Promotion of Testa Rupture during Garden Cress Germination Involves Seed Compartment-Specific Expression and Activity of Pectin Methylesterases [1][OPEN]

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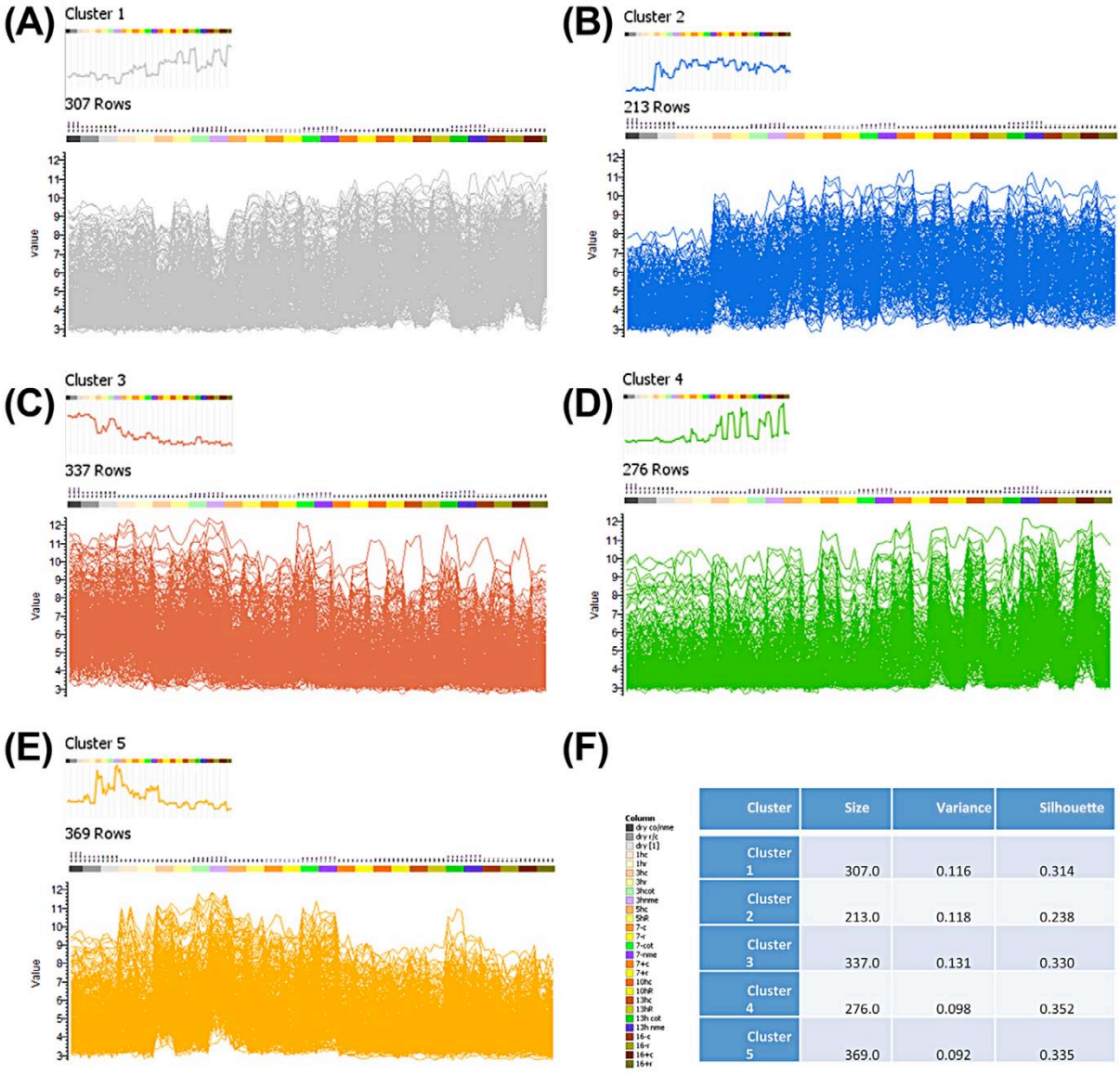
Supplemental Data

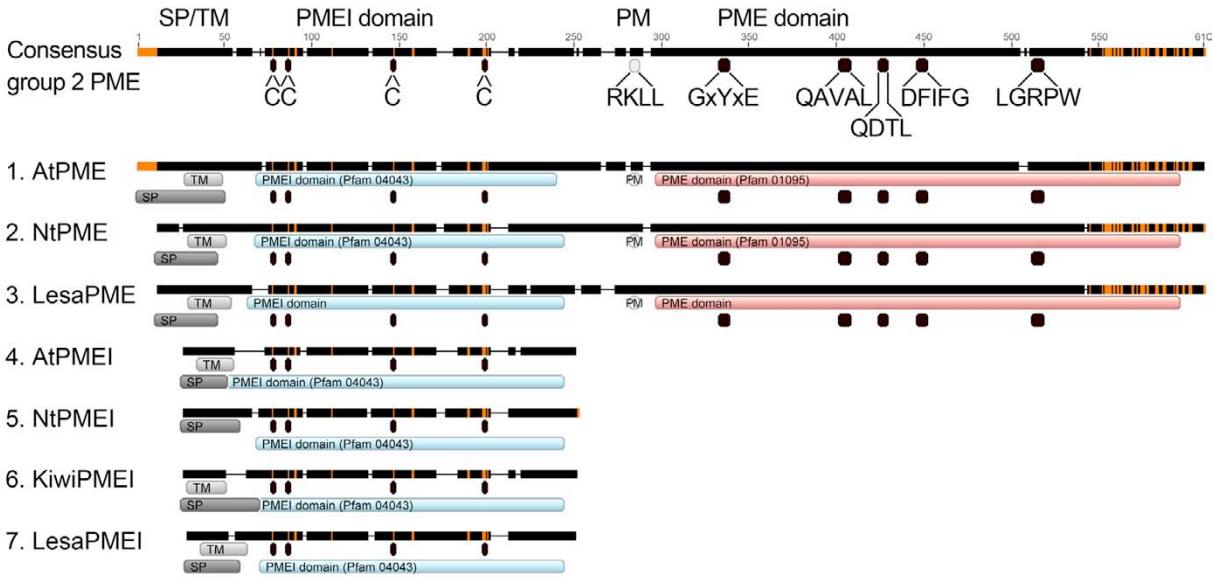


(F)

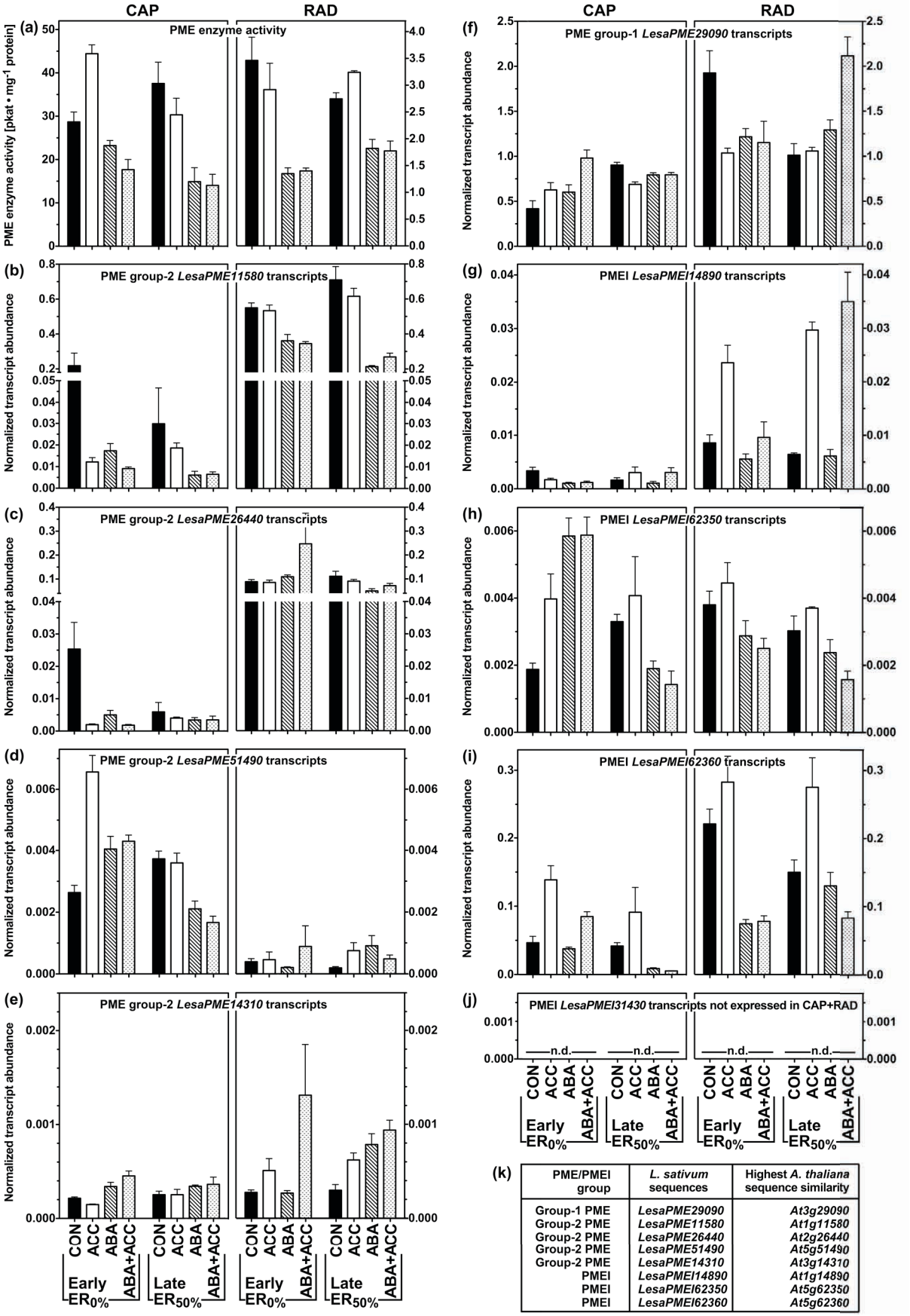
Cluster	Size	Variance	Silhouette
Cluster 1	307.0	0.116	0.314
Cluster 2	213.0	0.118	0.238
Cluster 3	337.0	0.131	0.330
Cluster 4	276.0	0.098	0.352
Cluster 5	369.0	0.092	0.335

Supplemental Figure S1. A cluster analysis of the *Lepidium sativum* differentially regulated transcriptome over time and seed compartments showed five clearly distinct clusters **(A to E).** Masked and normalised spot intensity values were used as input for the analysis. The members of the clusters are represented in a parallel plot in the large panels. The top bar of the parallel plot shows the chips/columns in the temporal sequence of the germination time course from dry (left) to 16h with ER (right). In the bar above the plot all chips are separated by type into: dry seed chips in grey, RAD in yellow, CAP in red, COT in green and NME in purple. The analysis was performed with the Genedata Analyst. **(F)** A table showing the calculated silhouette, the silhouette value, and the number of genes in each cluster. **(A)** The first cluster shows a relatively low but stable expression during early germination and then a rise for the later stages of germination with a tendency towards higher relative expression in the embryo (RAD and COT) compared to the endosperm (CAP and NME).

(B) Cluster 2 is the smallest cluster out of the five with just over 200 genes, showing a distinct pattern with no expression in dry seeds and at one hour after sowing, then a slight peak at three hours followed by continuous expression in all seed compartments. **(C)** Cluster 3 shows a pattern with high expression in dry seeds, 1h imbibed seeds, and for NME and COT at 3h after sowing. Transcript abundance subsequently declines. **(D)** The fourth cluster is endosperm specific with a small peak at 7h before TR, high expression in the CAP and lower expression in the NME. RAD and COT show only very low or no expression, and has the best fitting silhouette score of all clusters. **(E)** Cluster five shows low expression in dry seeds and at 1 h after sowing apart from a small increase in the CAP. The expression peaks at 3 h in CAP and NME, declines slowly until 7 h after sowing, then increases again in COT and NME, followed by a further decline toward the end of germination.



Supplemental Figure S2. Amino acid sequence alignment of PMEs and PMEIs showing characteristic features of the different groups. *LesaPME11580* (group 2 PME) and *LesaPMEI14890* (PMEI) with characteristic previously identified PMEs and PMEIs of other species: (1) Arabidopsis PME (Ac. NP_175787), (2) Tobacco PME (Ac. AY772945), (3) *Lepidium sativum PME11580* (Ac. JQ011281), (4) Arabidopsis PMEI (Ac. NP_175236), (5) Tobacco PMEI (Ac. AY594179), (6) Kiwi PMEI (Ac. AB091088) and (7) *L. sativum PMEI14890* (Ac. JQ011282). The conserved cysteine residues in the inhibitory domain and also the functional motifs in the PME domain are marked with black boxes. All PMEs have a processing motif between the PME (red) and PMEI domain (blue), which is RRLL for Arabidopsis and tobacco and RKLL for *L. sativum*. Other structural motifs according to Pelloux et al. (2007): SP, signal peptide; TM, transmembrane domain; PM, processing motif.
Note that we fully cloned and analysed the cDNAs for the *L. sativum* PME group 2 homolog of Arabidopsis *At1g11580*, which we named *LesaPME11580*. All other cloned *L. sativum* PME cDNAs were named following the same principle. We partially cloned and analysed PME group 2 *LesaPME26440*, *LesaPME14310*, and *LesaPME51490*. The GenBank accession numbers for all cDNAs are listed in Supplemental Table S4. The typical inhibitory domain (PMEI, Pfam04043) was identified for the predicted protein sequence of *LesaPME11580*, as was a pectin esterase domain (Pfam01095). According to Markovic and Janecek (2004), the PME domain harbors 5 amino acid motifs important for enzyme activity: GxYxE, QAVAL, QDTL, DFIFG and LGRPW. All these motifs were present at conserved positions in *LesaPME11580* as well as a typical PM sequence (RKLL) between the PME and PMEI domains. The PMEI domain shows the four conserved cysteine residues proposed to be fundamental for the inhibitory activity of the protein domain, as well as variable N-termini with SP/TM as described by Pelloux et al. (2007).

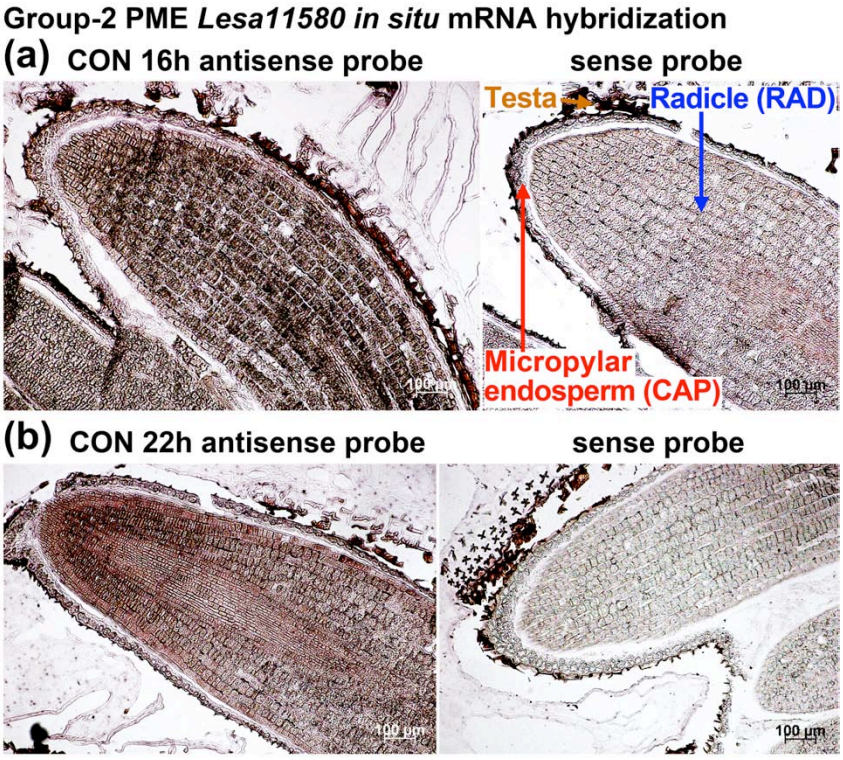


(k)

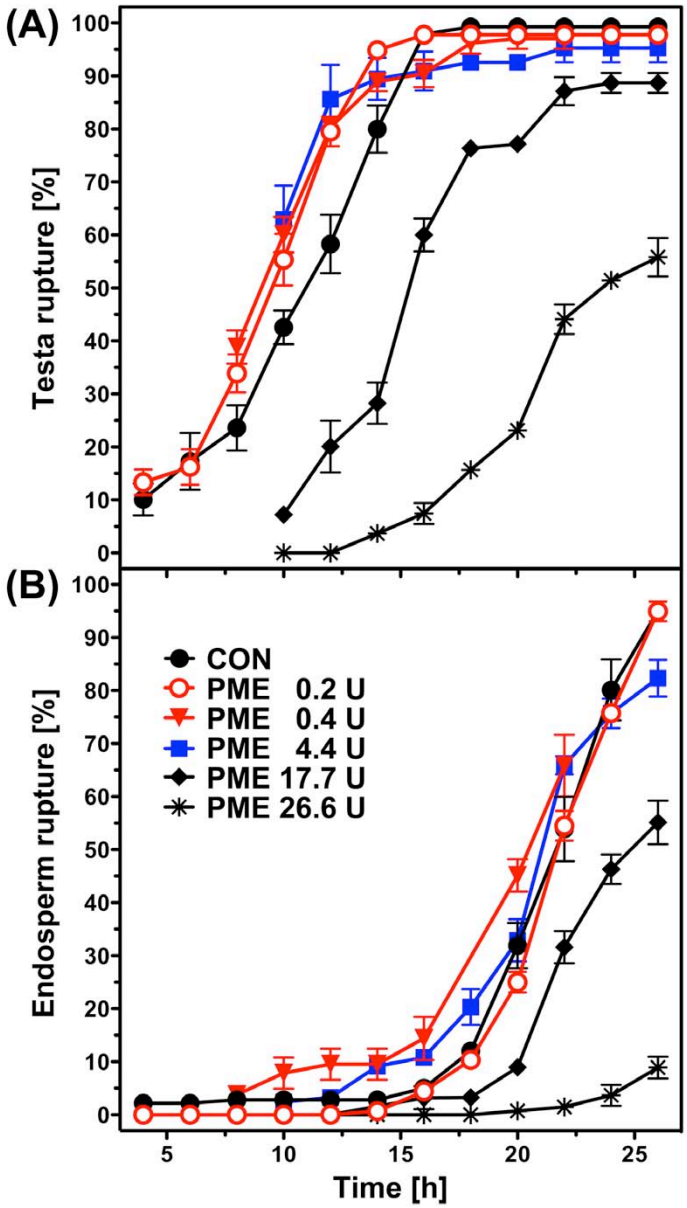
PME/PMEI group	*L. sativum* sequences	Highest *A. thaliana* sequence similarity
Group-1 PME	*LesaPME29090*	*At3g29090*
Group-2 PME	*LesaPME11580*	*At1g11580*
Group-2 PME	*LesaPME26440*	*At2g26440*
Group-2 PME	*LesaPME51490*	*At5g51490*
Group-2 PME	*LesaPME14310*	*At3g14310*
PMEI	*LesaPMEI14890*	*At1g14890*
PMEI	*LesaPMEI62350*	*At5g62350*
PMEI	*LesaPMEI62360*	*At5g62360*

Supplemental Figure S3. Spatial and temporal analysis of transcript abundances of novel *Lepidium sativum (Lesa)* PMEs (group 1 and group 2) and PMEIs in germinating *L. sativum* seeds by qRT-PCR. **(A)** PME enzyme activities as determined in Figure 5; note that scales for CAP and RAD are different. **(B-J)** Normalized transcript abundances of selected PMEs and PMEIs, as indicated. Seeds were imbibed without (CON, control) or with ABA (5 µM) or ACC (1 mM; direct ethylene precursor). Micropylar endosperm (CAP) and the lower 1/3 of the radicle/hypocotyl axis (RAD) were excised from seeds; results for CAP (*left*) and RAD (*right*) are displayed on identical scales. *Early germination:* seeds after TR, but prior to ER (16h). *Late germination:* seeds at $ER_{50\%}$, which was ca. 22h for CON and ACC, ca. 65h for ABA, and ca. 50h for ABA+ACC. Only unruptured CAPs were sampled. *Lesa17210, Lesa04320* and *Lesa20000* (Graeber et al. 2011) were used as references genes for the qRT-PCR normalization as described in the methods. Mean values ± SE for four biological replicates.

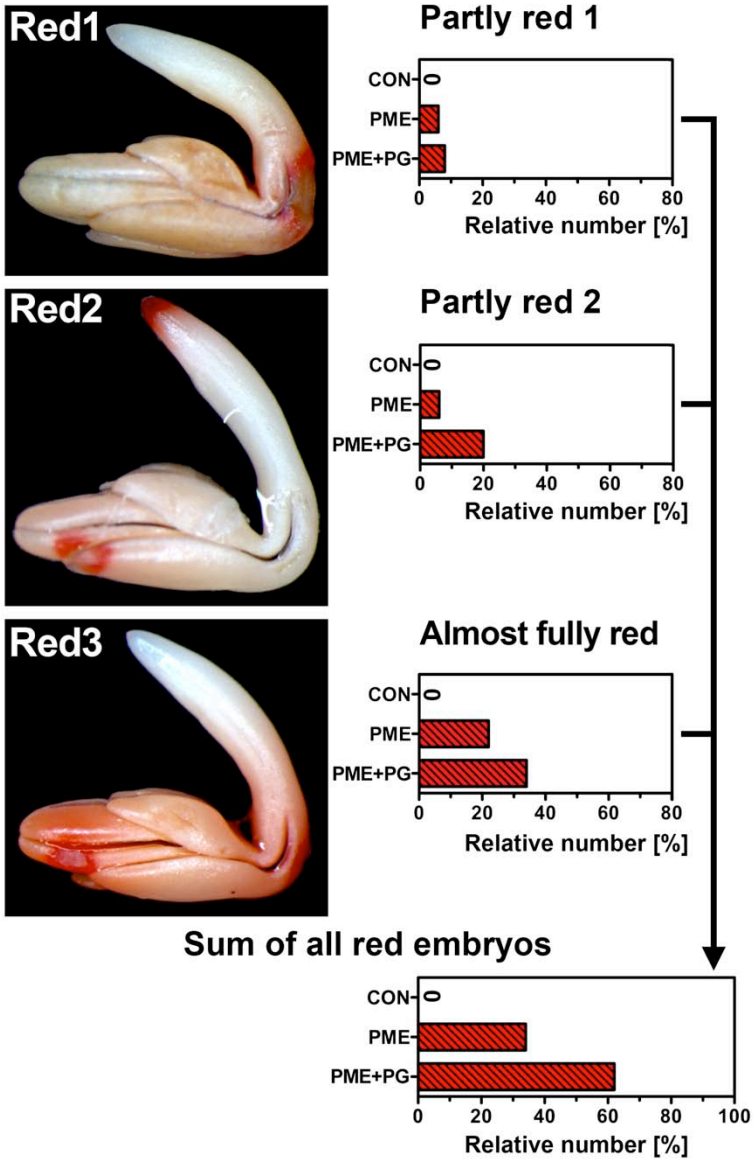
Note that in the RAD during early germination ACC, ABA and ABA/ACC down-regulate the *LesaPME29090* transcript up to 50%. In contrast, in the CAP no hormonal regulation on the transcript level could be observed. The PMEI transcript abundances were very variable regarding their expression patterns. *LesaPMEI62360* had the highest transcript abundances of the four analysed and was, except for the CAP during early germination, down-regulated by ABA. *LesaPMEI14890* exhibited interesting hormonal regulation in the RAD as it was up-regulated by ACC, but not down-regulated by ABA.



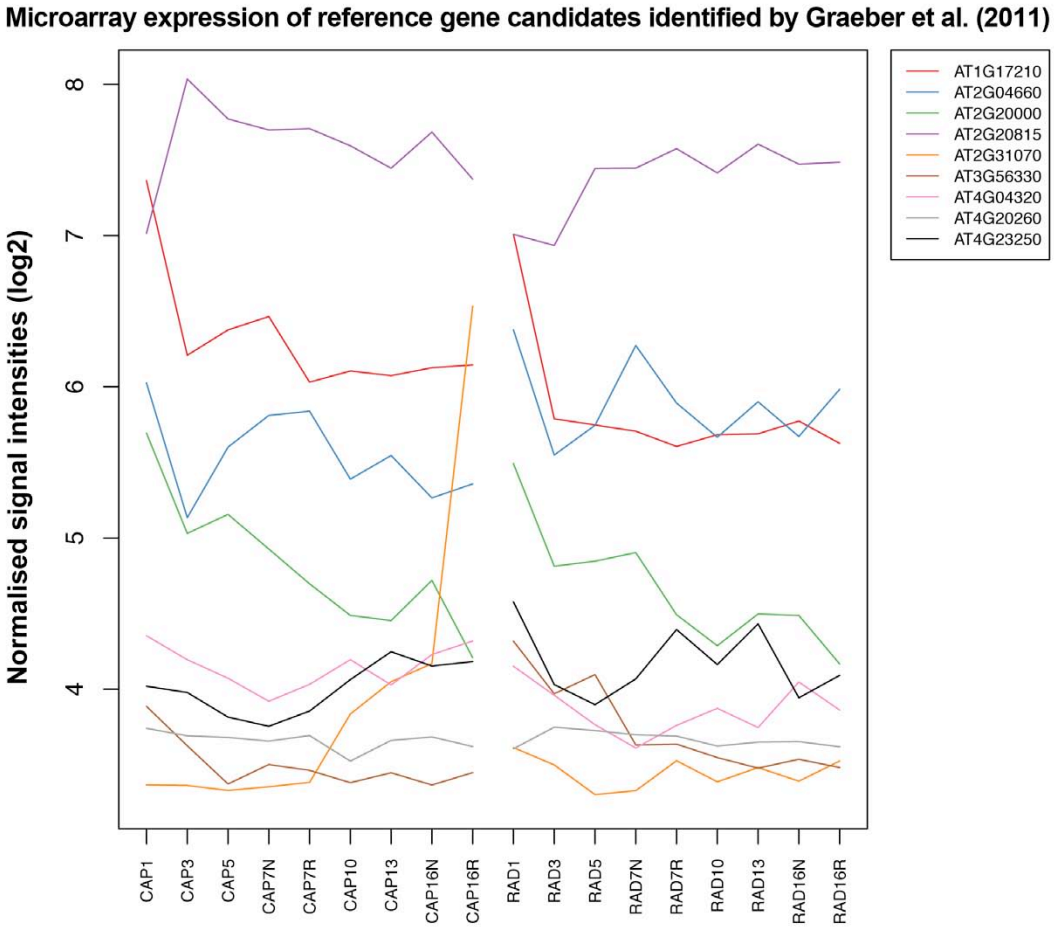
Supplemental Figure S4. Detection of *Lepidium sativum* PME group-2 *LesaPME11580* transcripts via mRNA *in situ* hybridization. Seeds were imbibed in water embedded and the hybridization was carried out as described in the methods. **(A)** Early germination: 16h, completed TR and prior to ER. **(B)** Late germination: $ER_{50\%}$, presented at 22h.



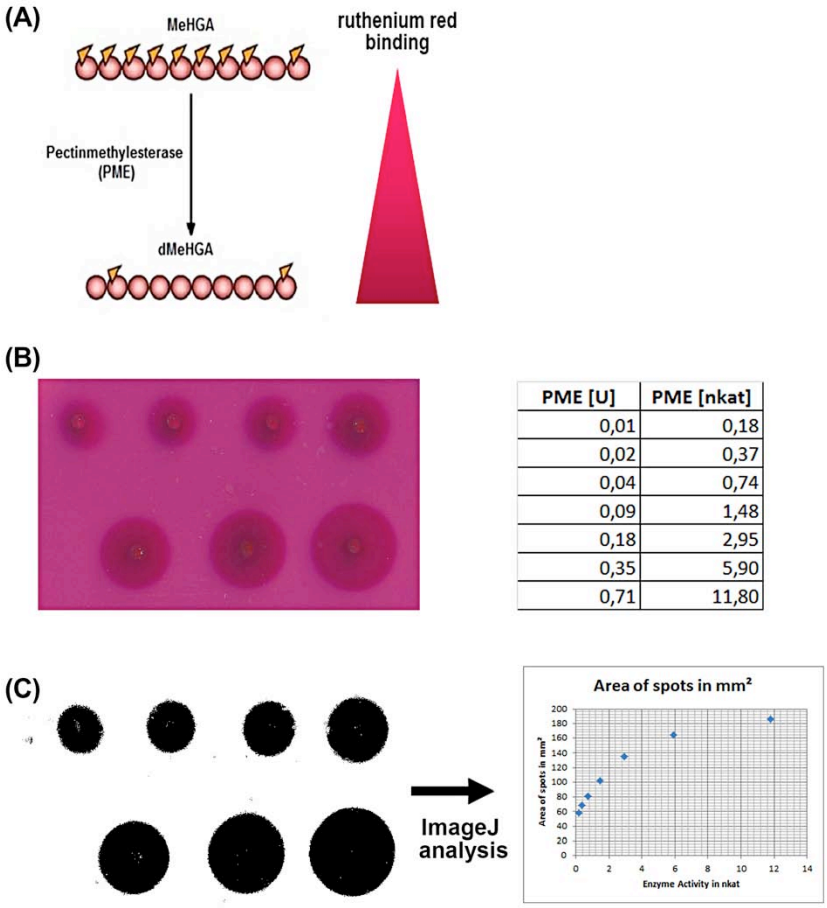
Supplemental Figure S5. The effect of exogenous treatments of *Lepidium sativum* seeds with PME on testa and endosperm rupture. Treatment of imbibed seeds with relatively high amounts (18 and 27 U, i.e. 3.0 and 4.4 U/ml, respectively) of orange peel PME delayed TR **(A)** and ER **(B)**. In contrast, low amounts (0.2, 0.4 and 4.4 U, i.e. 0.03, 0.07 and 0.7 U/ml, respectively) promoted TR **(A)**, but did not appreciably affected ER **(B).** Seeds were imbibed at 18ºC in continuous light; mean values ±SE of four biological replicates are shown.



Supplemental Figure S6. The effect of exogenous treaments of *Lepidium sativum* seeds with PME and pectin degradation by polygalacturonase (PG) on testa permeability using the tetrazolium assay. Seeds were imbibed for 9 h in tetrazolium salt assay solution without (CON) or with 0.2 U PME or PME+PG added. Embryos were excised and classified into five staining groups: **(1)** pale (no staining, testa impermeable for tetrazolium salts, see Figure 8B), **(2)** yellow (low testa permeability, see Figure 8B), and three groups of red embryos (partly or fully red): **(3)** partly red 1 (red staining at cotyledon base), **(4)** partly red 2 (red staining at radicle tip), **(5)** almost fully red (except radicle). Relative numbers based on 50 embryos for each series are presented. The sum of red stained embryos is also presented in Figure 8. Red embryo staining is indicative for increased testa permeability.



Supplemental Figure S7. Expression of the Arabidopsis probesets corresponding to the *Lepidium sativum* reference gene candidates identified in Graeber et al. (2011) which are present in the masked data of the heterologous ATH1 microarray hybridisations. Of the 15 reference genes identified there, 1 is not present on the Affymetrix array and 5 are masked in the data we used here. Of the 5 masked genes, 4 have at least one significant probe at the FDR of 0.05 but less than 3 probes remaining, so are masked for that reason. The nine reference gene candidates include the three most stable reference genes (LesaG17210 (At1G17210), LesaG20000 (At2G20000), LesaG04320 (At4G04320)) for which the geometric mean was used for normalising our qRT-PCR analysis of CAP and RAD RNA from fully imbibed seeds. In agreement with our qRT-PCR results (this work and Graeber et al. 2011) the microarray expression of most of the 9 genes is fairly stable in fully imbibed seeds (i.e. excluding the 1h values) and the three reference genes used show the same order in signal intensity (LesaG17210 high, LesaG20000 medium, LesaG04320 low) as in transcript abundance determined by qRT-PCR. For abbreviations (x-axis) see Figure 1.



PME [U]	PME [nkat]
0,01	0,18
0,02	0,37
0,04	0,74
0,09	1,48
0,18	2,95
0,35	5,90
0,71	11,80

Supplemental Figure S8. As PME enzyme activity assay we used the gel diffusion assay described by Downie et al. (1998). **(A)** The assay is based on the stronger binding of ruthenium red to de-methylesterfied HG (Me-HG) compared to highly methlesterified HG. **(B)** Staining of agar supplemented with Me-HG after exposure to different concentrations of commercially available PME. The Units of PME activity applied are shown in the table. **(C)** Digital analysis of the size of the demethylesterified darker stained circle on the photograph, and the standard curve derived from it.

GO Term	Details	GO Group	Support	List size	Genes
GO:0009826	Unidimensional cell growth	Biological Process	3	40	AT1G17060, AT2G06850, AT3G45970
GO:0010411	Xyloglucan metabolic process	Biological Process	2	40	AT4G30280, AT1G65310
GO:0006820	Anion transport	Biological Process	2	40	AT3G62270, AT3G06450
	Other	Biological Process	12		
GO:0016798	Hydrolase activity, acting on glycosyl bonds (MF)	Molecular Function	5	40	AT4G30280, AT2G06850, AT3G23730, AT1G65310, AT5G65730
GO:0005507	Copper ion binding (MF)	Molecular Function	3	40	AT1G64640, AT4G22010, AT4G12420
GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds (MF)	Molecular Function	3	40	AT5G08370, AT3G23730, AT5G65730
GO:0016788	Hydrolase activity, acting on ester bonds (MF)	Molecular Function	3	40	AT3G48610, AT5G55050, AT3G10870
GO:0016762	Xyloglucan:xyloglucosyl transferase activity (MF)	Molecular Function	3	40	AT2G06850, AT3G23730, AT5G65730
GO:0080039	Xyloglucan endotrans-glucosylase activity (MF)	Molecular Function	2	40	AT4G30280, AT1G65310
GO:0033946	Xyloglucan-specific endo-beta-1,4-glucanase activity (MF)	Molecular Function	2	40	AT4G30280, AT1G65310
GO:0015301	Anion:anion antiporter activity (MF)	Molecular Function	2	40	AT3G06450, AT3G62270
	Other	Molecular Function	5	40	

Supplemental Table S1. Overrepresentation analysis of GO terms for genes differentially up-regulated after testa rupture compared to before testa rupture in the CAP 7 h after sowing. "Other" in the table refers to single gene groups which were listed as overrepresented and condensed to the other listing.

Note that using GO enrichment analyses for CAP and RAD, we found three time points (Fig. 1) with a high number of differentially regulated genes: one was right after the end of imbibition at 3 h after sowing compared to seeds after 1 h of sowing, the other two time points with high amounts of differentially regulated genes were at the phase transition time points (7 h for TR and 16 h for ER) when comparing testa or endosperm ruptured seeds, respectively to their non-ruptured counterparts. As the late stage of germination is well studied (as reviewed by Kucera et al., 2005; Finch-Savage and Leubner-Metzger 2006; Holdsworth et al. 2008) we chose to focus on testa rupture for our analyses as this is the first visible manifestation of germination. Genes upregulated in the CAP (Supplemental Table S1) in samples with test rupture (+TR) compared to samples with intact testa (-TR) were enriched in Gene Ontology (GO) terms for xyloglucan metabolic processes, one-dimensional cell growth, and anion transport (Supplemental Table S1). Enrichment was calculated compared to all Arabidopsis genes that are present on the ATH1 chip. The GOs for "molecular function" showed overrepresentation of hydrolases acting on cell wall-related targets as well as copper ion binding, while metabolic processes of germination enhancing hormones (Kucera et al., 2005) were overrepresented in the "biological process" GO group.

GO Term	Details	GO Group	Support	List size	Genes
GO:0005975	Carbohydrate metabolic process	Biological Process	2	5	AT3G23730, AT1G11545
GO:0006073	Cellular glucan metabolic process	Biological Process	2	5	AT3G23730, AT1G11545
GO:0042545	Cell wall modification	Biological Process	1	5	AT1G02810
GO:0009831	Plant-type cell wall modification involved in multidimensional cell growth (BP)	Biological Process	1	5	AT1G10550
GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	Molecular Function	3	5	AT1G11545, AT1G10550, AT3G23730
GO:0016762	Xyloglucan:xyloglucosyl transferase activity	Molecular Function	3	5	AT1G11545, AT1G10550, AT3G23730
GO:0016798	Hydrolase activity, acting on glycosyl bonds	Molecular Function	3	5	AT1G11545, AT1G10550, AT3G23730
GO:0004857	Enzyme inhibitor activity	Molecular Function	1	5	AT1G02810
GO:0030599	Pectinesterase activity	Molecular Function	1	5	AT1G02810

Supplemental Table S2. Overrepresentation analysis of GO terms for genes differentially up-regulated after testa rupture compared to before testa rupture in the RAD 7 h after sowing. "Other" in the table refers to single gene groups which were listed as overrepresented and condensed to the other listing.

See the legend of Supplemental Table S1 for details on the time points for the GO enrichment analyses. The RAD samples also showed an overrepresentation of cell-wall related GO terms (Supplemental Table S2). Specifically, the xyloglucan endotransglycosylase/hydrolase activity term reoccurs, and pectinesterase activity and enzyme inhibitor activity joined the overrepresented terms.

Parameter	Value	Units
$[MeHG]_{CAP,0}$	8797.0552	mg μm^{-3}
$[dHG]_{CAP,0}$	0.6963	mg μm^{-3}
$[G1]_{CAP,0}$	0.0007	mg μm^{-3}
$[G2]_{CAP,0}$	0.0181	mg μm^{-3}
$[PMEI]_{CAP,0}$	39.2946	mg μm^{-3}
$[iG2]_{CAP,0}$	2.3048	mg μm^{-3}
$[PMEI:G1]_{CAP,0}$	2.6749	mg μm^{-3}
$[PMEI:G2]_{CAP,0}$	2.8807	mg μm^{-3}
$[MeHG]_{RAD,0}$	6771.1779	mg μm^{-3}
$[dHG]_{RAD,0}$	0.6219	mg μm^{-3}
$[G1]_{RAD,0}$	11.0852	mg μm^{-3}
$[G2]_{RAD,0}$	8.1561	mg μm^{-3}
$[PMEI]_{RAD,0}$	0.3689	mg μm^{-3}
$[iG2]_{RAD,0}$	0.2982	mg μm^{-3}
$[PMEI:G1]_{RAD,0}$	7.6944	mg μm^{-3}
$[PMEI:G2]_{RAD,0}$	0.9789	mg μm^{-3}
α_1	0.1265	μm^3 mg^{-1} s^{-1}
α_2	0.0259	μm^3 mg^{-1} s^{-1}
ζ_1	0.9966	μm^3 mg^{-1} s^{-1}
ζ_2	0.0602	μm^3 mg^{-1} s^{-1}
ζ_3	0.9906	μm^3 mg^{-1} s^{-1}
C	0.3438	

Supplemental Table S3. Parameter values for the mathematical model (Fig. 6), from fitting the model to PME enzyme activity data (Fig. 5). All values are accurate to four decimal places, a subscripted zero denotes the value at t = 0 in the indicated compartment (CAP or RAD). C is dimensionless.

Group	Putative Arabidopsis ortholog	*Lepidium sativum (Lesa)* name	*Lepidium sativum* GenBank accession number	*Lepidium sativum* specific qRT-PCR primer	
				forward primer (5'-3')	reverse primer (5'-3')
PME-group1	At3g29090	LesaPME29090	JK693824	CTCGGTTGGCAGGATACATT	CCCTGAGATTTGCAGTGGAT
PME-group2	At1g11580	LesaPME11580	JQ011281	ATGCTTGTTGGTGACGGCAA	GAATCCATATGTCTTGCGCC
PME-group2	At2g26440	LesaPME26440	JK693823	CACTCCTTCCGTTTTCTCGT	TGTTGGGGCTTATGTTGATG
PME-group2	At5g51490	LesaPME51490	JK693825	ATCCTACCGGCTCCTGATCT	CAGACCAAACACCGAACCTT
PME-group2	At3g14310	LesaPME14310	JK693822	CGAACACAGGAGCAGGGG	ACCGAGCGAGAAGGGGAAAC
PMEI	At1g14890	LesaPMEI14890	JQ011282	GGCTTACCTCTCCAAACTCTC	CTCCGTCTTCCATCTCCTC
PMEI	At5g62360	LesaPMEI62360	JK693827	GAGACTGCGTCGAGGAGTT	CCCAAGTCTGTATATCGCTTA
PMEI	At5g62350	LesaPMEI62350	JK693826	CCCTGCCTTATGTGTCCACT	ACGCAATCTTTGATGGCTTC

Supplemental Table S4. Gene bank accession numbers for the cloned LesaPME/PMEI cDNAs from *Lepidium sativum* seeds and primer sequences for LesaPME/PMEI qRT-PCR. For reference gene LesaG17210, LesaG20000 and LesaG04320 primer see Graeber et al. (2011).