect of α-amanitin and cycloheximide on radicle growth

**Supplemental Table S2.** Normalised mean abundance of transcripts from genes identified as “present” and used in the analysis of +ABA-arrays (19,794 genes).

**Supplemental Table S3.** Normalised mean abundance of transcripts from genes indentified as “present” and used in the analysis of –ABA-arrays (22,025 genes).

**Supplemental Table S4.** Proportional representation in functional categories of expressed (“present”) genes on +ABA-arrays.

**Supplemental Table S5.** Proportional representation in functional categories of genes with expression on +ABA-arrays that is unique to each tissue/time combination.

**Supplemental Table S6.** Genes on +ABA-arrays that show differential expression between tissues.
Supplemental Figure S1. Heat maps showing the relative abundance of transcripts from genes encoding cell wall modification proteins.

Supplemental Figure S2. Heat maps showing the relative abundance of transcripts from genes encoding proteins associated with posttranslational modification.

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Figure legends

**Figure 1.** Progression of endosperm CAP weakening both with and without the addition of ABA. 
**A.** Endosperm rupture progresses more quickly without ABA, but the mean force required to puncture the CAP is the same in both treatments before and after weakening. **B.** The distributions of force required to puncture the CAP are the same in both treatments.

**Figure 2.** The effect of transcription (α-amanitin) and translation (cycloheximide) inhibitors on the progress of autolysis in isolated CAPs. A, schematic cross-section of a *Lepidium sativum* seed. B, Isolated CAP (micropylar endosperm). C, Examples of initial autolysis: CAP tip abscission (upper) and hole formation (lower). D. Progressed autolysis. E, The effect of inhibitors on the progress of autolysis in a population of CAPs isolated at 18h imbibition. Isolated CAPs were incubated for the times indicated without or with inhibitors (α-AM = α-amanitin; CHX = cycloheximide). F. The effect of inhibitors on the progress of autolysis (initial + progressed lysis) in a population of CAPs isolated after 10 and 18 h of seed imbibition; subsequent incubation as indicated.

**Figure 3.** The results of principal component analysis (PCA) applied to the expression of all the *Lepidium* FR1 gene homologs represented in the +ABA microarrays. (A) Principle component 1 and 2 accounted for 42 and 20 % of the variance, respectively. (B) Principle component 2 and 3 accounted for 20 % and 12 % of the variance, respectively.

**Figure 4:** Hormone signalling in *Lepidium sativum* during CAP weakening. A. Schematic to illustrate ABA and GA signalling pathways. B. Heat maps showing the relative abundance of transcripts from genes involved in ABA, GA and environmental signalling. †, *, **, *** indicate that transcript numbers are significantly different between the tissues on +ABA-arrays at P<0.1, <0.05, <0.01, <0.001 respectively. Genes not present in the data sets are coloured grey.

**Figure 5:** Relative abundance of transcripts in the CAP, RAD or NME for genes related to cell wall modification. A logarithmic scale is used to quantitatively indicate if a transcript is more abundant in the CAP (below the x-axes, negative value, p≤0.1), RAD, or NME (above the x-axes, positive value, p≤0.1). A, alpha-expansins, B, XTH group 1, C, XTH group 2, C, XTH group 3, D, beta-1,4-mannanase and mannan synthase, E, alpha-galactosidase and beta-mannosidase. Key identifies genes and which arrays (+ABA- or –ABA-arrays, left or right part of graphs, respectively) the data is from.
Figure 6. Endo-β-1,4-mannanase enzyme activity during CAP weakening. A. Time course of endo-β-1,4-mannanase enzyme activity in separate tissues. B. Endo-β-1,4-mannanase enzyme activity in the RAD, CAP, NME and cotyledons at three time points. C. The pattern of LesaMAN7 transcript expression in different tissues during CAP weakening. Note in B and C values at 36 h are measured separately in tissues from seeds with and without CAP rupture. GenBank accession numbers for LesaMAN7 and LesaACT7 are HQ436349 and HQ436350 respectively.

Figure 7. Heat maps showing relative abundance of transcripts from genes encoding proteases that are differentially expressed between seed tissues. ′, *, **, *** indicate that transcript numbers are significantly different between the tissues on +ABA-arrays at P<0.1, <0.05, <0.01, <0.001 respectively.

Figure 8. The effect of inhibitors of specific proteases (Pepstatin, Pefabloc, E64) and a proteosome (MG132) inhibitor on the progression of CAP autolysis. Initial autolysis = hole formation or tip abcission (Fig. 2C); progressed autolysis = autolysis of the whole CAP (Fig. 2D).
Table I. Numbers of probes on the array that were considered to be significantly “expressed”. Values shown are numbers of “expressed” probes that are unique to the RAD and CAP at each time point and the number of probes that are expressed in both tissues (Common). The numbers of probes that are uniquely expressed in any time/tissue combination are shown in parenthesis.

|     | RAD       | Common | CAP       |
|-----|-----------|--------|-----------|
| 8h  | 4087 (513)| 9416   | 917 (154) |
| Common | 10327     | 8045   | 9153      |
| 18h | 2004 (229)| 9515   | 2542 (298)|
| Common | 10073     | 8482   | 10304     |
| 30h | 1903 (145)| 9479   | 2328 (285)|
| Common | 10493     | 8552   | 10271     |
| 96h | 2601 (322)| 9809   | 2228 (321)|
| Tags                                | RAD  |     |     |     | CAP  |     |     |     |
|------------------------------------|------|-----|-----|-----|------|-----|-----|-----|
| Dormancy related                   | 8    | 18  | 30  | 96  | 8    | 18  | 30  | 96  |
| Germination related                | 2    | 3   | 3   | 2   | 0    | 0   | 2   | 3   |
| ABA                                | 10   | 16  | 22  | 26  | 8    | 6   | 11  | 12  |
| Auxin                              | 14   | 14  | 14  | 27  | 10   | 13  | 16  | 12  |
| Brassinosteroid                    | 0    | 0   | 1   | 1   | 2    | 3   | 3   | 2   |
| Cytokinin                          | 6    | 3   | 2   | 6   | 1    | 1   | 2   | 2   |
| Ethylene                           | 6    | 7   | 7   | 11  | 2    | 5   | 10  | 8   |
| Gibberellin                        | 6    | 5   | 5   | 5   | 2    | 5   | 3   | 3   |
| Jasmonic acid                      | 2    | 6   | 3   | 5   | 0    | 1   | 0   | 1   |
| Seed storage proteins/Late Embryogenesis Abundant | 1    | 4   | 4   | 7   | 6    | 10  | 12  | 10  |
| Inhibition of protein degradation  | 1    | 1   | 1   | 1   | 0    | 1   | 0   | 0   |
| Protein degradation                | 13   | 19  | 24  | 37  | 18   | 22  | 28  | 35  |
| Heat Shock                         | 2    | 5   | 6   | 6   | 7    | 10  | 14  | 17  |
| Cell-wall modification             | 19   | 18  | 15  | 24  | 12   | 18  | 19  | 22  |
| Cell cycle related                 | 7    | 1   | 9   | 7   | 6    | 14  | 14  | 22  |
| Cytoskeleton                       | 6    | 5   | 8   | 6   | 8    | 8   | 14  | 13  |
| Translation associated             | 4    | 1   | 3   | 7   | 63   | 86  | 111 | 137 |
| DNA repair                         | 1    | 2   | 3   | 6   | 2    | 4   | 7   | 5   |
| Respiration                        | 0    | 0   | 0   | 0   | 0    | 1   | 1   | 3   |
| Electron Transport                 | 1    | 2   | 2   | 3   | 0    | 0   | 1   | 2   |
| Pentose phosphate pathway          | 2    | 2   | 4   | 3   | 0    | 0   | 0   | 0   |
| Glycolysis and gluconeogenesis     | 6    | 9   | 10  | 8   | 0    | 0   | 1   | 4   |
| Krebs cycle                        | 2    | 3   | 4   | 4   | 1    | 0   | 1   | 1   |
| Beta oxidation                     | 2    | 2   | 4   | 4   | 0    | 0   | 0   | 0   |
| Stress                             | 36   | 55  | 53  | 53  | 17   | 24  | 38  | 45  |
| Photosynthesis/chloroplast related | 48   | 60  | 73  | 134 | 34   | 44  | 69  | 121 |
| Unannotated                        | 1    | 2   | 1   | 4   | 2    | 2   | 4   | 3   |
| Unclassified                       | 346  | 484 | 496 | 828 | 339  | 438 | 593 | 718 |
| Total genes in lists               | 545  | 736 | 783 | 1238| 555  | 736 | 1000| 1226|
| Total genes classified above       | 199  | 252 | 287 | 410 | 216  | 298 | 407 | 508 |
| Percentage classified              | 36.5 | 34.2| 36.7| 33.1| 38.9 | 40.5| 40.7| 41.4|

**Table II:** The numbers of genes classified in functional categories of the GO-based seed-specific TAGGIT workflow (Carrera et al., 2007) that were up-regulated in the tissue shown at each time point for the +ABA-arrays.
Figure 1. Progression of endosperm CAP weakening at 18 °C both with and without the addition of ABA. A. Endosperm rupture progresses more quickly without ABA, but the mean force required to puncture the CAP is the same in both treatments before and after weakening. B. The distributions of force required to puncture the CAP are the same in both treatments.
Figure 2. The effect of transcription (α-amanitin) and translation (cycloheximide) inhibitors on the progress of autolysis in isolated CAPs. A, schematic cross-section of a *Lepidium sativum* seed. B, Isolated CAP (micropylar endosperm). C, Examples of initial autolysis: CAP tip abscission (upper) and hole formation (lower). D, Progressed autolysis. E, The effect of inhibitors on the progress of autolysis at 18 °C in a population of CAPs isolated at 18h imbibition. Isolated CAPs were incubated for the times indicated without or with inhibitors (α-AM = α-amanitin; CHX = cycloheximide). F. The effect of inhibitors on the progress of autolysis (initial + progressed lysis) at 18 °C in a population of CAPs isolated after 10 and 18 h of seed imbibition; subsequent incubation as indicated.
Figure 3. The results of principal component analysis (PCA) applied to the expression of all the Lepidium FR1 gene homologs represented in the +ABA microarrays. (A) Principle component 1 and 2 accounted for 42 and 20 % of the variance, respectively. (B) Principle component 2 and 3 accounted for 20 % and 12 % of the variance, respectively.
Figure 4: Hormone signalling in Lepidium during CAP weakening. A. Schematic to illustrate ABA and GA signalling pathways. B. Heat maps showing the relative expression levels of genes involved in ABA, GA and environmental signalling. *, **, *** indicate that transcript numbers are significantly different between the tissues on +ABA-arrays at P<0.1, <0.05, <0.01, <0.001 respectively. Genes not present in the data sets are coloured grey.
Figure 5: Relative abundance of transcripts in the CAP, RAD or NME for genes related to cell wall modification. A logarithmic scale is used to quantitatively indicate if a transcript is more abundant in the CAP (below the x-axes, negative value, p≤0.1), RAD, or NME (above the x-axes, positive value, p≤0.1). A, alpha-expansins, B, XTH group 1, C, XTH group 2, C, XTH group 3, D, beta-1,4-mannanase and mannan synthase, E, alpha-galactosidase and beta-mannosidase. Key identifies genes and which arrays (+ABA- or –ABA-arrays, left or right part of graphs, respectively) the data is from.
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