Molecular mechanisms of seed dormancy release by gas plasma-activated water technology

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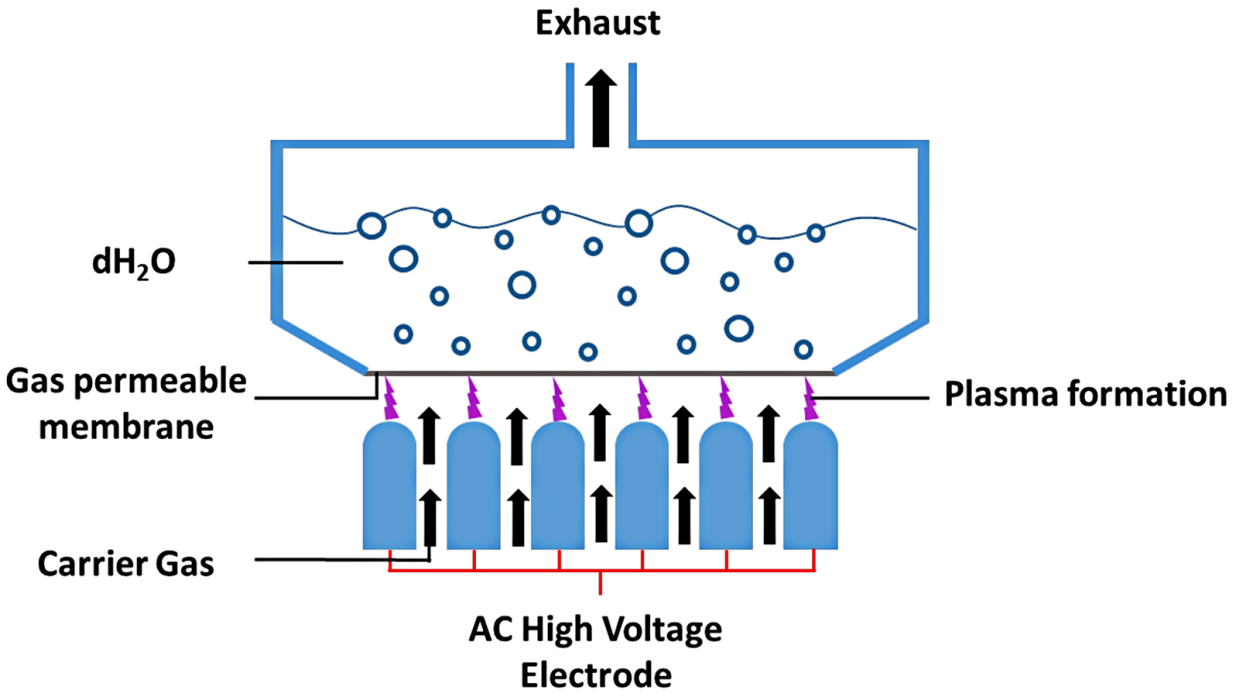
Supplementary data



Supplementary Fig. S1 Diagram of the bubble reactor used to produce gas plasma-activated water (GPAW). The bubble reactor includes 12 high voltage AC electrodes in a dielectric material fixed below a gas permeable stainless-steel membrane. Above the membrane is a tank containing 100 ml of deionised (dH_2O; water purifier system Select Purewater 300, Purite Ltd., Trevose, Pennsylvania, USA). Carrier gas flows past the electrode, and then through the membrane and dH_2O. For activation, plasma is formed between the electrodes and the membrane within the carrier gas and then flows through the membrane bubbling up through the water to produce the GPAW. Major chemical species produced with the bubble reactor were quantified (Figure 2). The non-equilibrium chemistry triggered by atmospheric pressure plasmas in contact with water is complex (Bruggeman *et al.*, 2016; Lu *et al.*, 2016) and here we highlight only some of the key pathways that lead to the formation of the reactive species that have been identified to play a concerted role in the release of physiological dormancy of seeds, namely NO_3^-, $^{\bullet}OH$, H_2O_2 and $^{\bullet}NO$.

$^{\bullet}OH$ (hydroxyl radical):

Although reactive plasma species such as O, $^1O_2$, $^{\bullet}H$ and $HO_2^{\bullet}$ as well as VUV radiation can dissociate water molecules and produce hydroxyl radicals (Bruggeman *et al.*, 2016), hydroxyl radicals in plasma systems are primarily formed at the gas liquid interface by electron impact dissociation [1] of water molecules (Vasko *et al.*, 2014):

$$e + H_2O \rightarrow e + {}^{\bullet}OH + {}^{\bullet}H \qquad [1]$$

$^{\bullet}OH$ radicals are short-lived and therefore they do not contribute to the $^{\bullet}OH$ radicals observed hours after the plasma treatment. Instead, in GPAW, $^{\bullet}OH$ radicals keep being produced well after the plasma treatment has ended via secondary reactions such as Fenton reactions when metal ions are present [2], quenching of hydrogen peroxide by long lived species such as ozone [3] and decomposition of peroxynitrite [4], which forms in the water as a result of reactions of reactive oxygen and nitrogen species species (Bruggeman *et al.*, 2016; Lukes *et al.*, 2014):

$$Fe^{2+} + 2H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + HO_2^{\bullet} + H_2O \quad [2]$$

$$O_3 + H_2O_2 \rightarrow HO_2^{\bullet} + {}^{\bullet}OH + O_2 \quad [3]$$

$$O{=}NOOH \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}OH \quad [4]$$

H_2O_2 (hydrogen peroxide):

The main reaction leading to the formation of H_2O_2 is the recombination of hydroxyl radicals [5] (Vasko *et al.*, 2014; Winter *et al.*, 2014):

$$2\,{}^{\bullet}OH \rightarrow H_2O_2 \quad [5]$$

Unlike hydroxyl radicals, hydrogen peroxide is fairly long-lived and can be detected in GPAW long after the plasma treatment has ended. Reactions contributing to the decay over time of H_2O_2 include the ozone and iron catalysed decomposition reactions (2 and 3) and in acidic conditions, the reaction with nitrite ions to form peroxynitrite [6] (Lukes *et al.*, 2014):

$$NO_2^- + H_2O_2 + H^+ \rightarrow O{=}NOOH + H_2O \quad [6]$$

NO_2^- (nitrite) and NO_3^- (nitrate):

Nitrites and nitrates are formed in plasma-treated water through dissolution of nitrogen oxides, nitrous acid and nitric acid formed in the plasma by gas-phase reactions of dissociated N_2, O_2 and H_2O [7-10] (Bruggeman *et al.*, 2016; Lukes *et al.*, 2014; Sakiyama *et al.*, 2012):

$$HNO_3 \rightarrow NO_3^- + H^+ \quad [7]$$

$$HNO_2 \rightarrow NO_2^- + H^+ \quad [8]$$

$${}^{\bullet}NO_2 + {}^{\bullet}NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad [9]$$

$${}^{\bullet}NO + {}^{\bullet}NO_2 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad [10]$$

The relative concentrations of NO_3^-, NO_2^- and H_2O_2 in GPAW under acidic conditions is regulated by peroxynitrite, which favours the formation of nitrate over nitrite and hydrogen peroxide over time [4,6] (Lukes *et al.*, 2014).

$^{\bullet}NO$ (nitric oxide):

Nitric oxide is produced in the gas plasma as a result of the dissociation of N_2 and O_2 and can partly dissolve in water before it is converted into other NO_x species [11-13] (Sakiyama *et al.*, 2012):

$$N_2^* + O \rightarrow {}^{\bullet}NO + {}^{\bullet}N \quad [11]$$

$$O_2 + {}^{\bullet}N \rightarrow {}^{\bullet}NO + O \quad [12]$$

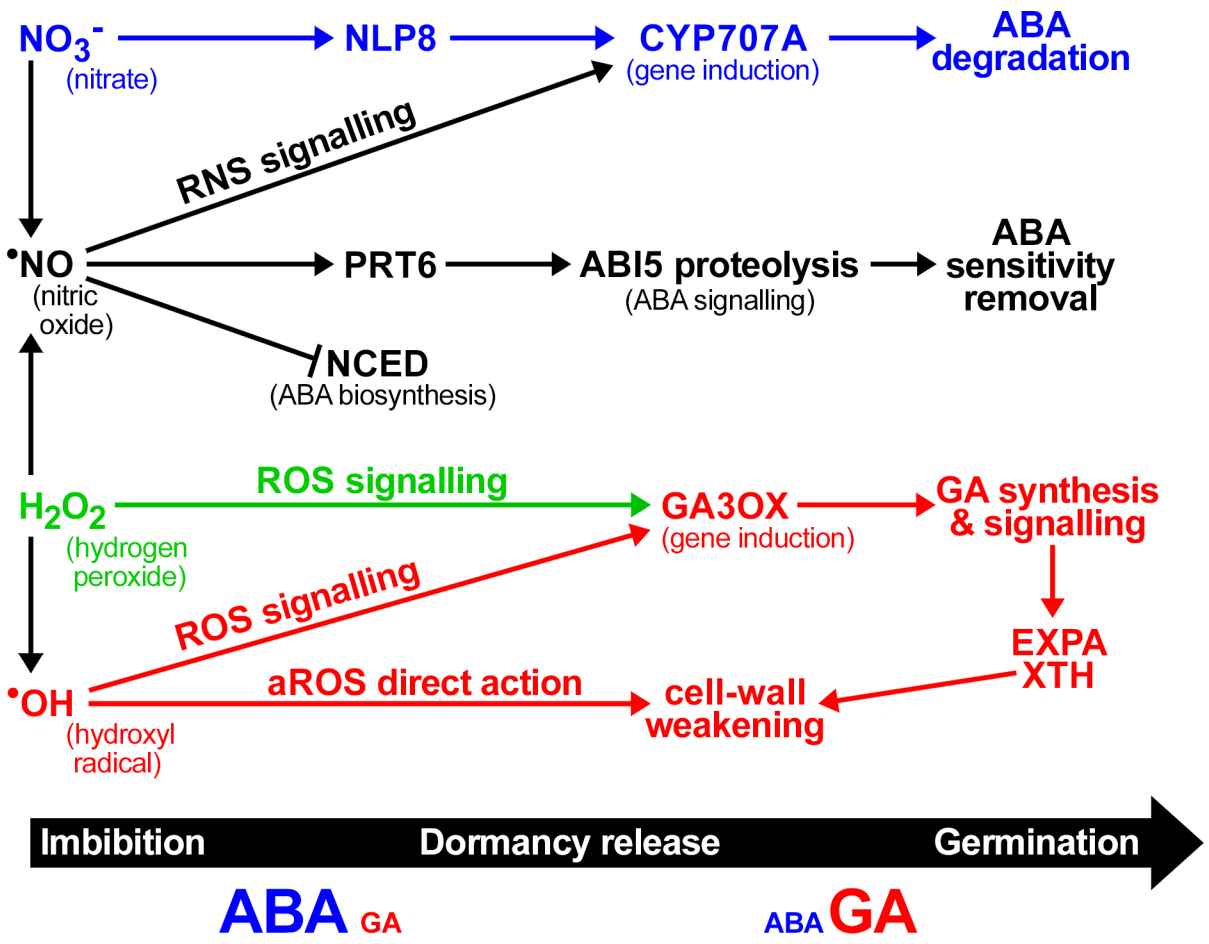
$${}^{\bullet}N + {}^{\bullet}OH \rightarrow {}^{\bullet}NO + {}^{\bullet}H \quad [13]$$

Besides direct solvation, $^{\bullet}NO$ is also produced at the liquid interface and inside water by reduction of plasma generated nitrogen dioxide [14,15] (Jablonowski *et al.*, 2018):

$${}^{\bullet}NO_2 + O \rightarrow {}^{\bullet}NO + O_2 \quad [14]$$

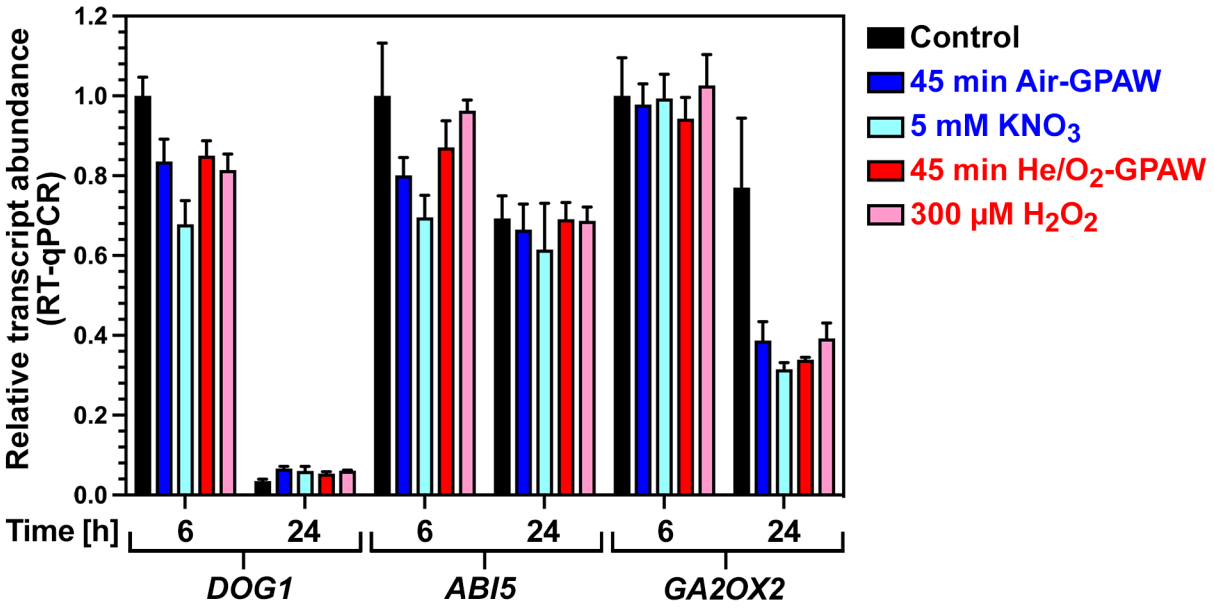
$${}^{\bullet}NO_2 + O_3 \rightarrow {}^{\bullet}NO + 2O_2 \quad [15]$$

Seed dormancy releasing pathways of major chemical species in GPAW

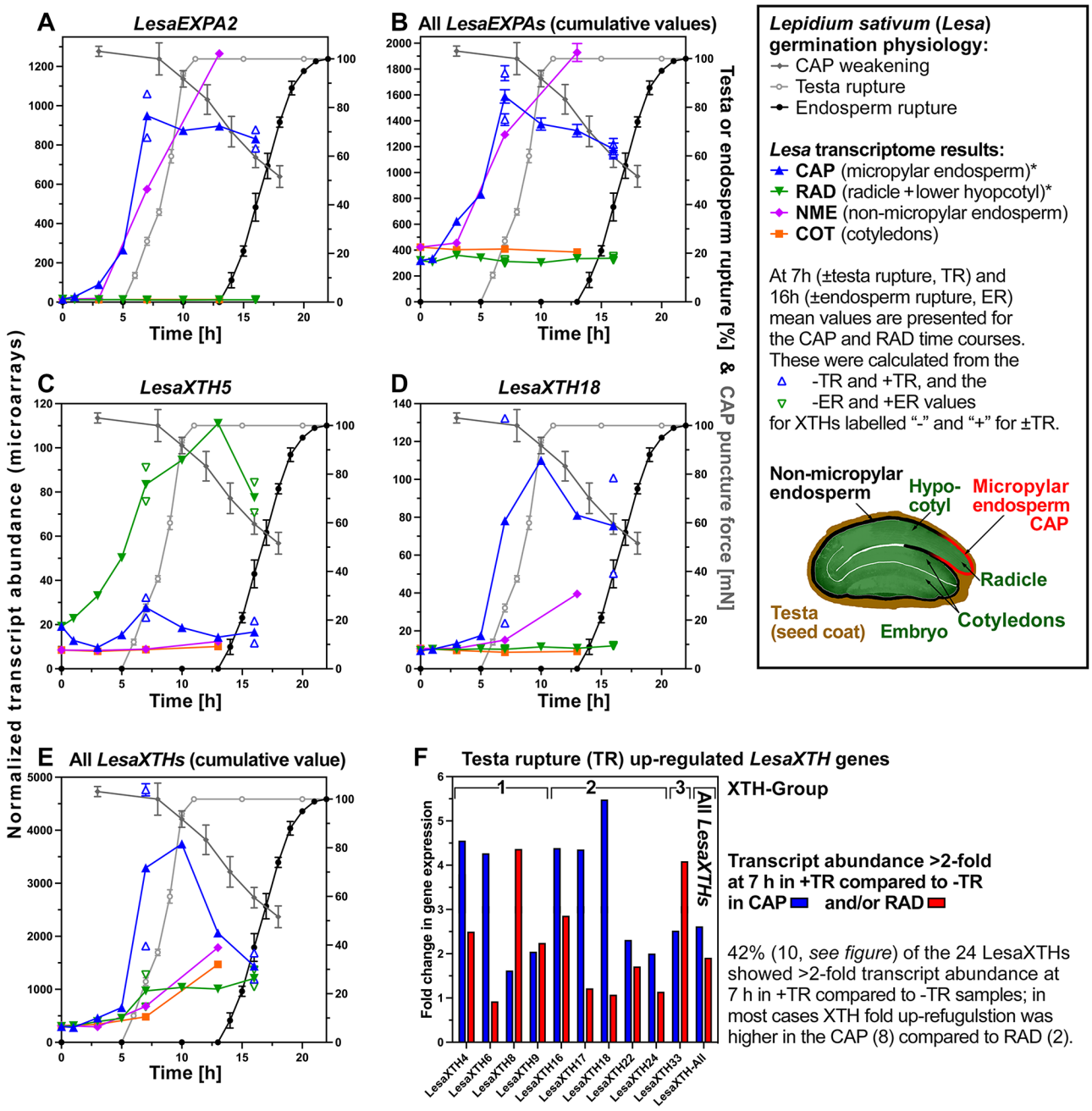


Supplementary Fig. S2 Simplified schematic presentation of ROS and RNS signalling pathways in plants. Major chemical species produced in GPAW include NO_3^-, $^{\bullet}NO$, H_2O_2 and $^{\bullet}OH$ (Figure 2, Supplementary Figure S1) which are also produced *in planta* and are known for their signalling roles (Nonogaki, 2017) and direct chemical actions on cell walls (Müller *et al.*, 2009). In brief, in imbibed seeds the *CYP707A2* gene encoding ABA 8'-hydroxylase to catalyse ABA degradation , is known to be induced by NO_3^- via the NLP8 master regulator (Duermeyer *et al.*, 2018; Nonogaki, 2017; Yan *et al.*, 2016). RNS signalling by $^{\bullet}NO$ which is known to be generated in planta (Kolbert *et al.*, 2019; Liu and Zhang, 2009) also leads to reduced ABA biosynthesis and by signalling via E3 ubiquitin ligase PRT6 (as depicted in the simplified scheme) and other components of the N-end rule pathway (Holdsworth *et al.*, 2020; Holman *et al.*, 2009) or by *S*-nitrosylation (not presented in the simplified scheme) (Albertos *et al.*, 2015) to the removal of ABA sensitivity by ABI5 proteolysis. ROS signalling by $^{\bullet}OH$, H_2O_2 and other ROS leads in seeds to the induction of the *GA3OX* genes to catalyse the biosynthesis of bioactive GA (Bailly, 2019; Liu *et al.*, 2010). High H_2O_2 concentrations (5-10 mM) are required for the very early up-regulation of *GA3OX1* and *CYP707A2* genes in imbibed seeds, low H_2O_2 concentrations (< 1mM) are less effective (Liu *et al.*, 2010). Apoplastic ROS (aROS) produced in the cell wall of seed compartments are involved in embryo expansion growth and micropylar endosperm weakening (Graeber *et al.*, 2014; Müller *et al.*, 2009; Steinbrecher and Leubner-Metzger, 2017; Zhang *et al.*, 2014). Experimentally produced $^{\bullet}OH$ (Fenton reaction) for example caused a ca. 50% decrease in the *L. sativum* CAP puncture force within one hour (Müller *et al.*, 2009). Expansins (EXPA) and xyloglucan endo-transglycosylases/hydrolases (XTH) including through their xyloglucan

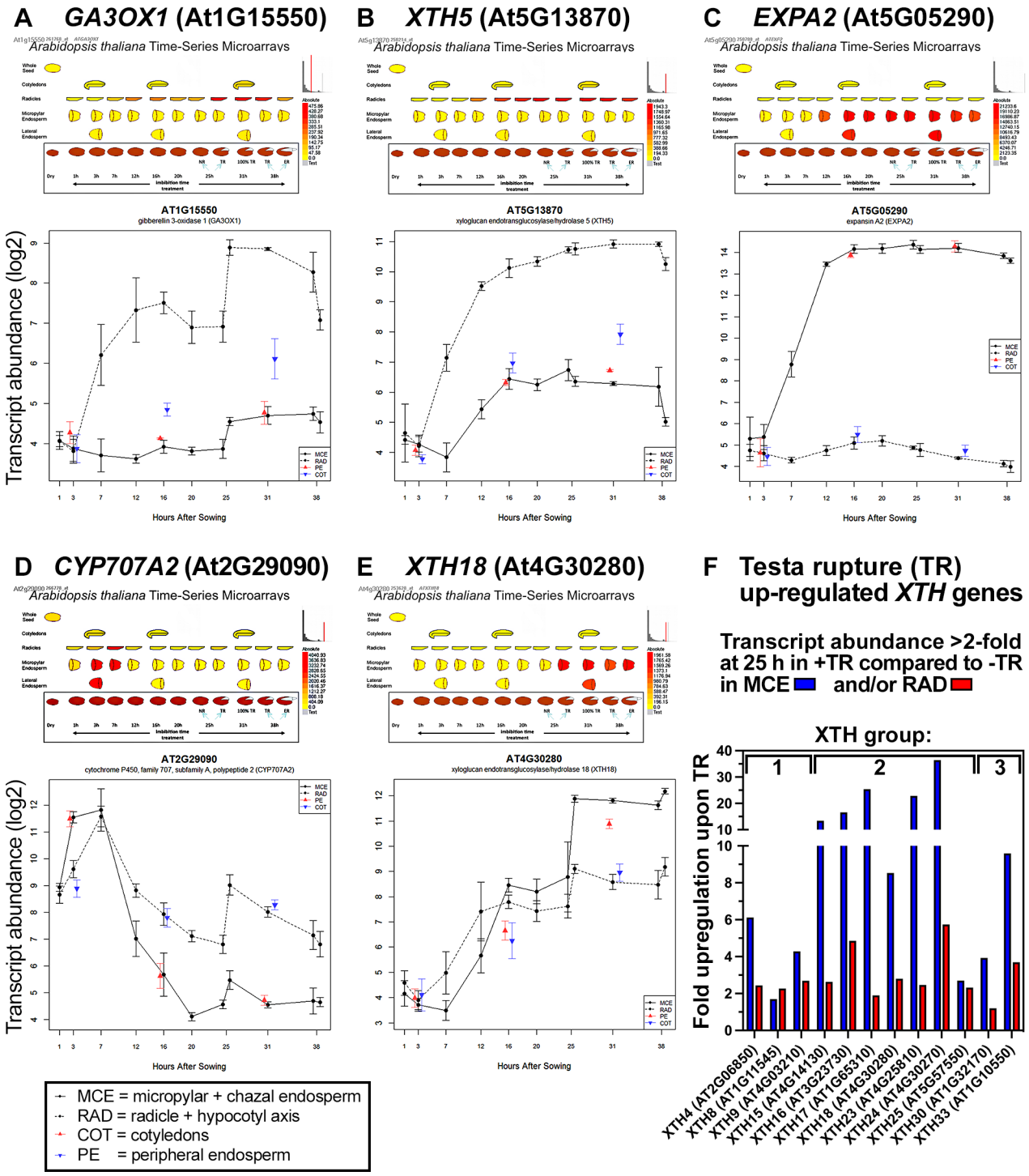
endo-transglycosylase enzyme activity (XET) enzyme activity are involved in promoting testa rupture and enhanced endosperm CAP weakening (Chen *et al.*, 2002; Graeber *et al.*, 2014; Steinbrecher and Leubner-Metzger, 2017; Voegele *et al.*, 2011). Due to the altered balance in GA and ABA metabolism and sensitivity release dormancy and shift the seed state towards the germination programme (Finch-Savage and Leubner-Metzger, 2006).



Supplementary Fig. 3 GPAW-induced gene expression associated with dormancy release and germination. RT-qPCR analyses of *Arabidopsis thaliana* C24 seed transcript abundances at 6 h and 24 h, as indicated, for key genes encoding the dormancy master regulator (*DOG1*), a transcription factor conferring seed ABA sensitivity (*ABI5*), and a GA inactivation enzyme (*GA2OX2*) known to be involved in dormancy and germination. Relative mean ± SEM values compared to the 6-h control (set to 1 for each gene) are presented for the control, Air-GPAW, He/O_2-GPAW, NO_3^- and H_2O_2 treatments. Relative mean ± SEM values compared to the 6-h control are presented.



Supplementary Fig. S4 Spatiotemporal expression of cell wall remodelling genes in germinating *Lepidium sativum* seeds. Transcriptome analysis (microarrays) of *EXPA* and *XTH* gene expression in *L. sativum* FR14 seed compartments (Scheler *et al.*, 2015) as specified in the legend. (A) *LesaEXPA2*, for which the expression is endosperm-specific. (B) Cumulative values for all 18 detected *LesaEXPA* genes (*EXPA1,2,4,6,7,8,9,10,11,12,13,14,15,16,17,18,20,21*). (C) *LesaXTH5*. (D) *LesaXTH18*. (E) Cumulative values for all 24 detected *LesaXTH* genes (*XTH1,4,5,6,8,9,10,13, 15,16,17,18,19,20,22,23,24,25,27,28,30,31,32,33*). (F) Interestingly, and in agreement with a role of in promoting endosperm weakening and testa rupture, most of the *XTH* genes are expressed in the endosperm and about half of the *XTH* genes are differentially expressed in that they are, as upon GPAW treatment, up-regulated upon testa rupture in *L. sativum* and *A. thaliana* (Supplementary Figures S4F and S5).



Supplementary Fig. S5 Spatiotemporal expression of cell wall remodelling genes in germinating *Arabidopsis thaliana* seeds. Transcriptome analysis (microarrays) of *EXPA, XTH*, and hormone-related gene expression in *A. thaliana* seed compartments (Dekkers *et al.*, 2013) as specified in the legend. (A) *AtGA3OX1*. (B) *AtXTH5*. (C) *AtEXPA2*. (D) *AtCYP707A2*. (E) *AtXTH18*. (F) *Arabidopsis thaliana XTH* genes up-regulated upon testa rupture; note that Dekkers et al. (2013) identified 503 genes in the endosperm and 283 genes in the radicle which are upregulated by testa rupture. Transcript abundances (log2) (Dekkers *et al.*, 2013) from the eFP browser (Winter *et al.*, 2007) are presented.

Supplementary Table S1 Primer sequences used for RT-qPCR

Gene name	Gene ID		Primer sequences (5'-3')	Annealing Temp (°C)[b]	Reference
CYP707A2	At2g29090	Fw	CGTCTCTCACATCGAGCTCCTT	60	[1]
		Rev	CCAAAAGTCCATCAACACCCTC		
GA3OX1	At1g15550	Fw	TCCGAAGGTTTCACCATCACT	60	[2]
		Rev	TCGCAGTAGTTGAGGTGATGTTG		
NCED2	At4g18350	Fw	GCGGCTGAGCGTGCATTAA	60	[3]
		Rev	GGGAATAATTCCCGGCAATCT		
NCED9	At1g78390	Fw	GGAAAACGCCATGATCTCACA	60	[3]
		Rev	AGGATCCGCCGTTTTAGGAT		
XTH5	At5g13870	Fw	CACGTCGATGGATGTGAAGCT	64	[4]
		Rev	CTTTCTGATCCCACCAACGTTT		
EXPA1	At1g69530	Fw	AACGCACACGCCACATTCTAC	64	[5]
		Rev	CGTGTTGGTTCCATAGCCTTG		
EXPA2	At5g05290	Fw	CATAAACTCCGACGACAACG	64	[6]
		Rev	TACCCACAAGCACCACCCAT		
EXPA8	At2g40610	Fw	GCTCAAAAACACAGTCGTGGC	64	[5]
		Rev	CGTTACCTGGAAGGAAAGGCT		
DOG1	At5g45830	Fw	GAGCTGATCTTGCTCACCGATGTAG	60	[7]
		Rev	CCGCCACCACCTGAAGATTCGTAG		
ABI5	At2g36270	Fw	CAGCTGCAGGTTCACATTCTG	60	[2]
		Rev	CACCCTCGCCTCCATTGTTAT		
GA2OX2	At1g30040	Fw	CCTAAAACCTCCGCCGTTTT	60	[2]
		Rev	CCTTCATGTACTCCTCCACCGA		
Hobbit[a]	At2g20000	Fw	ACAAGACACTACAACGCATGGTAC	60	[7]
		Rev	TCTCTAGTGCTTCCTCACTTCTCTTC		
TIP41-Like[a]	At4g34270	Fw	GTGAAAACTGTTGGAGAGAAGCAA	60	[8]
		Rev	TCAACTGGATACCCTTTCGCA		[9]

[a] Reference gene; [b] Annealing temperature used in qPCR assays; [c] References: [1] (Kushiro *et al.*, 2004), [2] (Ogawa *et al.*, 2003), [3] (Seo *et al.*, 2004), [4] (Liu *et al.*, 2010), [5] (Sanchez-Montesino *et al.*, 2019), [6] (Yan *et al.*, 2014), [7] (Nakabayashi *et al.*, 2012), [8] (Czechowski *et al.*, 2005), [9] This study.

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