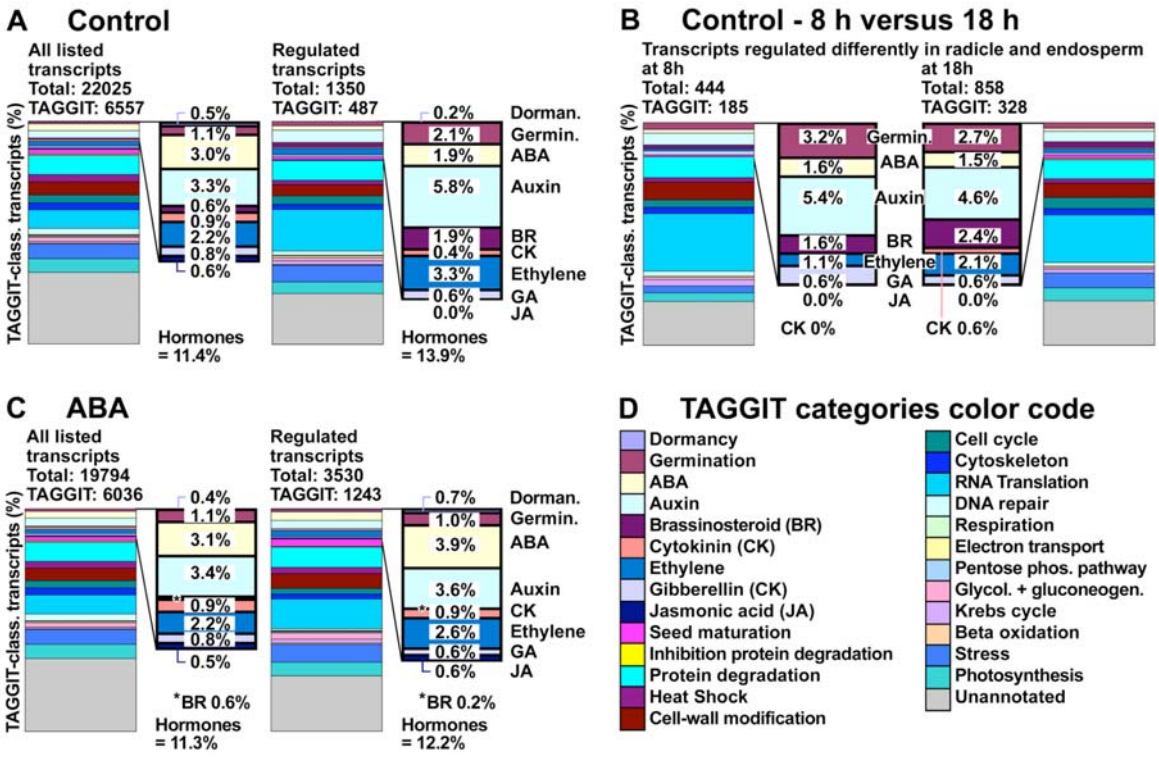
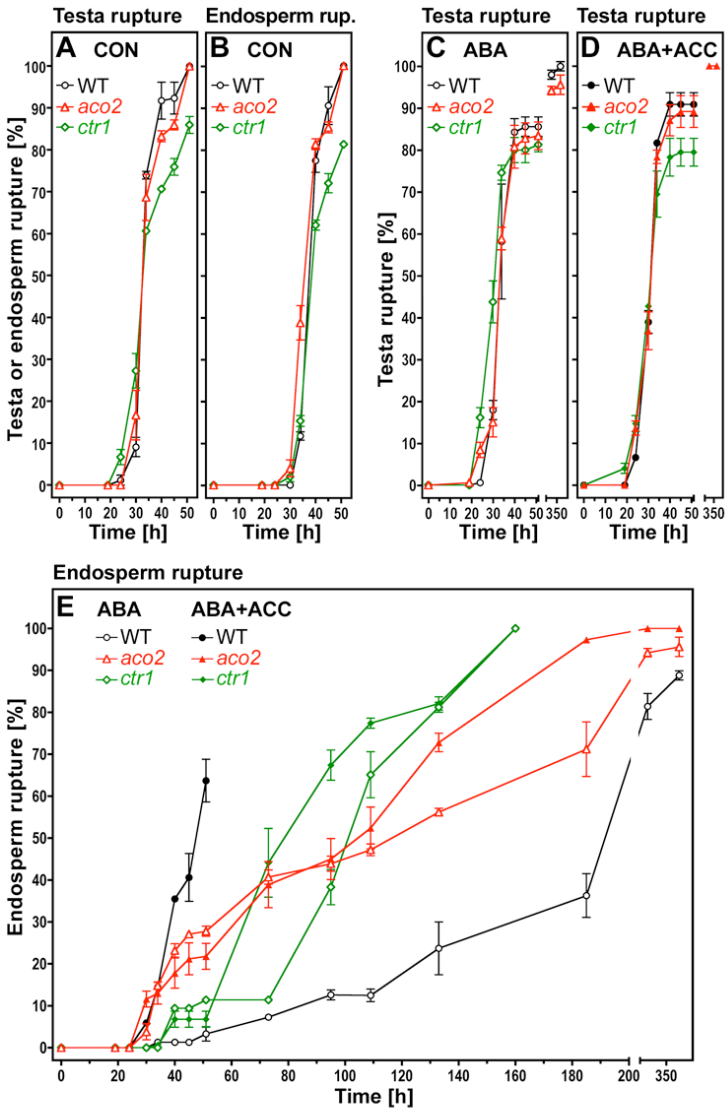
Supplemental Data. Linkies et al. (2009). Ethylene interacts with abscisic acid to regulate endosperm rupture during germination; a comparative approach using *Lepidium sativum* (cress) and *Arabidopsis thaliana.*

Supplemental Figure 1



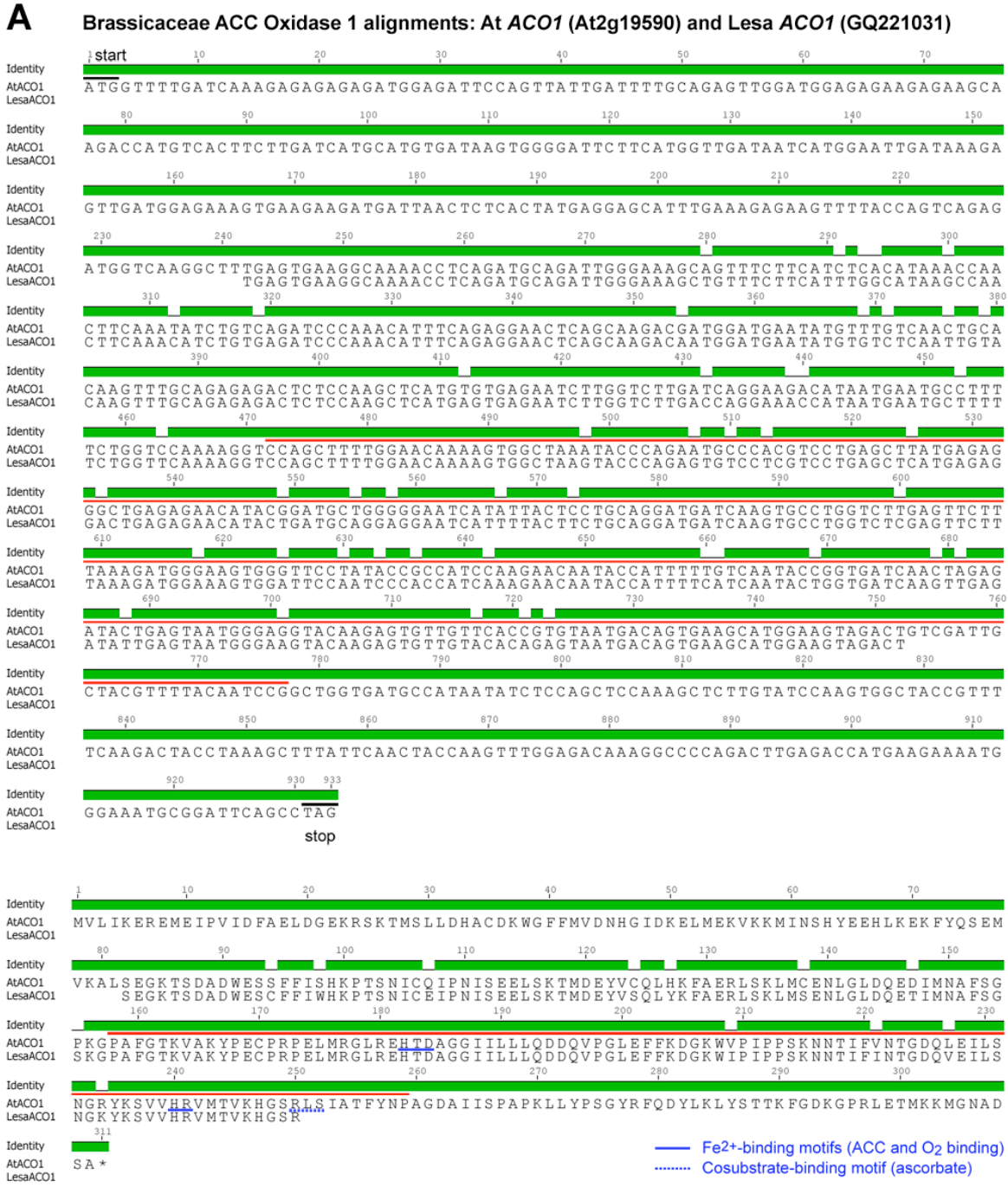
Supplemental Figure 1. TAGGIT analysis of functional categories in the *Lepidium sativum* FR1 transcriptome datasets. The seed-specific TAGGIT workflow (Carrera et al. 2007; Holdsworth et al., 2008) was applied to Lepidium transcriptome datasets to provide proportional representations of genes in functional categories. **(A, B)** Comparison of CON array lists: **(A)** All transcripts belonging to TAGGIT functional categories (6557 of 22025 transcripts, Supplemental Dataset 3 online) and significantly regulated transcripts belonging to TAGGIT functional categories (487 of 1350 transcripts, Supplemental Dataset 5 online). **(B)** Transcripts belonging to TAGGIT functional categories that are regulated in a significantly different way in the radicle and endosperm at 8 h and 18 h (CON-array, Supplemental Dataset 5 online). **(C)** Comparison of ABA array lists: All transcripts belonging to TAGGIT functional categories (6036 of 19794 transcripts, Supplemental Dataset 4 online) and significantly regulated transcripts belonging to TAGGIT functional categories (1243 of 3530 transcripts, Supplemental Dataset 6 online). **(D)** Key to the colour representation of TAGGIT functional categories (Holdsworth et al., 2008). **(A-C)** The TAGGIT analysis suggests that ethylene-related transcripts are important for germination and for counteracting the ABA inhibition. Supplemental Datasets 3-6 provide lists of the transcripts from which the subsets belonging to different TAGGIT functional categories can be extracted by sorting for the TAGGIT column. For example, the numbers (percentages) of transcripts in the functional category 'ethylene' are for 'all transcrips' and 'regulated transcripts' in **(A)** 144 (2.2%) and 16 (3.3%) for CON and in **(B)** 131 (2.2%) and 32 (2.6%) for ABA.

Supplemental Figure 2

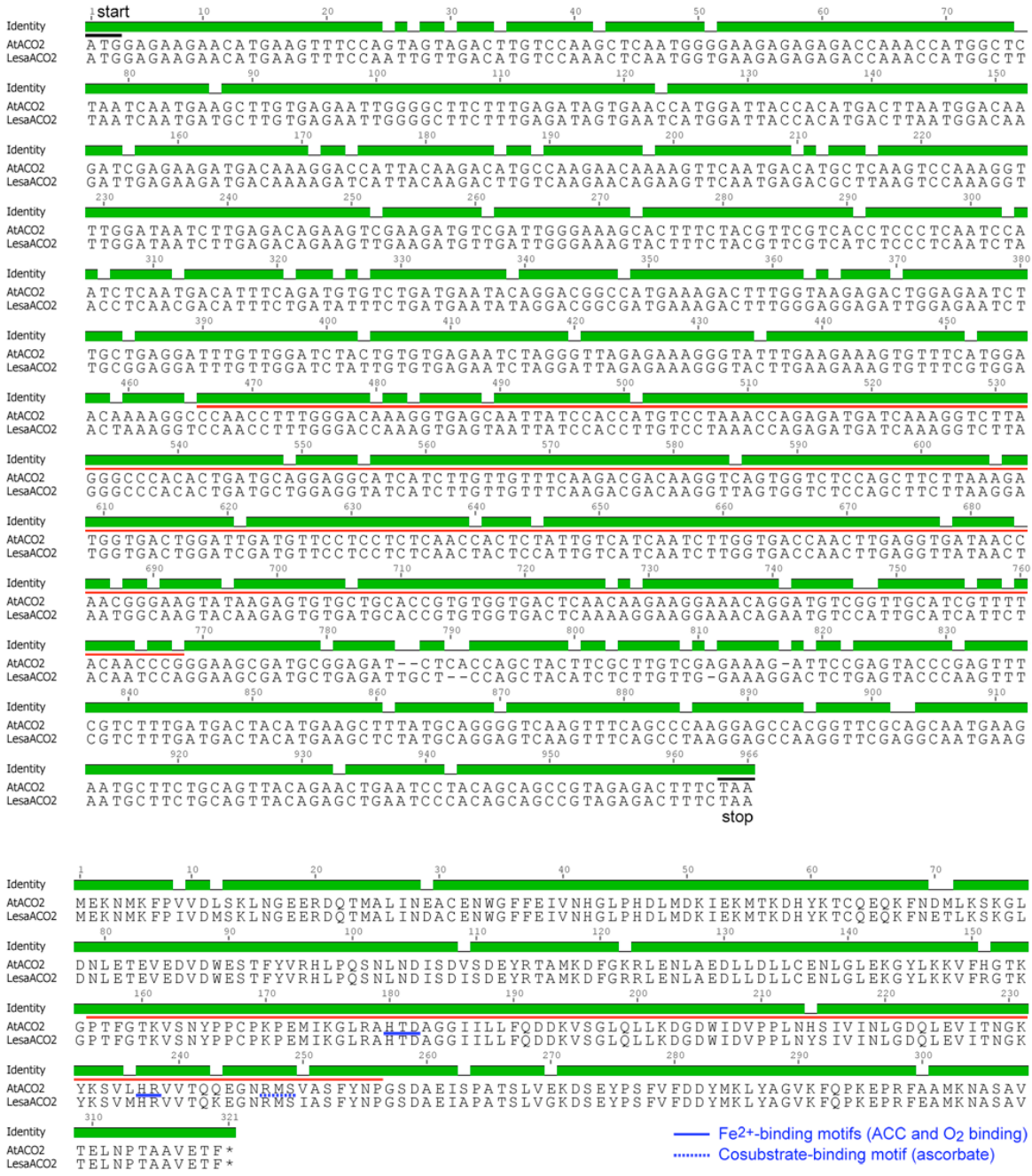


Supplemental Figure 2. The effect of ACC and ABA on the germination of *Arabidopsis thaliana*: Wild type (Col) and ethylene-related mutants (*aco2, ctr1*). (A-E) Time course analyses of testa and endosperm rupture of wild type (WT), the ACO2-deficient mutant *aco2* and the loss-of-function signaling mutant *ctr1*. **(A)** Testa and **(B)** endosperm rupture under optimal conditions (CON, no hormone addition to the medium). **(C,D)** Testa rupture of seeds incubated in the presence of **(C)** ABA or **(D)** ABA+ACC. **(E)** Endosperm rupture of seeds incubated in the presence of ABA or ABA+ACC. Incubation conditions: continuous light, 24ºC, no cold-stratification. Medium additions, as indicated: 1 µM ABA, 1 mM ACC. Mean values ± SE of three x 50 seeds are presented.

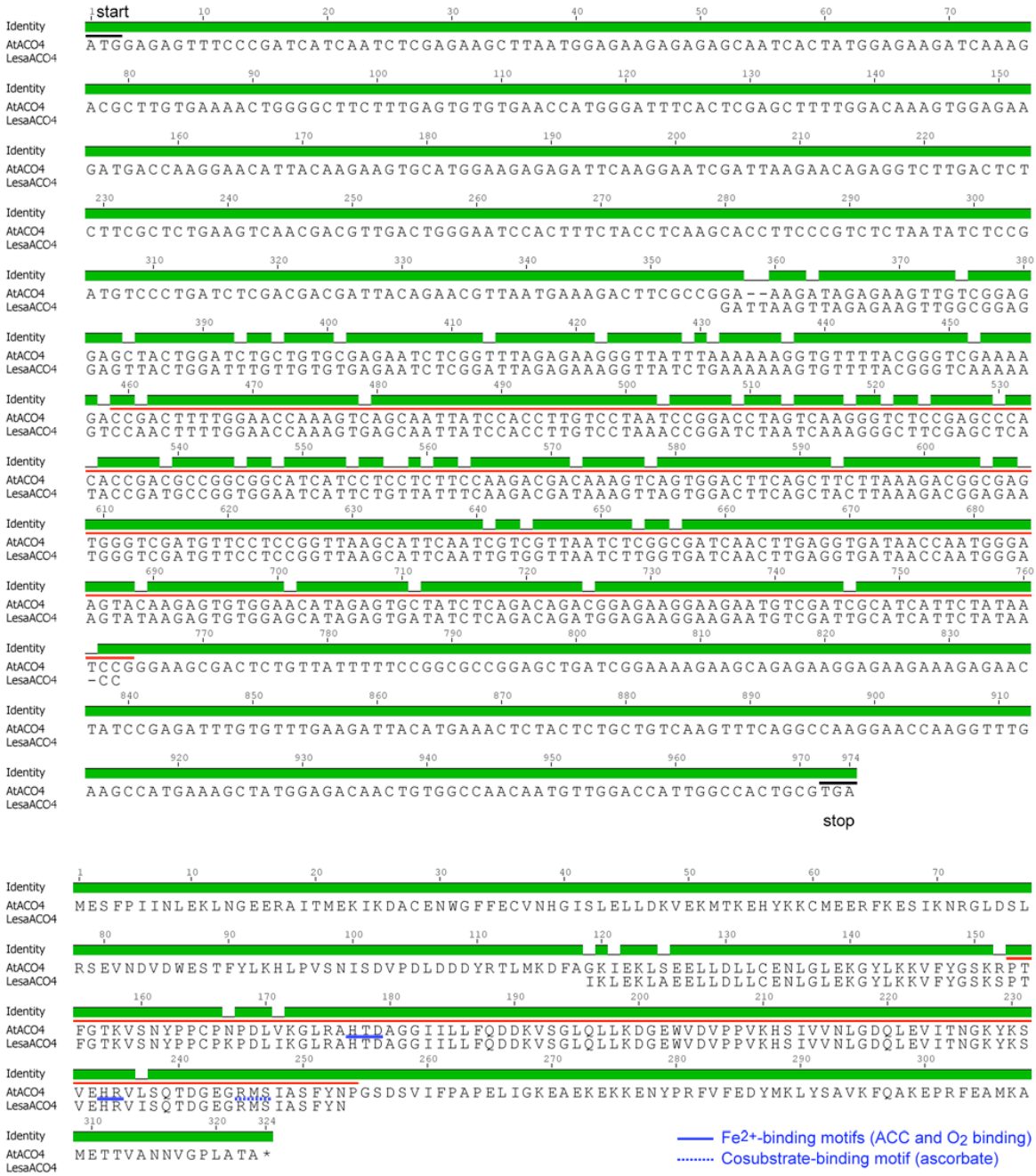
Supplemental Figure 3



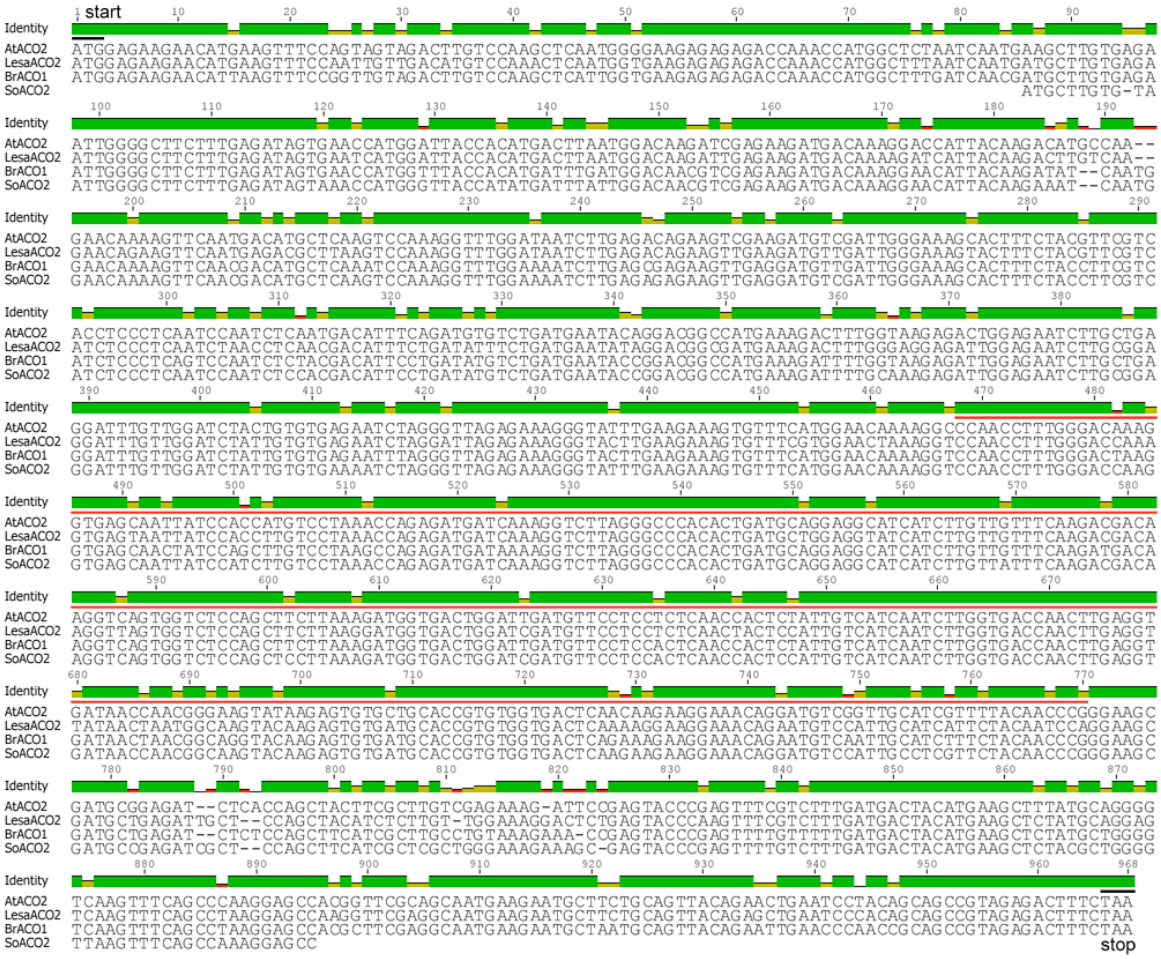
B **Brassicaceae ACC Oxidase 2 alignments: At *ACO2* (At1g62380) and Lesa *ACO2* (GQ221032)**



C **Brassicaceae ACC Oxidase 4 alignments: At *ACO4* (At1g05010) and Lesa *ACO4* (GQ221033)**

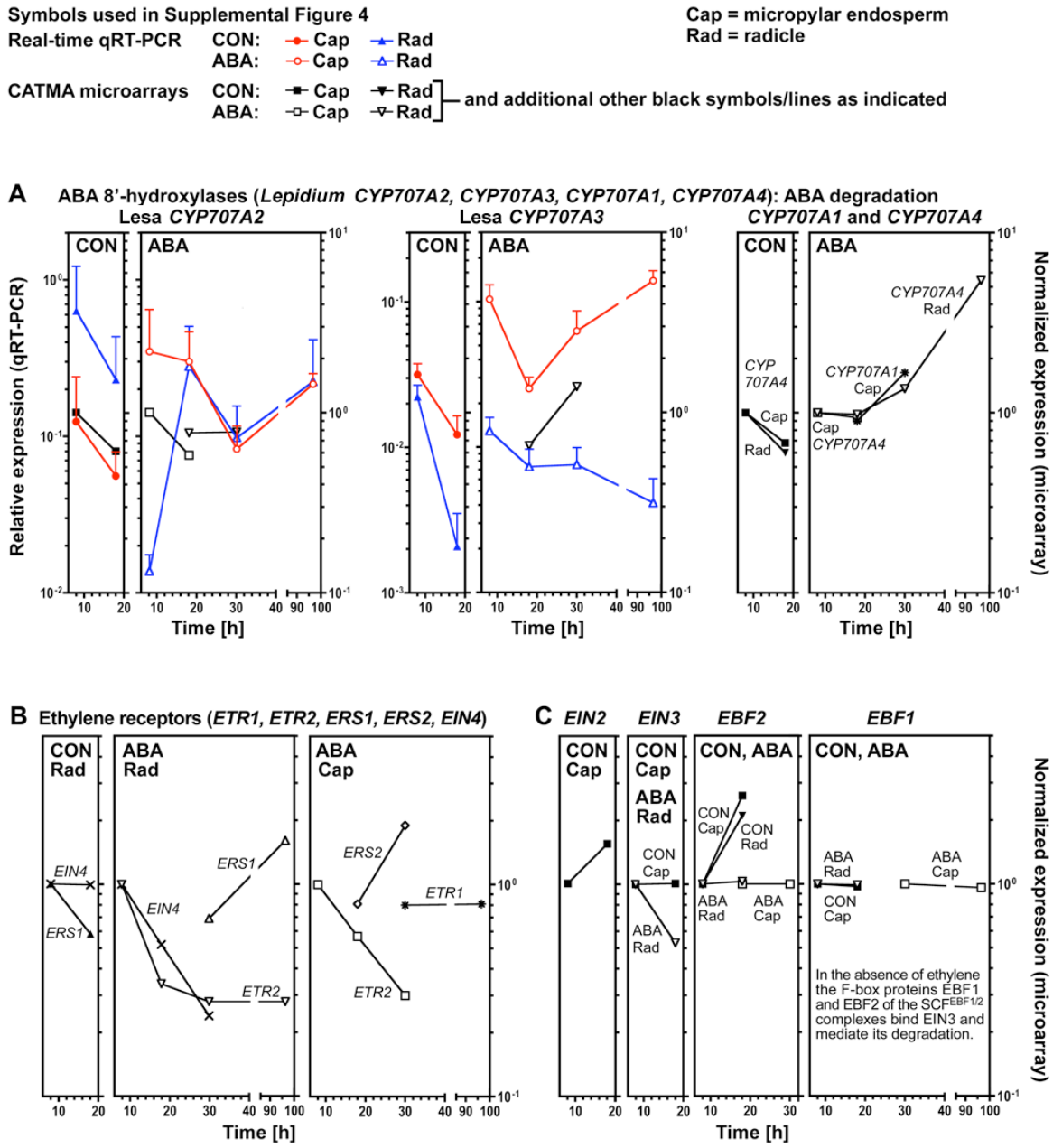


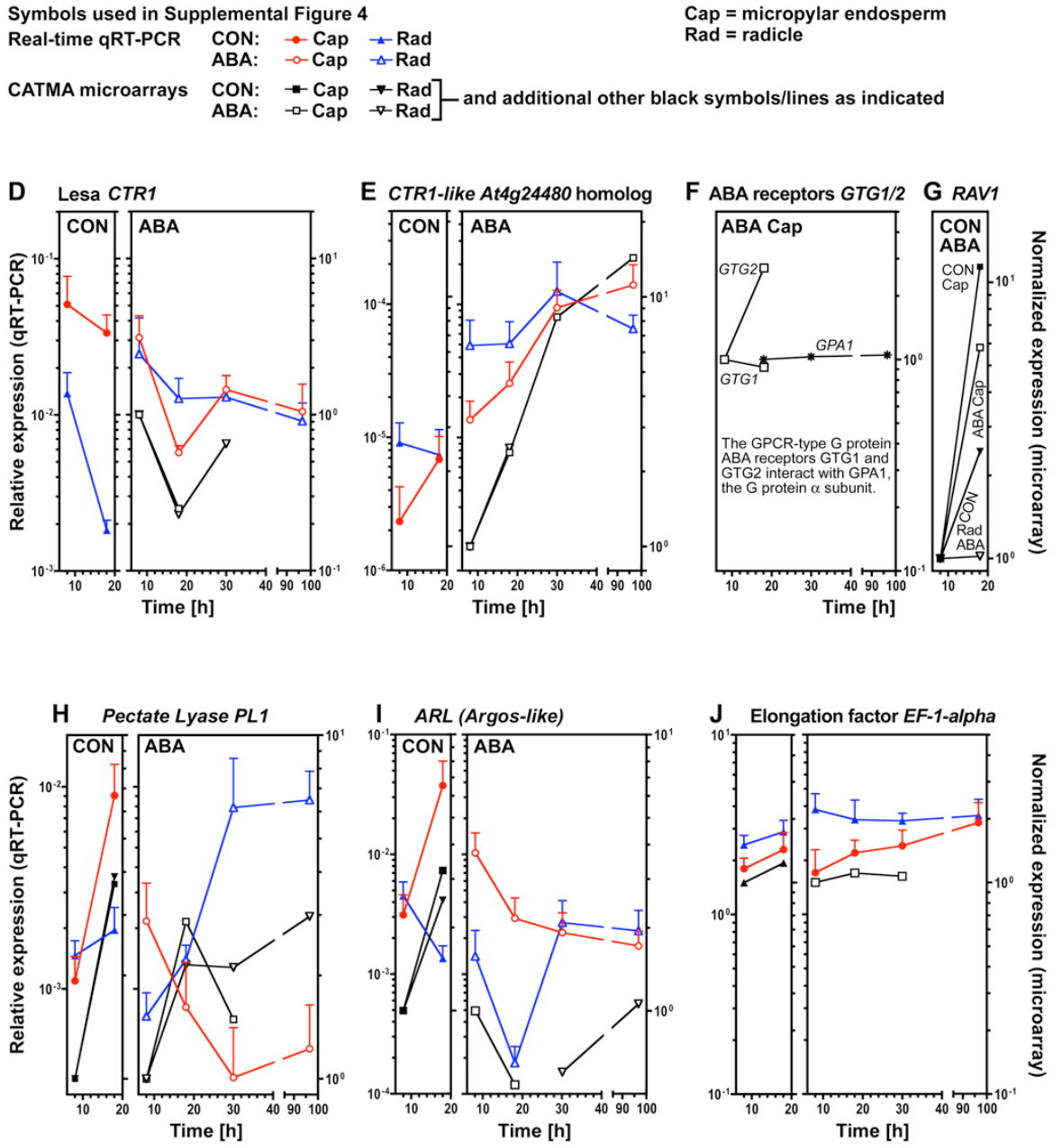
D **Brassicaceae ACC Oxidase 2 orthologous cDNA alignments:**
Lineage I (Arabidopsis, Lepidium): At *ACO2* (At1g62380) and Lesa *ACO2* (GQ221032)
Lineage II (Brassica, Sisymbrium): Br *ACO1* (AJ309322) and So *ACO2* (EU689115)



Supplemental Figure 3. Brassicaceae ACC oxidase (ACO) sequence comparisons. (A-C) Alignments of *Arabidopsis thaliana* Col and *Lepidium sativum* FR14 mRNA (cDNA) and amino acid sequences of the ACO orthologs. The conserved InterPro (www.ebi.ac.uk/interpro/) domain regions of 2OG-Fe(II)-oxygenases are underlined in red. The start and stop codons in the cDNA sequences are underlined and marked as start and stop in black. The conserved active binding site motifs for the substrates ACC and O_2 as well as the cosubstrate binding site for ascorbate (Seo et al. 2004) are underlined in blue in the amino acid sequences and indicate that these proteins are functional ACOs. **(A)** Arabidopsis ACO1 (At2g19590) and Lepidium *ACO1* (GQ221031). **(B)** Arabidopsis *ACO2* (At1g62380) and Lepidium *ACO2* (GQ221032). **(C)** Arabidopsis *ACO4* (At1g05010) and Lepidium *ACO4* (GQ221033). The corresponding Arabidopsis *ACOs* and Lepidium *ACOs* are true orthologs based on the highest BLAST hits and the molecular phylogenetic analysis. **(D)** Alignment of Brassicaceae *ACO2* orthologs which show transcript expression pattern in seeds (Fig. 7F) that are associated with germination responses. Brassicaceae lineage I (*Arabidopsis thaliana* (At), *Lepidium sativum* (Lesa)) and lineage II (*Brassica rapa* (Br), *Sisymbrium officinale* (So)) cDNA sequences are presented. At least three independent cDNA clones were sequenced for each Lepidium ortholog.

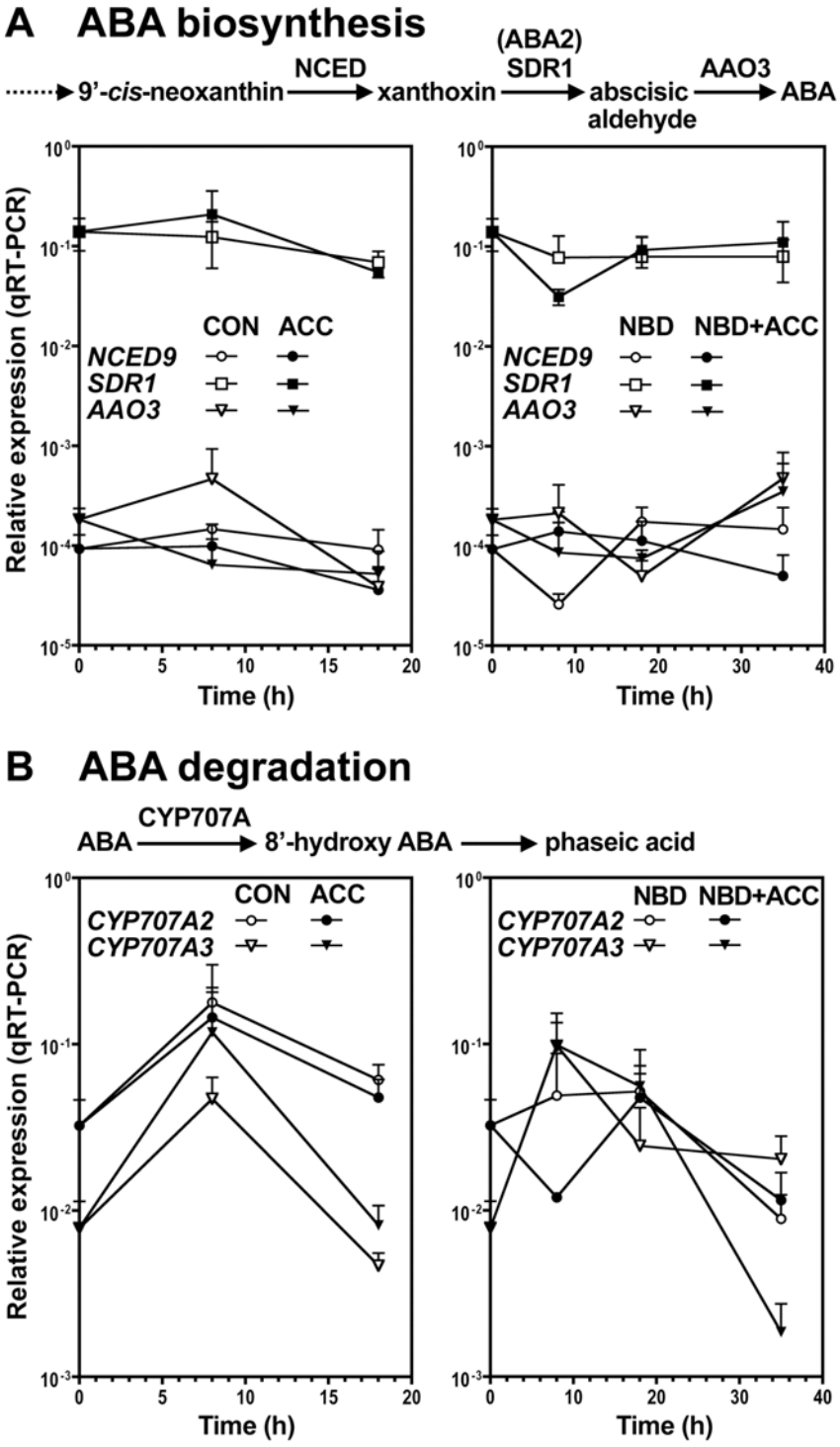
Supplemental Figure 4




Symbols used in Supplemental Figure 4
Real-time qRT-PCR
CON: Cap Rad
ABA: Cap Rad
CATMA microarrays
CON: Cap Rad
ABA: Cap Rad
and additional other black symbols/lines as indicated
Cap = micropylar endosperm
Rad = radicle
D Lesa CTR1
E CTR1-like At4g24480 homolog
F ABA receptors GTG1/2
G RAV1
CON
ABA
Relative expression (qRT-PCR)
Normalized expression (microarray)
Time [h]
ABA Cap
GTG2
GPA1
GTG1
The GPCR-type G protein ABA receptors GTG1 and GTG2 interact with GPA1, the G protein α subunit.
CON Cap
ABA Cap
CON Rad
ABA
H Pectate Lyase PL1
I ARL (Argos-like)
J Elongation factor EF-1-alpha

Supplemental Figure 4. Analysis of transcript expression by qRT-PCR and microarray analysis in specific seed tissues of *Lepidium sativum* FR1 during germination. Time course transcript expression data are presented for endosperm caps (Cap) and radicles (Rad) dissected from seeds incubated at 24ºC in continuous light in medium without (CON) or with 10 µM ABA added. For qRT-PCR relative $\Delta\Delta C_t$ expression values based on the comparison with validated constitutive transcripts are presented. Lepidium transcripts named with the prefix 'Lesa' were analysed by qRT-PCR primers designed on the basis of their cloned cDNA sequences. Lepidium transcripts without this prefix in their name were analysed with a qRT-PCR primer design based on Arabidopsis cDNA sequences. Primer sequences for the qRT-PCR are presented in Supplemental Table 1 online. **(A)** ABA 8'-hydroxylases: Four *CYP707A* genes are known in Arabidopsis and all provided expression results in the Lepidium seed arrays. Two Lepidium cDNAs were cloned (Lepidium *CYP707A2* and *CYP707A3*) and on the basis of their sequence analyses, represent putative Lepidium orthologs of Arabidopsis *CYP707A2* and *CYP707A3*, respectively. **(B)** Ethylene receptors. **(C)** Ethylene signaling components. **(D)** Ethylene signaling repressor *CTR1* (*Constitutive Triple Response1*); the cDNA of the putative Lepidium ortholog *CTR1* was cloned. **(E)** *CTR1-like* serine/threonine protein kinase. **(F)** GPCR-type G protein ABA receptors *GTG1* and *GTG2* and their interacting G protein α subunit *GPA1*. **(G)** *RAV1*, an AP2/EREBP-type transcription factor with an ABI3/VP1-like domain. **(H)** *PL1*, *Pectate lyase1*. **(I)** *ARL, Argos-like*, putative cell expansion gene. **(J)** *EF-1-alpha*, Transcription elongation factor 1-alpha. Mean values +SE of four independent biological RNA samples obtained from 1000 endosperm caps or 100 radicles from seeds with ruptured testa, but intact endosperm are presented for the qRT-PCR results. Normalized microarray differences are presented for comparison.

Supplemental Figure 5



Supplemental Figure 5. Analysis of the transcript expression of key ABA metabolism genes, in whole *Lepidium sativum* FR14 seeds, by qRT-PCR following treatment with ACC or NBD. Time course transcript expression data are presented for whole seeds incubated at 24ºC in continuous light in absence (CON) or presence of the ethylene precursor ACC (1 mM), the ethylene action inhibitor NBD (100 µl/l applied via the gas phase), or the combination NBD+ACC. For qRT-PCR, relative $\Delta\Delta C_t$ expression values based on the comparison with validated constitutive transcripts are presented. Primer sequences for the qRT-PCR are presented in Supplemental Table 1 online. **(A)** qRT-PCR for transcripts of key regulatory genes for ABA biosynthesis: *NCED9* = *NINE-cis-EPOXYCAROTENOID DIOXYGENASE9*, *SDR1* = *SHORT-CHAIN DEHYDROGENASE REDUCTASE1* (also known as *ABA2*), AAO3 = ABSCISIC ALDEHYDE OXIDASE3. **(B)** qRT-PCR for transcripts of key regulatory genes for ABA degradation: The Lepidium ABA 8'-hydroxylases *CYP707A2* and *CYP707A3* were analysed. Mean values +SE of three independent biological RNA samples are presented.

Supplemental Methods

aRNA labelling and CATMA microarray hybridization

The *Lepidium sativum* FR1 (Freiburg1) aRNA was labelled and the CATMA microarrays were hybridized according to the method described in Lim et al. (2007). Briefly, 5 µg of aRNA was reverse transcribed using random nonamers (Invitrogen, UK) and SuperScript II (Invitrogen, UK). The Cy3- and Cy5-labelled cDNA probes were prepared, whereby amino allyl-dUTP was incorporated during cDNA synthesis followed by chemical labeling of the amino allyl-modified cDNA using CyDye NHS-esters (Amersham Biosciences, NJ, USA). Reactions were incubated at 42ºC for 2.5 h and terminated by the addition of 2 µL NaOH and an additional incubation at 37ºC for 15 min. The labelled cDNA was neutralised by adding 10 µL 2 M MOPS buffer and purified using the Qiagen PCR Purification Kit (Qiagen, UK). Cy3- and Cy5-labelling efficiency was quantified using a nanodrop ND Spectrophotometer (NanoDrop Technologies, Rockland, DE). An aliquot, containing 40 pmol of Cy dye label, was used for subsequent microarray hybridisations.

The CATMA microarray slides were prehybridised in 60 mL prehybridisation buffer (1% (w/v) BSA, 5 x SSC buffer, 0.1% (w/v) SDS) at 42ºC for 2 h. Cy3- and Cy5-labelled cDNAs were freeze-dried and resuspended in 70 µL of hybridisation buffer (25% (v/v) formamide, 5 x SSC, 0.1% (w/v) SDS, 0.5% (w/v) yeast tRNA (invitrogen, UK). The microarray slides were washed five times in water and twice in isopropanol and dried by centrifugation at 1500 rpm for 2 min. The cDNA probes were denatured by incubation at 95ºC for 5 min and applied directly to the microarray slides that were held in a hybridisation chamber (Corning Life Sciences, Netherlands). The microarrays were covered with a coverslip (Sigma Aldrich, UK) and hybridised overnight at 42 ºC in a hybridisation oven. The microarrays were then subjected to the following washings: once in Wash Solution 1 (2x SSC, 0.1% (w/v) SDS) pre-warmed to 42ºC for 5 min; once with Wash solution 2 (0.1 x SSC, 0.1% (w/v) SDS) at room temperature for 10 min; and five times in Wash solution 3 (0.1 x SSC only) at room temperature for 10 min. The arrays were then transferred to isopropanol for a few seconds and centrifuged at 1500 rpm for 2 min. All washes were performed on an orbital shaker with vigorous shaking. The microarrays were then 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/).

Lepidium and Arabidopsis Genomic DNA Extraction and Labeling for Hybridisation to CATMA Microarrays

Genomic DNA was extracted from *L. sativum* FR1 and *Arabidopsis thaliana* Cvi leaf tissue using a modified CTAB extraction protocol. Approximately 100 mg of leaf tissue was homogenised in liquid nitrogen and ground fully in 300 µL of CTAB-Buffer B (100 mM Tris/HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA and 2% (w/v) hexadecyltrimethyl ammoniumbromide (CTAB)). The homogenate was incubated at 65ºC for 15 min and spun in a microcentrifuge at 13000 rpm for 2

min to remove cell debris. The supernatant was extracted twice with an equal volume of chloroform. The genomic DNA was precipitated by adding an equal volume of CTAB-Buffer C (50 mM Tris/HCl (pH 8.0), 10 mM EDTA and 1% (w/v) CTAB) and incubated at room temperature for 2 h. The genomic DNA was pelleted by centrifugation at 13000 rpm for 10 min. The DNA pellets were then resuspended in 400 µL 1 M CsCl and precipitated once more by the addition of 800 µL 100% ethanol. The DNA was pelleted by centrifugation at 13000 rpm for 10 mins. The DNA pellets were washed twice with 70% (v/v) ethanol, air dried and re-suspended in TE (10mM Tris/HCl (pH 7.5), 1 mM EDTA) and RNase A (20 µg/µL).

Lepidium and Arabidopsis genomic DNA was fluorescently labelled using the BioPrime Array CGH Genomic Labelling System (Invitrogen, UK). Briefly, 20 µg of genomic DNA was mixed with 2.5x Random Primers Solution (final volume 41 µL) and denatured at 95ºC for 5 minutes and immediately placed on ice. For each labelling reaction 5 µL of 10x dCTP mix, 3 µL of Cy3-dCTP or Cy5-dCTP CyDye NHS-esters (Amersham Biosciences, www5.amersham biosciences.com) and 1 µL of Exo-Klenow fragment were added. The reactions were incubated at 3 ºC for 2 h and the reaction was stopped by adding 5 µL of Stop buffer (0.5 M EDTA). The labelled DNA was purified according to the BioPrime Array CGH Genomic Labelling System. The reaction volumes was adjusted to 100 µL with TE, to which 400 µL Purification Buffer A was added and this mixture was vortexed. They were then loaded on to the column provided with the Kit and centrifuged at 11000 x g for 1 min at room temperature, and the flow-through was discarded. Two-hundred microliters of Purification Buffer B was added to the column and the column was centrifugated at 1000 x g for 1 min at room temperature and the flow-through was discarded. 50 µL dH_2O was then added to the column and the column incubated at room temperature for 1 min. The labelled DNA was recovered by centrifugation at 11000 x g for 1 min at room temperature. Cy3- and Cy5-labelling efficiency was quantified using a NanoDrop ND Spectrophotometer (NanoDrop Technologies, Rockland, DE). Cy3-labelled Arabidopsis genomic DNA and Cy5-labelled Lepidium genomic DNA (reciprocal labelling were also performed) were freeze-dried together for 2 h and re-suspended in hybridisation buffer.

Lepidium and Arabidopsis Genomic DNA hybridisation to CATMA Microarrays

Prehybridisation, hybridisation and washing of the CATMA version 3 microarrays is the same as outlined for the RNA microarrays above. For the genomic DNA microarrays, two independent genomic DNA preps from *A. thaliana* Cvi and *L. sativum* FR1 were labelled with both Cy3- and Cy5-dyes and hybridizing as described above for the aRNA to the CATMA v3 arrays. Thus two biological and two technical replicates were compared. The microarrays were then 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, www.biodiscovery.com/).

Determination of Lepidium genes "present" on the microarray

Spot intensity data from the genomic DNA microarrays determined using Imagene were analysed using the limma package in Bioconductor (Smyth et al. 2005). Background correction

was performed using the 'normexp' method, which is analogous to RMA. Within array normalisation (Smyth et al. 2003) was performed using print tip loess and between array normalisation using quantile normalisation on the 'A' values. For the two species separately, the normalised values for each probe were then compared to those for the 912 empty spots 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 “present”. The 21527 probes out of the 30343 spotted on the microarray (70.9%) were identified as having significant hybridization for Lepidium and therefore classified as being “present”. The equivalent number for Arabidopsis ecotype Cvi was 28146 (93.0%).

Lim, P.O., Kim, Y., Breeze, E., Koo, J.C., Woo, H.R., Ryu, J.S., Park, D.H., Beynon, J., Tabrett, A., Buchanan-Wollaston, V., and Nam, H.G. (2007). Overexpression of a chromatin architecture-controlling AT-hook protein extends leaf longevity and increases the post-harvest storage life of plants. The Plant Journal **52,** 1140-1153.

Smyth, G.K. (2005). Limma: linear models for microarray data. In: Bioinformatics and Computational Biology Solutions using R and Bioconductor, R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, and W. Huber, eds (New York: Springer), pp. 397-420.

Smyth, G.K., and Speed, T.P. (2003). Normalization of cDNA microarray data. Methods **31,** 265-273.

Supplemental Table 1. Primer list for transcript expression analysis by qRT-PCR.

Gene name	Description	Primer specificity [a]	Accession Number [b]	Forward Primer: name, sequence (5'→3')	Reverse Primer: name, sequence (5'→3')
LesaACO1	ACC oxidase 1	Lepidium	GQ221031 (At2g19590)	qPAt2g19590-F CGCTTCTGTACTCACATCATA	qPAt2g19590-R CTGAATCAGCAAGATTCTGAC
LesaACO2	ACC oxidase 2	Lepidium	GQ221032 (At1g62380)	Ls-aco2-qP-F3 AGACTTTGGGAAGAGATTGGAG	Ls-aco2-qP-R3 AGGTTGGACCTTTAGTTCCAC
LesaACO4	ACC oxidase 4	Lepidium	GQ221033 (At1g05010)	Ls-aco3-qP-F2 GGTTAAGCATTCAATTGTGGT	Ls-aco3-qP-R1 CATCTGTCTGAGATATCACTCT
LesaCYP707A2	ABA 8'-hydroxylase CYP707A2	Lepidium	GQ221028 (At2g29090)	Lesa-cypA2-F2 AAGAGCTTTCATGCCGGATTC	Lesa-cypA2-R2 GAGATTAGTTCCATCCCATGAA
LesaCYP707A3	ABA 8'-hydroxylase CYP707A3	Lepidium	GQ221029 (At5g45340)	Lesa-cypA3-F ATCAACACCCTCGAACACATG	Lesa-cypA3-R TCAATTTCAGTGGCCTCCTCTT
LesaCTR1	Constitutive Triple Response 1	Lepidium	GQ221030 (At5g03730)	Ls-ctr1-qP-F1 GATCACAGGTTGAATAACCAG	Ls-ctr1-qP-R1 CACTCGATTGTCTCTGCAAC
CTR1-like	Putative CTR1-like serine/ threonine protein kinase	Arabidopsis	At4g24480	At4g24480-F TGGTTGGAGCAGTTGCATTC	At4g24480-R CAGCAAGCTTCCATTAGAGAT
PL1	Pectate lyase 1	Arabidopsis	At1g04680	At1g04680-F CTTCAACCGCAAGTTAACACA	At1g04680-R CAAACAACCAACAACACTTCG
ARL	Agros-like, putative cell expansion gene	Arabidopsis	At2g44080	At2g44080-F TCGGACATTGTCGTCGCAG	At2g44080-R CCAACAAGCACAACCATTGAT
AAO3	Abscisic aldehyde oxidase 3	Arabidopsis	At2g27150	aao3-f1 GTTGGAGCTGCCTTACAAGC	aao3-r1 TGAATGCTCCATGAAGACAG
NCED9	Nine-*cis*-epoxycarotenoid dioxygenase 9	Arabidopsis	At1g78390	nced9-f1 TCGGTTAGCTACGCTTGTCG	nced9-r1 GTCCGTGAAGCTCTCCAATT
SDR1	Short-chain dehydro-genase reductase 1	Arabidopsis	At1g52340	sdr1-f1 TGAGTGAGTTCGAGATGACC	sdr1-r1 ACCTCCCACACTACATAAGG
EF1a	Translation elongation factor 1-alpha	Arabidopsis	At5g60390	ef1-F TGAGCACGCTCTTCTTGCT	ef1-R GTGGCATCCATCTTGTTACA
ACT7	Actin 7	Arabidopsis	At5g09810	act7-F GGTCGTACAACCGGTATTGT	act7-R GAAGAGCATACCCCTCGTA

[a] Primer were designed within the GST regions of the CATMA array probes for either the Lepidium or the Arabidopsis cDNA sequence.
[b] Accession numbers in brackets are the corresponding Arabidopsis orthologs of the Lepidium genes.