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

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Highlight: Gas plasma-activated water agri-technologies mimick the multitude of environmental signals to enhance seed germination and growth required for the sustainable intensification of food production.

Abstract

Developing innovative agri-technologies is essential for the sustainable intensification of global food production. Seed dormancy is an adaptive trait which defines the environmental conditions in which the seed is able to germinate. Dormancy release requires sensing and integration of multiple environmental signals, a complex process which may be mimicked by seed treatment technologies. Here, we reveal molecular mechanisms by which non-thermal (cold) atmospheric gas plasma-activated water (GPAW) releases the physiological seed dormancy of *Arabidopsis thaliana*. GPAW triggered dormancy release by synergistic interaction between plasma generated reactive chemical species (NO_3^-, H_2O_2, $^{\bullet}NO$, $^{\bullet}OH$) and multiple signalling pathways targeting gibberellin and abscisic acid (ABA) metabolism and the expression of downstream cell wall remodelling genes. Direct chemical action of GPAW on cell walls resulted in premature biomechanical endosperm weakening. The germination responses of dormancy signalling (*nlp8*, *prt6, dog1*) and ABA metabolism (*cyp707a2*) mutants varied with GPAW composition. GPAW removes seed dormancy blocks by triggering multiple molecular signalling pathways combined with direct chemical tissue weakening to permit seed germination. Gas plasma technologies therefore improve seed quality by mimicking permissive environments in which sensing and integration of multiple signals leads to dormancy release and germination.

Keywords: abscisic acid metabolism, *Arabidopsis thaliana*, endosperm weakening, gas plasma-activated water, nitrogen signalling, non-thermal atmospheric gas plasma technology, plant hormone signalling, reactive oxygen species, seed dormancy,

Introduction

"Plasma agriculture" is a rapidly emerging field in which pre-planting and post-harvest applications using non-thermal atmospheric gas plasma are developed into environment-friendly agri-technologies for the sustainable production of food (Bourke *et al.*, 2018; Ito *et al.*, 2018; Ranieri *et al.*, 2021). Seeds are the beginning (sowing) and the end (harvesting) of many food chains important to human existence. High-quality crop seeds are the delivery systems of technological advances in plant breeding and seed treatment to agriculture and food chains (Finch-Savage and Leubner-Metzger, 2006; Nonogaki, 2017; Yan *et al.*, 2014). Gas plasma is often defined as "the fourth state of matter" due to its high energetic state. In non-thermal (cold, non-equilibrium) atmospheric plasma reactive species including free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced at ambient temperature and atmospheric pressure (Graves, 2012; Lu *et al.*, 2016; Lukes *et al.*, 2014; Park *et al.*, 2013; Ranieri *et al.*, 2021; Takamatsu *et al.*, 2014; Zhou *et al.*, 2020). The formation of specific reactive species is dependent on the gas admixture, flow of the gas, plasma frequency and temperature. The non-thermal plasma's gaseous phase has demonstrated its potential as an effective method for decontaminating surfaces of heat-sensitive food products and plant seeds by inactivating microbial pathogens (Bourke *et al.*, 2018; Ito *et al.*, 2018; Stepanova *et al.*, 2018). During these gas-phase treatments of dry seeds effects on germination performance were also noted and attributed to direct physical changes (etching) of the seed coat surface resulting in improved wetting, permeability and water uptake (Bafoil *et al.*, 2019; Bormashenko *et al.*, 2015; Ling *et al.*, 2015; Zhou *et al.*, 2016). Progress in applying these as innovative sustainable seed technologies requires knowledge about the underpinning molecular mechanisms, as well as a clear distinction between the target seed quality traits dormancy, germination and subsequent seedling growth.

A technological application distinct from using it in the 'gaseous' form is to utilise plasma chemistry for the treatment of biological materials through 'activating' water. Gas plasma-activated water (GPAW) is produced by a gas plasma discharge at the gas-liquid interface (Bruggeman *et al.*, 2016; Liu *et al.*, 2019; Lukes *et al.*, 2014; Takamatsu *et al.*, 2014; Zhou *et al.*, 2020). In the case of air as carrier gas, this initiates the formation of transient ROS and RNS species such as hydroxyl ($^{\bullet}OH$) and nitric oxide ($^{\bullet}NO$) radicals, which react to form more stable compounds such as hydrogen peroxide (H_2O_2), nitrite (NO_2^-) and nitrate (NO_3^-). Treatment of non-dormant seeds with GPAW enhanced their germination speed as well as the growth of the emerged seedlings (Bafoil *et al.*, 2018; Liu *et al.*, 2019; Park *et al.*, 2013; Zhou *et al.*, 2016). Works with *Arabidopsis thaliana* demonstrated that GPAW increased the permeability of the seed coat (testa) and slightly promoted testa rupture of non-dormant wild type (Col-0) seeds (Bafoil *et al.*, 2018; Bafoil *et al.*, 2019). However, neither were the chemical species in the GPAW which cause these effects identified, nor were the underpinning molecular mechanisms studied in these works with non-dormant *A. thaliana* seeds. Seed dormancy is an innate seed property that defines the environmental conditions in which a seed is able to germinate (Finch-Savage and Leubner-Metzger, 2006). Several well distinguished seed dormancy classes are known and include the physiological dormancy of *A. thaliana*. The molecular mechanisms of physiological dormancy induction during seed maturation on the mother plant and its release after shedding are well known (Bailly, 2019; Duermeyer *et al.*, 2018; Graeber *et al.*, 2012; Nambara *et al.*, 2010; Nonogaki,

2017; Shu *et al.*, 2016; Steinbrecher and Leubner-Metzger, 2017; Yan *et al.*, 2014), but it is not known if GPAW can release physiological dormancy of mature seeds of any species. Due to their larger size and similar seed structure, the seeds of *Lepidium sativum*, a Brassicaceae relative of *Arabidopsis*, provide a model system for the direct biomechanical measurement of endosperm weakening (Graeber *et al.*, 2014; Lee *et al.*, 2012; Müller *et al.*, 2009; Steinbrecher and Leubner-Metzger, 2017).

In this study we compared the effects of GPAW (produced in a bubble reactor; Supplementary Fig. S1) treatment on physiologically dormant and after-ripened (non-dormant) *A. thaliana* seeds. We demonstrate that depending on the type of GPAW used defined RNS and ROS in GPAW caused the dormancy release and stimulated germination via altering the metabolism and signalling of the antagonistically acting hormones abscisic acid (ABA) and gibberellins (GA). We found that GPAW altered the expression of the associated genes and mutant analysis revealed the signalling pathways involved. Further to this, biochemical and biomechanical analysis demonstrated that GPAW promoted micropylar endosperm (CAP) weakening by direct and indirect mechanisms. Therefore, GPAW technology targets mechanisms conserved among plants, opening an enticing prospect for the agricultural industry and the sustainable intensification of food production.

Materials and methods

Seed material and germination assays

Plant materials used in this study were *Arabidopsis thaliana* (L.) Heynh. C24, Col-0, mutants *cyp707a2-1* (Kushiro *et al.*, 2004), *dog1-2* (Nakabayashi *et al.*, 2012), *nlp8-2* (Yan *et al.*, 2016), and *prt6-1* (Holman *et al.*, 2009). The *nlp8-2* mutant was kindly provided by Eiji Nambara (University of Toronto, Canada). The *prt6-1* mutant (SAIL 1278_H11) was obtained from the Nottingham Arabidopsis Stock Centre, UK. All the mutants were in Col-0 background. All plants were grown at 20°C (16/8 hour day/night cycle) until flowering, and were then transferred to 16 ºC (16/8 hour day/night cycle) to establish higher primary dormancy as previously described (Nakabayashi et al., 2012) except C24 which remained at 20 °C. Post-harvest, seeds were dried for two days on silica gel (15% relative humidity) before being stored at -20 ºC to maintain primary dormancy. Seeds were defrosted at room temperature 1 h prior to assays. For after-ripening, seeds were stored at 21 ºC and 33% relative humidity for up to 6 weeks. For germination assays, approximately 60 seeds in triplicate were imbibed in 600 µl of deionised (dH_2O; water purifier system Select Purewater 300, Purite Ltd., Trevose, Pennsylvania, USA) and autoclaved water (control) or the specified GPAW (45 min discharged) within a 6-cm Petri dish with a single filter paper (MN 713, Macherey-Nagel, Düren, Germany), incubated in the growth chamber set at 20 °C in constant white light (~100 µmol m^{-2} s^{-1}) (MLR-352 Environmental Test Chamber, Panasonic, Bracknell, UK). Radicle emergence was scored and germination percentage was graphed with mean ± SEM and statistically compared through ANOVA and Tukey's analysis using Prism 7.01 software (GraphPad Software, Inc., USA). *Lepidium sativum* L. FR14 seeds ('Keimsprossen', Juliwa) (Scheler *et al.*, 2015) were propagated, and harvested seeds were dried under 15% relative humidity before stored at -20 ºC until used in the experiment.

Dielectric barrier discharge gas plasma-activated water (GPAW) production

The plasma reactor engineered to produce GPAW (Supplementary Fig. S1) consists of 12 high voltage AC electrodes covered in a dielectric material fixed below a gas permeable stainless-steel membrane (Wright *et al.*, 2018). Above the membrane is a tank containing 100ml of dH_2O. A carrier gas (Air or He-O_2 admixture) flows past the electrodes at 1 SLPM (standard litre per minute), and then through the membrane and H_2O. For activation, plasma is formed between the electrodes and the membrane within the carrier gas. The non-thermal atmospheric gas plasma after-glow then flows through the membrane bubbling up through the water, facilitating radical and ion diffusion into the water. To produce Air-GPAW air was used as carrier gas with the plasma sustained at 15.8 kV and 27.1 kHz. To produce He/O_2-GPAW a helium (98%) and oxygen (2%) mixture was used as carrier gas with the plasma sustained at 8.5kV and 29.3 kHz. In both cases, the plasma was modulated with an on-time of 100 ms and a duty cycle of 30%. Compressed air (UN1002, BOC Ltd, Guildford, UK), helium (UN1046, N4.6, BOC Ltd, Guildford, UK) and oxygen (UN1072, N5.0, BOC Ltd, Guildford, UK) gases were used for all treatments and both their mixture and flow rate were controlled through Alicat MC-series mass flow controllers (Alicat Scientific, Tucson, Arizona, USA). Voltage and frequency measurements were recorded using a Tektronix P6015A high voltage probe (Tektronix, Oregon, USA) and a TBS1102B digital oscilloscope (Tektronix, Oregon, USA).

GPAW chemical analysis

H_2O_2 was quantified colourimetrically using the titanium sulphate method (Eisenberg, 1943). The peroxotitanium (IV) complex formed by the reaction of H_2O_2 with titanyl ions under acidic conditions was quantified by measuring the absorbance at 407 nm. A standard curve constructed using H_2O_2 solution (30%, Sigma-Aldrich, Missouri, USA) was used to calculate a molar extinction coefficient. NO_2^- and NO_3^- were quantified simultaneously using Griess and vanadium (III) chloride (VCl_3) reagents in an assay described in detail by Garcia-Robledo et al. (2014). $^{\bullet}OH$ radicals were quantified through the hydroxylation of the chemical probe terephthalic acid; the resultant 2-hydroxy terephthalic acid (HTA) is fluorescent (excitation 315 nm, and emission at 425 nm). $^{\bullet}OH$ radical synthetic rate was quantified both during plasma discharge and post-discharge. Standards of HTA (Sigma-Aldrich) were used for quantification and conversion to molar units (Sahni and Locke, 2006). For measurement during discharge, 100 ml of 2 mM HTA, dissolved in 10 mM phosphate buffer (pH 6.8) was placed in the reactor chamber, and fluorescence was recorded from 300 µl samples at timed intervals. For post discharge measurements, GPAW samples were removed from the reactor and combined with double concentration HTA reagent (4 mM HTA, 20 mM phosphate buffer, pH 6.8) in a 1:1 ratio, total volume 2 ml, sealed in a 6-cm petri dish, incubated at 21ºC in constant light; and 300 µl samples were removed at timed intervals. For measurement samples were placed in UV transparent 96 well plates (UV transparent, Costar® 3635, Corning Inc., New York, USA) and fluorence was measured (excitation 315 nm, and emission at 425 nm) using a Spark™ Multimode Plate Reader (Tecan Group Ltd., Männedorf, Switzerland).

RNA extraction, cDNA synthesis and RT-qPCR analysis

For each sample, 10 mg of whole seeds were collected at specified times, frozen in liquid nitrogen and stored at -80ºC. RNA extraction was performed using the CTAB method (Chang *et al.*, 1993) with the following modifications. Chloroform extraction was repeated 3 times before LiCl precipitation, additionally repeated 3 times following dissolving the RNA in SSTE, and extracted RNA was treated with DNase (Qiagen, Manchester, UK) according to manufacturer's instruction. RNA quantity and purity were measured using a Spark™ Multimode Plate Reader (Tecan Group Ltd., Männedorf, Switzerland), and only samples with absorbance ratios of at least 2.0 (260/280 nm) and 2 (260/230 nm) were used for cDNA synthesis. RT-qPCR analysis was conducted as described (Graeber *et al.*, 2011). In brief, cDNA was synthesised with random pentadecamers from 1 µg total RNA using superscript III reverse transcriptase (Invitrogen, Paisley, UK) in a 20 µl volume and diluted 20-fold. qPCR was performed with ABsolute qPCR SYBR mix (Thermo Fisher Scientific, Oxford, UK) on a Biorad CFX96 system (Bio-Rad Laboratories, Watford, UK) with 140 nM gene specific primer sets (Supplementary Table S1). The PCR program was as follows: 15 min at 95°C, followed by 50 cycles of 15 sec at 95°C, 30 sec at 60 or 64°C, and 30 sec at 72°C, and then melt curve analysis was performed. Two technical replicates were performed on five biological replicates for each sample. The expression values were normalised against the geometric mean of two reference genes, *HBT* (At2g20000) and *TIP41-Like* (At4g34270), and relative expression values were shown as fold-change against indicated samples using the $2^{-\Delta\Delta Ct}$ method (Graeber *et al.*, 2011; Livak and Schmittgen, 2001).

Biomechanical analysis

Puncture force measurements of *Lepidium sativum* FR14 micropylar endosperm (CAP) tissue was conducted using a custom-made machine as described earlier (Graeber *et al.*, 2014; Lee *et al.*, 2012; Steinbrecher and Leubner-Metzger, 2017). Seeds were imbibed for 1 h in 6 cm Petri dishes with two filter papers (4007130050 Macherey-Nagel) and 1.5 ml of H_2O in constant light at 20 ºC. The 1-hour imbibed seeds were then dissected in moist conditions to obtain the micropylar endosperm (CAP) tissues. The isolated CAPs were then incubated for 2 h in 1.5 ml treatment solution (control (dH_2O), Air-GPAW, or He/O_2-GPAW) in constant light at 20 ºC. The 3-hour CAPs were then fixed into a metal mould before a metal probe (diameter 0.3 mm) was driven (0.7 mm min^{-1}) through the CAPs. The force it took to rupture the CAPs was recorded with the displacement. The CAP puncture force (tissue resistance) was determined as the maximal force from the displacement-force curve, and the CAP tissue elasticity was calculated as the slope of the linear portion of the displacement-force curve (Fig. 5).

XET enzyme activity assays

Lepidium sativum FR14 seeds were imbibed in dH_2O and incubated as described above. CAP tissues were prepared from 1, 3, and 10 h imbibed seeds for monitoring the XET activities in CAP in germination processes. Another set of CAP tissues dissected from 1 h

imbibed seeds (isolated CAP) were further incubated for 2 h in 1.5 ml treatment solution (water for the control, Air-GPAW, or He/O_2-GPAW) in constant light at 20 ºC for monitoring direct effects of GPAW on CAP. Total protein was extracted from 22 CAPs per sample and the XET enzyme activities were assayed using the method by Fry (1997) with the modifications described in Holloway et al. (2021) except for the following changes. All protein samples were adjusted to 2 µg $µl^{-1}$, 10 µg protein samples were used for each reaction, and was incubated in darkness at 20 ºC for 4 h. Matrices were measured dry, twice, before native protein loading (fluorescence t=0) and post-incubation plus washing and drying (fluorescence t=4 h). The percentage of transglycosylated XLLG-SR to the xyloglucan substrate, after blank subtraction (extraction buffer loaded matrix) was used as the relative XET activity value.

Results

GPAW interferes with the seed hormone metabolism to release physiological dormancy and promote germination

Freshly harvested (FH) mature seeds of *A. thaliana* (Fig. 1A) have physiological dormancy which means that they do not germinate when imbibed under favourable conditions (Graeber *et al.*, 2012). The dormancy maintenance in imbibed FH seeds is achieved, at least in part, by enhanced ABA biosynthesis and signalling (Fig. 1B). In agreement with this, FH *A. thaliana* Col-0 and C24 seed populations were dormant in our experiments when imbibed in water (control). The maximum germination percentages (G_{MAX}) remained low at 10-15% even after prolonged incubation (Fig. 1C,D). In contrast to water, imbibition in Air-GPAW caused dormancy release and resulted in high G_{MAX} values of 80-90% (Fig. 1C,D). He/O_2-GPAW also caused dormancy release of a fraction of the seed population resulting in a G_{MAX} of ca. 40% (Fig. 1D). The release of physiological seed dormancy in the imbibed state by cold-stratification is known to be achieved by increased GA biosynthesis (Ogawa *et al.*, 2003) and ABA degradation via ABA 8'-hydroxlase encoded by the *CYP707A2* gene (Kushiro *et al.*, 2004). In agreement with a key role of CYP707A2 in the GPAW-mediated dormancy release, Air-GPAW treatment of non-germinating (FH) *cyp707a2* mutant seeds did not result in full dormancy release as observed in the wild type (Fig. 1C). Release of physiological dormancy can also be achieved in the dry state during after-ripening (AR) storage (Fig. 1B). Non-dormant (AR) *A. thaliana* seed populations fully germinated when imbibed in water (control) with no or only small G_{MAX} increases upon GPAW treatment (Fig. 1C). In contrast to the small effect on AR seed, the finding that GPAW treatment released the physiological dormancy of FH seeds resulting in a large increase in G_{MAX} (Fig. 1) was intriguing and triggered follow-up experiments to identify the dormancy-releasing compounds in GPAW and to investigate the underpinning molecular mechanisms.

The GPAW produced for this study was standardised using a bubble reactor (Supplementary Fig. S1), and the active species generated under two preparation regimes, Air-GPAW and He/O_2-GPAW, were quantified (Fig. 2). As the water was exposed to plasma during GPAW production, primary reactions led to an increase of the concentration of nitrate (NO_3^-) in Air-GPAW (Fig. 2A), and as expected, negligible nitrate production in He/O_2-GPAW (Fig. 2B).

Hydrogen peroxide (H_2O_2) increased to reach 388 µM in He/O_2-GPAW (Fig. 2B) and was >10-fold lower in Air-GPAW (Fig. 2A). The highly reactive hydroxyl radical ($^{\bullet}OH$) increased to ca. 60 µM steady-state concentration in both, Air-GPAW (Fig. 2A) and He/O_2-GPAW (Fig. 2B), while nitrite (NO_2^-) was produced in Air-GPAW and absent in He/O_2-GPAW. The two types of GPAW, therefore, shared the $^{\bullet}OH$ production, but differed in that NO_3^- accumulated over discharge time only in Air-GPAW and H_2O_2 only in He/O_2-GPAW. Post-discharge, the concentration of active species in the water evolved over time due to secondary reactions. This included initially a sharp drop in the very short-lived $^{\bullet}OH$ radical (half-life 1 nsec), followed by secondary $^{\bullet}OH$ steady-state production. During a GPAW incubation period of up to 100 h, the concentration of H_2O_2 remained at 100-300 µM in both GPAWs (Fig. 2C,D). However, the two GPAWs differed in that NO_3^- remained high at ca. 6 mM and $^{\bullet}OH$ secondary production was very slow (0.01 µM/h) leading to <1 µM $^{\bullet}OH$ concentrations in Air-GPAW (Fig. 2C), while in He/O_2-GPAW NO_3^- remained very low and a >10-fold faster (0.14 µM/h) $^{\bullet}OH$ secondary production led to >10 µM $^{\bullet}OH$ concentrations. The observed seed dormancy release with GPAW (Fig. 1) may therefore be achieved by NO_3^-, H_2O_2, $^{\bullet}OH$ (Fig. 2) and other ROS/RNS species derived from these (including nitric oxide ($^{\bullet}NO$) radicals; Supplementary Fig. S2) produced *in vitro* (Lukes *et al.*, 2014; Takamatsu *et al.*, 2014; Zhou *et al.*, 2020) and/or *in planta* (Albertos *et al.*, 2015; Kolbert *et al.*, 2019; Müller *et al.*, 2009).

GPAW-mediated dormancy release involves signalling of reactive species to induce genes for GA biosynthesis, ABA degradation, and cell-wall remodelling

Fig. 3 shows that GPAW-mediated dormancy release caused changes in the expression patterns of key genes in GA and ABA metabolism, as well as in downstream genes encoding cell-wall remodelling proteins (CWRP) known to be required for germination (Finch-Savage and Leubner-Metzger, 2006; Graeber *et al.*, 2012; Nambara *et al.*, 2010). The full dormancy release of imbibed FH C24 seeds by Air-GPAW (Fig. 3A) was associated with the very early (6 h) up-regulation of *GA 3-oxidase 1* (*GA3OX1*; biosynthesis of bioactive GA) and *CYP707A2* (ABA degradation) and down-regulation of 9-*cis*-epoxycarotenoid dioxygenase *NCED2* and *NCED9* (ABA biosynthesis) transcript abundances (Fig. 3C). The partial dormancy release of imbibed FH C24 seeds by He/O_2-GPAW (Fig. 3B) was also associated with the down-regulation of *NCED2* and *NCED9* at 6 h, but the up-regulation of *GA3OX1* and *CYP707A2* was slower and became evident only at 24 h (Fig. 3D). From studies of dormant and non-dormant *A. thaliana* seeds it is known that these key genes are regulated early during imbibition to control the GA/ABA balance (Bethke *et al.*, 2007; Graeber *et al.*, 2014; Liu *et al.*, 2010; Nakabayashi *et al.*, 2005; Preston *et al.*, 2009), which is decisive in the control of germination by dormancy maintenance or release (Fig. 1B). Nitrate treatment resulted in very similar expression patterns as Air-GPAW (Fig. 3C) suggesting that nitrate signalling may be involved in the dormancy release by the ca. 5 mM NO_3^- in the Air-GPAW (Fig. 2A). The dormancy release caused by nitrate treatment was, however, only partial (G_{MAX} ca. 40%; Fig. 3A), suggesting that in addition $^{\bullet}OH$ (Fig. 2) and other ROS and RNS pathways may be involved (Supplementary Fig. S2) to achieve the full dormancy release.

In agreement with this, He/O_2-GPAW treatment caused partial dormancy release associated with similar but slower changes in the *GA3OX1*, *CYP707A2*, and *NCED2/9* transcript abundances (Fig. 3). The ca. 10-fold lower nitrate concentration in He/O_2-GPAW is too low to alone trigger the dormancy release (Fig. 4), but $^{\bullet}OH$, H_2O_2 (Fig. 2) or other ROS signalling

may be involved. It is known that high H_2O_2 concentrations (5-10 mM) fully release *A. thaliana* dormancy in association with very early up-regulation of *GA3OX1* and *CYP707A2* expression in imbibed seeds (Liu *et al.*, 2010). In contrast to this, low H_2O_2 concentrations (< 1mM) (Liu *et al.*, 2010) or 300 µM (Fig. 3B) are less effective. In addition, the treatments induced the expression of CWRP genes such as expansins and XTHs (*XTH5*, *EXPA1*, *EXPA2*, *EXPA8*) responsible for endosperm weakening (Fig. 3C,D). Taken together, these findings demonstrate that GPAW treatment alters the expression of GA and ABA metabolism genes, and suggest that the expected change in the GA/ABA balance and resulting downstream CWRP gene expression may cause the GPAW-mediated dormancy release. To further investigate which of the known major ROS and RNS signalling pathways (Supplementary Fig. S2) are involved in these GPAW responses, we utilised specific *A. thaliana* mutants in dose response experiments (Fig. 4).

Mutant analysis reveals that GPAW acts to release dormancy via several key signalling pathways involved in the control of germination by physiological dormancy

Increased Air-GPAW discharge time leading to elevated production of major chemical species (Fig. 2) resulted in a dose-dependent increase in physiological dormancy release of FH *A. thaliana* seeds (Fig. 4A). This GPAW dose response resulted in a partial dormancy release (G_{MAX} ca. 60%) for the 15 min discharge time to full dormancy release (G_{MAX} ca. 90%) for 45 min discharge time. A dose-dependent response was also evident for nitrate, which reached partial dormancy release (G_{MAX} ca. 60%) at 3.5 mM KNO_3 in FH Col-0 seeds (Fig. 4A). Using 5 mM KNO_3 also only caused partial dormancy release (G_{MAX} ca. 40%) in FH C24 seeds (Fig. 3A). This dormancy release by nitrate treatment in about half of the population's seeds demonstrates that nitrate is indeed a major chemical involved in the GPAW-mediated seed dormancy release. However, other pathways must be involved in addition as for Air-GPAW full dormancy release of the seed population was achieved for C24 (Fig. 3A) and Col-0 (Fig. 4A). The control of germination by physiological dormancy includes the *DELAY OF GERMINATION 1 (DOG1)* gene, which encodes a dormancy-specific master regulator (Graeber *et al.*, 2012; Nakabayashi *et al.*, 2012; Nee *et al.*, 2017; Nishimura *et al.*, 2018; Nonogaki, 2017). Due to the absence of dormancy, FH *dog1* mutant seeds fully germinated and no germination improvement was achieved by either GPAW or nitrate treatment (Fig. 4B,F). In agreement with GPAW not acting via altering the expression of *DOG1* in imbibed FH seeds, no differences in the *DOG1* transcript abundances were observed in response to GPAW or nitrate (Supplementary Fig. S3). Dormancy can also be released by after-ripening (AR) storage (Fig. 1B) and consequently AR wild type seed populations fully germinated (Fig. 4F). In contrast to FH Col-0 seeds and as for non-dormant *dog1* seeds, treatment of AR Col-0 seeds with either GPAW or nitrate did not appreciably affect the G_{MAX} responses of AR seeds.

Nitrate signalling in seeds to release physiological dormancy and promote germination is achieved via the NIN-LIKE PROTEIN 8 (NLP8) transcription factor which binds to the nitrate-responsive *cis*-element (NTR) in the promoter region of nitrate-responsive genes including *CYP707A2* (Duermeyer *et al.*, 2018; Nambara *et al.*, 2010; Nonogaki, 2017). In agreement with this, FH seeds of the *nlp8* mutant were dormant and did not respond to nitrate treatment with increase in G_{MAX} because they were nitrate-insensitive (Fig. 4C). In contrast to their insensitivity to nitrate, the FH *nlp8* seeds responded to Air-GPAW treatment with dormancy

release resulting in a maximum increase in G_{MAX} of ca. 60% (Fig. 4C). This is direct evidence that the dormancy release by Air-GPAW is not only caused by nitrate signalling, but by additional mechanisms triggered by other chemical species (Supplementary Fig. S2). The dose response of the FH *nlp8* seeds differs from FH wild type (Col-0) seeds in that the maximum response (G_{MAX} ca. 60%) was already achieved with 15 min discharge time (Fig. 4F). In contrast to the FH Col-0 seed dose response where G_{MAX} further increased from ca. 60% (15 min) to ca. 90% (45 min), increased discharge time lowered the dormancy release response of the *nlp8* mutant, suggesting that multiple positive and negative pathways are involved (Fig. 4F). The response of FH *cyp707a2* mutant seeds to the treatments (Fig. 4D) has verified that the induction of the *CYP707A2* gene expression (Fig. 3) and thereby ABA degradation is a major requirement for dormancy release. While nitrate was completely ineffective in releasing their dormancy, with Air-GPAW a partial release of FH *cyp707a2* seed dormancy was achieved (G_{MAX} ca. 30%), suggesting that mechanisms independent of ABA degradation are also triggered by GPAW. After-ripening fully released the dormancy of *nlp8* and *cyp707a2* seeds, and the G_{MAX} values of AR seeds were therefore not appreciably affected by GPAW or nitrate (Fig. 4F). The dormancy-releasing activity of the Air-GPAW is therefore not exclusively achieved by nitrate signalling via the NLP8 pathway, but by via multiple pathways involving the concerted action of multiple chemical species in the GPAW.

Other major pathways involved in the control of physiological dormancy include RNS signalling (Supplementary Fig. S2) via nitric oxide ($^{\bullet}NO$), which is known to be generated in planta (Kolbert *et al.*, 2019; Nambara *et al.*, 2010; Nonogaki, 2017) and in GPAW (Lukes *et al.*, 2014; Takamatsu *et al.*, 2014; Zhou *et al.*, 2020). Signalling of $^{\bullet}NO$ in seeds includes crosstalk with ABA to increase ABA degradation, to inhibit ABA biosynthesis, and to remove ABA sensitivity by triggering proteolysis of the transcription factor ABA INSENSITIVE 5 (ABI5) (Albertos *et al.*, 2015; Bethke *et al.*, 2007; Holman *et al.*, 2009). The removal of the ABA sensitivity by $^{\bullet}NO$-signalling leading to proteasome-dependent ABI5 degradation can be achieved either by S-nitrosylation (Albertos *et al.*, 2015) or via the N-end rule pathway (Holman *et al.*, 2009). ABI5 degradation via the N-end rule pathway requires the E3 ubiquitin ligase PROTEOLYSIS 6 (PRT6) component and *prt6* mutant seeds of *A. thaliana* are therefore hypersensitive to ABA (Holman *et al.*, 2009). In agreement with this, we found that FH *prt6* seeds exhibited an altered sensitivity to nitrate and dormancy release was only observed with the highest nitrate concentration used (Fig. 4E). This resulted in a very different nitrate dose response (G_{MAX}) of the *prt6* compared to Col-0 FH seeds (Fig. 4F). In contrast to this, the dose response of FH *prt6* seeds to Air-GPAW treatment was very similar to Col-0 FH seeds (Fig. 4A,E). In addition, it appears that GPAW does not act on altering the expression of *ABI5* in imbibed FH seeds (Supplementary Fig. S3). Air-GPAW triggered dormancy release and germination in FH *nlp8* and *cyp707a2* seeds differed considerably to that of FH Col-0 seeds, whereas FH *prt6* seeds displayed a wild-type like dose response (Fig. 4F). The repression of germination through prevention of testa rupture in *A. thaliana* requires ABI5 for down-regulation of expansin *(EXPA)* gene expression (Barros-Galvao *et al.*, 2019). Removal of ABA sensitivity by ABI5 proteolysis together with the accumulation of bioactive GA due to the GPAW-mediated induction of *GA3OX1* (Fig. 3) and reduced expression of *GA2OX2* (Supplementary Fig. S3) will therefore trigger CWRP gene expression to stimulate endosperm weakening and germination.

Micropylar endosperm weakening caused directly by GPAW-generated ROS as well as indirectly by GPAW-induced expression of cell-wall remodelling genes

Endosperm weakening by cell wall loosening of the micropylar endosperm (CAP) tissue precedes the completion of germination of non-dormant seeds by radicle emergence (Steinbrecher and Leubner-Metzger, 2017). The endosperm (aleurone layer) contributes to the coat-imposed dormancy of *A. thaliana* seeds (Fig. 1A) and the dormancy release for example by $^{\bullet}$NO and GA treatment leads to *A. thaliana* CAP weakening (Bethke *et al.*, 2007). Testa rupture is known to be preceded by the induction of cell-wall remodelling (CWRP) genes in the endosperm of *L. sativum* (Scheler *et al.*, 2015) and *A. thaliana* (Dekkers *et al.*, 2013) to promote endosperm weakening and testa rupture (Steinbrecher and Leubner-Metzger, 2017). Due to their larger size, the seeds of the *Arabidopsis* close relative *Lepidium sativum* are highly suited for the direct biomechanical measurement of CAP weakening by the puncture force method (Graeber *et al.*, 2014; Müller *et al.*, 2009; Steinbrecher and Leubner-Metzger, 2017). Fig. 5a shows that the *L. sativum* CAP puncture force was >100 mN at 3-8 hours GPAW imbibed seeds and subsequently decreased to ca. 92 mN (10 h) and 70 mN (14 h) in association with the progression with testa rupture. This was associated with a rapid rise of the expansin (*EXPA*) and xyloglucan endo-transglycolases/hydrolases (*XTH*) transcript abundances in the CAP. Transcript accumulation of all *EXPA* and *XTH* genes in the CAP was 5-fold (at 7 h) and 8-fold (at 10 h), respectively (Fig. 5A). Transcript accumulation of *EXPA2* in the *L. sativum* CAP was >60-fold (Supplementary Fig. S4) and *EXPA2* is also induced exclusively in the endosperm in germinating *A. thaliana* seeds (Supplementary Fig. S5). Figure S4E and S4F show that most LesaXTH genes are mainly CAP-expressed, and that the cumulative transcript abundance of all LesaXTH genes is several-fold higher in the CAP as compared to the other seed compartments. We found that the accumulation of *XTH* transcripts in the *L. sativum* CAP (Fig. 5A) was accompanied by a 4-fold increase in xyloglucan endo-transglycosylase enzyme activity (XET, EC 2.4.1.207) in the CAP between 3 h and 10 h (Fig. 5B). The increased CAP XET activity at 10 h is consistent with a role of XTHs in the subsequent CAP weakening and testa rupture (Fig. 3a). In contrast to the enhanced CAP XET activity between 3h and 10 h, there was no difference in the CAP XET activity between the 1 h and 3 h timepoints (Fig. 5B). Consistent with this, the onset of CAP weakening was evident between 8 h and 10 h, and no decrease in CAP puncture force was evident until 8 h (Fig. 5A).

In addition to ROS signalling and CWRPs (Supplementary Fig. S2), apoplastic ROS (aROS) produced in the plant cell wall can also act directly by chemical scission of backbone polysaccharides resulting in cell wall loosening to enhance embryo elongation growth (Bailly, 2019; Müller *et al.*, 2009) and endosperm weakening (Müller *et al.*, 2009; Steinbrecher and Leubner-Metzger, 2017; Zhang *et al.*, 2014). Experimentally produced $^{\bullet}$OH for example caused a ca. 50% decrease in the *L. sativum* CAP puncture force within one hour (Müller *et al.*, 2009). To test if the $^{\bullet}$OH and other ROS/RNS produced in Air-GPAW and He/O_2-GPAW (Fig. 2) cause CAP weakening, we incubated isolated *L. sativum* CAPs in GPAW and biomechanically analysed their responses (Fig. 5). Fig. 5c shows that the puncture force of CAPs incubated for 3 h in water (control) remained high (113.1±4.9 mN) and therefore no CAP weakening had occurred at the 3 h time point. In contrast to this, incubation of isolated CAPs in GPAW resulted in a significant decrease in the CAP puncture force at the 3 h time point with 70.5±3.6 mN for Air-GPAW and 80.9±2.3 mN for He/O_2-GPAW (Fig. 5c). The XET enzyme activities of these GPAW-treated CAPs did however not differ from the control at 3 h

(Fig. 5B). We therefore conclude that GPAW can cause CAP weakening very early in imbibed seeds by direct chemical action of the ROS produced in the GPAW. The observed premature CAP weakening caused by direct chemical action of the GPAW was not only associated with a decreased CAP puncture force, but also with an increased CAP elasticity compared to the control (Fig. 5C). Biological materials are structurally complex composites and a decrease in puncture force has been observed in many endospermic seeds (Steinbrecher and Leubner-Metzger, 2017), but a change in the slope of the strain-stress curves (Fig. 5D), i.e. the CAP elasticity, has not been previously reported for seeds. Whether the increase in the CAP elasticity caused by reactive species in the GPAW (direct chemical scission of cell wall polysaccharides) is specific for the GPAW or also occurs in seeds upon abiotic stress is not known. Taken together, the GPAW-induced dormancy release is caused by multiple biochemical and biomechanical mechanisms which include the induction of endosperm weakening and the control of radicle emergence by ROS/RNS-mediated signalling and direct action.

Discussion

GPAW are an emerging seed treatment agri-technology to release physiological dormancy and stimulate germination by multiple molecular signalling pathways

Most crop species and their wild relatives produce seeds which are either non-dormant or physiologically dormant in their mature state at harvest (Finch-Savage and Leubner-Metzger, 2006; Holloway *et al.*, 2021; Nambara *et al.*, 2010). Examples for the latter include Brassicaceae crops such as oilseed rape, the *Brassica* vegetables and *L. sativum* sprouts, and *A. thaliana* as their wild relative. We demonstrate here using dormant seeds of *A. thaliana* that GPAW treatment releases the physiological dormancy by triggering multiple key signalling pathways which gear the hormonal control towards the germination programme and by direct chemical action resulting in premature endosperm weakening. Depending on the carrier gas (air or He/O_2) and the discharge treatment time (bubble reactor, Supplementary Fig. S1), the GPAW used to imbibe the seeds differed qualitatively and quantitatively in the cocktail of reactive chemical species they contain (secondary reactions in Fig. 2). Major chemical species quantified included NO_3^- (only in Air-GPAW), H_2O_2 and the $^{\bullet}OH$ radical (10-fold higher concentration in He/O_2-GPAW compared to Air-GPAW). These, and in addition the $^{\bullet}NO$ generated from NO_3^- and H_2O_2, are known to be produced by seeds in response to environmental cues to release physiological dormancy via well-established molecular signalling pathways (Supplementary Fig. S2). We demonstrate here by using specific *A. thaliana* mutants that GPAW triggers multiple of these pathways which interact synergistically to cause the observed strong molecular and physiological responses. These include the NLP8-mediated nitrate signalling pathway that induces *CYP707A2* gene expression as a key target (Duermeyer *et al.*, 2018; Kushiro *et al.*, 2004; Matakiadis *et al.*, 2009; Okamoto *et al.*, 2006; Yan *et al.*, 2016). Non-NLP8 RNS ($^{\bullet}NO$) signalling pathways to remove ABA sensitivity by ABI5 degradation are also involved (Albertos *et al.*, 2015; Bethke *et al.*, 2007; Holman *et al.*, 2009; Kolbert *et al.*, 2019; Nonogaki, 2017), as well as ROS signalling pathways that up-regulate GA biosynthesis (*GA3OX1*) and CWRP genes from the EXPA and XTH families (Barros-Galvao *et al.*, 2019; Chen *et al.*, 2002; Graeber *et al.*, 2014;

Herburger *et al.*, 2020; Sanchez-Montesino *et al.*, 2019; Steinbrecher and Leubner-Metzger, 2017, 2022; Voegele *et al.*, 2011).

GPAW-generated ROS cause micropylar endosperm (CAP) weakening by direct chemical action and by inducing EXPA and XTH gene expression

Direct biomechanical quantification of the CAP puncture force conducted in *L. sativum* seeds demonstrated that GPAW causes premature endosperm weakening that is detectable just 3h after imbibition with GPAW (Fig. 5). Endosperm weakening is a prerequisite for the completion of germination by radicle protrusion and is blocked in physiologically dormant seeds as component of the coat-imposed dormancy mechanism (Bethke *et al.*, 2007; Graeber *et al.*, 2014; Steinbrecher and Leubner-Metzger, 2017). In particular, endosperm weakening is inhibited by ABA, and promoted by GA and apoplastic ROS (aROS) (Bailly, 2019; Bethke *et al.*, 2007; Graeber *et al.*, 2014; Müller *et al.*, 2009; Steinbrecher and Leubner-Metzger, 2017; Yan *et al.*, 2014; Zhang *et al.*, 2014). Apoplastic ROS (aROS), including $^{\bullet}OH$ and $O_2^{\bullet-}$ are produced *in vivo* in the CAP cell walls of *L. sativum* and lettuce seeds, and direct $^{\bullet}OH$ attack of cell wall polysaccharides has been demonstrated to cause *in vivo* polysaccharide scission and therefore constitutes a more direct mechanism of CAP weakening (Müller *et al.*, 2009; Steinbrecher and Leubner-Metzger, 2017; Zhang *et al.*, 2014). It is also not known if GPAW treatment of seeds cause a similar weakening of the non-micropylar endosperm. It is however known that micropylar (CAP) and non-micropylar endosperm of *L. sativum* differs in the degree of the weakening in that it is only pronounced in the CAP and comparatively small in other regions of the endosperm (Lee *et al.*, 2012). Similar findings were made in endospermic seeds of other species (Steinbrecher and Leubner-Metzger, 2017). In addition seeds contain seed coats and in many cases fruit coats which may differ locally in their permeability for compound uptake (Hermann *et al.*, 2007; Scheler *et al.*, 2015; Steinbrecher and Leubner-Metzger, 2017) this could also differ for GPAW.

We found that the GPAW-induced premature CAP weakening at 3 h was not associated with the induction of XET enzyme activity (Fig. 5), but by the direct chemical action of $^{\bullet}OH$ radicals in Air-GPAW and He/O_2-GPAW (Fig. 2). Further progression of CAP weakening at ca. 10 h and later, however, involves the GPAW-enhanced up-regulation of XTH genes and accumulation of XET enzyme activity in the CAP (Fig. 5). Consistent with this, the GA-regulated *LeXET4* gene is induced during tomato CAP weakening (Chen *et al.*, 2002) and the *XTH18* and *XTH19* genes are expressed in *A. thaliana* and *L. sativum* in a GA-regulated manner (Graeber *et al.*, 2014; Voegele *et al.*, 2011). XTHs with XET enzyme activity can, however, also reinforce tissues and play roles in the coleorhiza-enforced dormancy in grasses, which is together with the endosperm-imposed dormancy of eudicot seeds an example for the convergent evolution of mechanical restraint by overlaying tissues (Holloway *et al.*, 2021; Yan *et al.*, 2014). Interestingly, and in agreement with a role in promoting endosperm weakening and testa rupture, most of the *XTH* genes are expressed in the endosperm upon GPAW treatment. About half of the *XTH* genes are differentially expressed in that they are up-regulated upon testa rupture in *L. sativum* (Supplementary Fig. S4) and *A. thaliana* (Supplementary Fig. S5). GPAW therefore acts by mimicking environmental cues which trigger the removal of the various layers of dormancy blocks to permit seed germination.

Practical applications

Innovations in seed treatment technologies support primary crop production and increasing yield potential by providing protection against pathogens and physiological enhancement to perform better in abiotic stress conditions. We demonstrated that the hallmark of GPAW action important for developing seed treatment and plant growth applications is the concerted action of the combined major chemical species produced to trigger multiple molecular direct (chemical weakening) and indirect (signalling pathways) mechanisms. GPAW thereby mimicks the multitude of environmental signals which are sensed and integrated by seeds. Among them signalling pathways which are conserved among plants (Supplementary Fig. S2) and allow translation of our findings to seeds of other species for practical applications. The knowledge of the underpinning mechanisms derived from our work is therefore crucial for the emerging "plasma agriculture" (Bourke *et al.*, 2018; Ito *et al.*, 2018; Ranieri *et al.*, 2021) in which non-thermal atmospheric gas plasma are developed into environment-friendly agri-technologies for the sustainable global food production.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Diagram of the bubble reactor used to produce GPAW.

Supplementary Fig. S2. Schematic presentation of ROS and RNS signalling pathways.

Supplementary Fig. S3. GPAW-induced gene expression associated in seeds.

Supplementary Fig. S4. Cell wall remodelling genes in germinating *L. sativum* seeds.

Supplementary Fig. S5. Cell wall remodelling genes in germinating *A. thaliana* seeds.

Supplementary Table S1. Primer sequences used for RT-qPCR

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Author contributions

G.G., K.N., T.S., S.K., F.I. and G.L.-M. conceived the project and designed the research. J.R. and F.I. designed and characterised the GPAW generator and G.G., J.R. and T.S. conducted the experiments. G.G., K.N., J.R., T.S. and G.L.-M. analyzed data. G.G. and G.L.-M. wrote the manuscript with input from all authors. All authors read and approved the manuscript

Conflict of interest

The authors declare no competing interests.

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Data availability

All data presented or analysed in this published article are available online through figshare https://doi.org/10.17637/rh.1937630 and the Supplementary data.

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Figure legends

Fig. 1 Seed dormancy release by gas plasma-activated water (GPAW). (A) Drawing of a mature *Arabidopsis thaliana* seed. (B) Visualisation of *A. thaliana* seed states and dormancy release pathways. The freshly harvested (FH) dry seed has physiological dormancy, which can be released either during imbibition, e.g. by cold-stratification, or be after-ripening (AR) storage. Abscisic acid (ABA) and gibberellin (GA) metabolism and signalling differ between dormancy maintenance and release to control germination in response to the environment. (C) The release of physiological dormancy of FH *A. thaliana* Col-0 seeds by treatment with Air-GPAW involves ABA degradation via the *CYP707A2* gene. Air-GPAW treatment of AR seeds does not significantly improve the maximal germination (G_{MAX}) of either wild type or *cyp707a2* mutant seeds. (D) The effects of Air-GPAW, He/O_2-GPAW, 5 mM KNO_3 and 300 µM H_2O_2 on the G_{MAX} of FH (dormant) *A. thaliana* C24 seeds. Discharge times of 45 min were used for the GPAW production (Supplementary Fig. S1). Mean ± SEM are presented for G_{MAX}.

Fig. 2 Chemical characterisation of gas plasma-activated water (GPAW). (A) Air-GPAW. (B) He/O_2-GPAW. *Top panels:* Time evolution of the concentrations of major chemical species produced in GPAW during the discharge treatment in the plasma bubble reactor; primary reactions (Supplementary Fig. S1). *Bottom panels:* Secondary reactions, chemistry after removal from the bubble reactor. Quantification of major chemical species as a function of the incubation time at ~22ºC after the plasma treatment. Note that Air-GPAW and He/O_2-GPAW differ considerably in their composition; mean ± SEM are presented.

Fig. 3 GPAW-induced gene expression associated with dormancy release and germination. (A) Dormancy release of freshly harvested (FH) *Arabidopsis thaliana* C24 seeds by Air-GPAW (45 min discharge time) and 5 mM KNO_3 mimicking the Air-GPAW's NO_3^- concentration (see Fig. 3a) as compared to the control (dH_2O). (B) Dormancy release of FH C24 seeds by He/O_2-GPAW (45 min discharge time) and 300 µM H_2O_2 mimicking the He/O_2-GPAW's H_2O_2 concentration (see Fig. 3b). (C) RT-qPCR analyses of seed transcript abundances at 6 h for key genes encoding enzymes for ABA degradation (*CYP707A2*), GA biosynthesis (*GA3OX1*), ABA biosynthesis (*NECD2/9*) and at 24 h for CWRP genes known to be involved in endosperm weakening and germination. Relative mean ± SEM values for the Air-GPAW and NO_3^- treatments are compared to the 6 and 24 h samples of the control series (set to 1 for each gene). (D) RT-qPCR analyses of seed transcript abundances for the He/O_2-GPAW and H_2O_2 treatments. Relative mean ± SEM values compared to the 6 and 24 h controls are presented.

Fig. 4 Germination responses of *Arabidopsis thaliana* mutant seeds to Air-GPAW treatment. (A) Germination responses of freshly harvested (FH) *A. thaliana* Col-0 seeds to Air-GPAW with increasing discharge time and the corresponding NO_3^- mimic with increasing concentration. (B) Germination responses of FH *dog1* mutant seeds. (C) Germination responses of FH *nlp8* mutant seeds. (D) Germination responses of FH *cyp707a2* mutant seeds. (E) Germination responses of FH *prt6* mutant seeds. (F) Dose responses to Air-GPAW and the corresponding NO_3^- mimic for the maximum germination (G_{MAX}) of FH compared to AR wild type and mutant

seeds. Mean ± SEM germination values over time of seed populations incubated in constant white light at 20 ºC are presented.

Fig. 5 Biomechanical and biochemical analysis of GPAW-induced endosperm weakening. (A) Time courses of micropylar endosperm (CAP) puncture force, testa and endosperm rupture, and expansin (*EXPA*) and xyloglucan endo-transglycolases/hydrolase (*XTH*) transcript abundances in the CAP of *Lepidium sativum* FR14 seeds during germination. For the CAP puncture force normalized values combined from two datasets (Graeber *et al.*, 2014; Linkies *et al.*, 2009) are presented. The CAP-specific relative cumulative expression values of *EXPA* and *XTH* genes were from the spatiotemporal transcriptome dataset of *L. sativum* seed germination (Scheler *et al.*, 2015); for individual genes and details see Supplementary Fig. S4. (B) Xyloglucan endo-transglycosylase (XET) enzyme activities of XTH proteins in the CAP after incubation of whole *L. sativum* seeds in dH_2O for the times indicated (*left panel*). Effects of incubating isolated CAPs in Air-GPAW (45 min) or He/O_2-GPAW(45 min) on the XET enzyme activities at 3 h (*right panel*). Note that only the 10-h CAP XET activity was statistically different while all of the other XET activity values were not significantly ("ns") different from each other. (C) Biomechanical analysis of the effects of Air-GPAW (45 min) or He/O_2-GPAW (45 min) on CAP endosperm weakening at 3 h. The CAP puncture force (tissue resistance) at 3 h (*left panel*) was determined as the maximal force from the displacement-force curve, and the CAP tissue elasticity at 3 h (*right panel*) was calculated as the slope of the linear portion of the displacement-force curve. (D) Example displacement-force curve and images of the biomechancial assay with CAP prior to rupture (intact) and CAP post rupture by the metal probe of the biomechanics device.

Figure 1

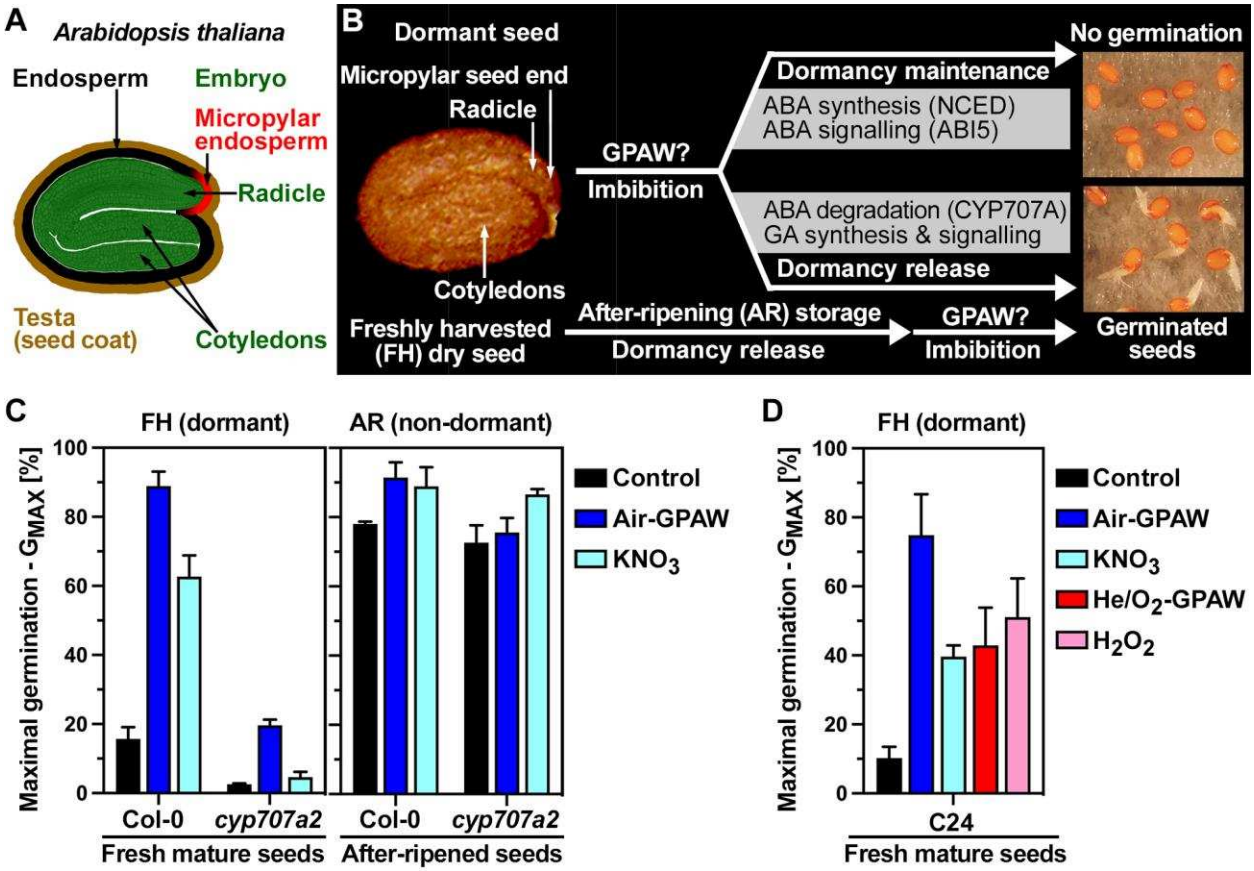


Figure 2

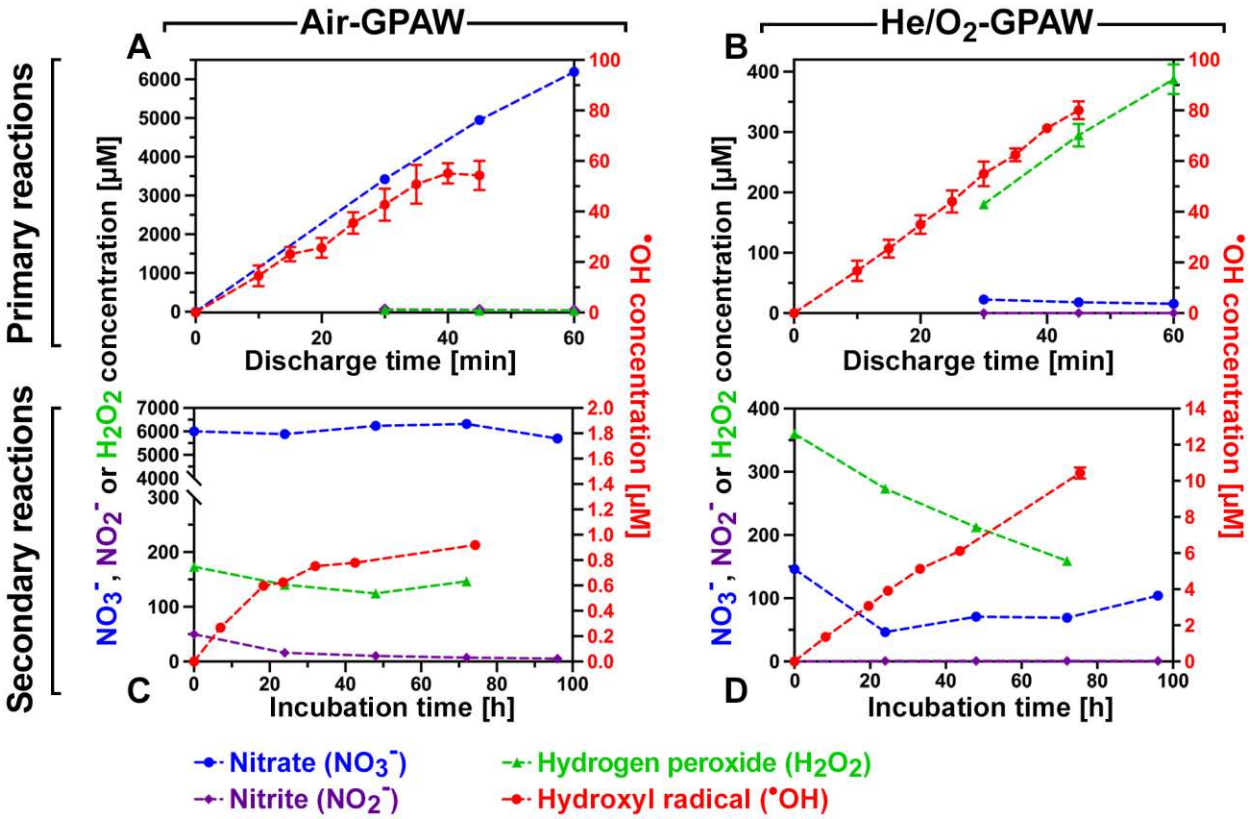


Figure 3

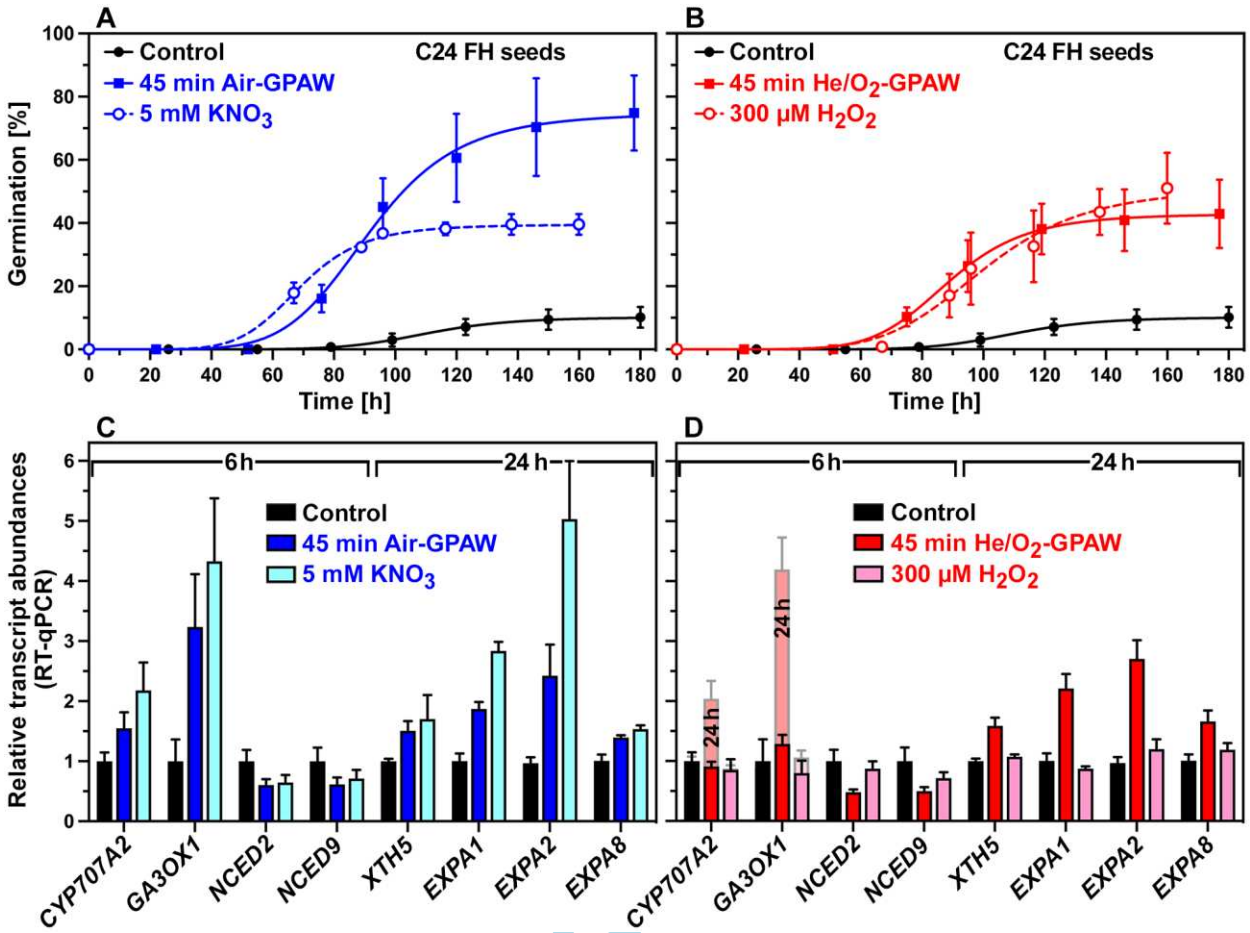


Figure 4

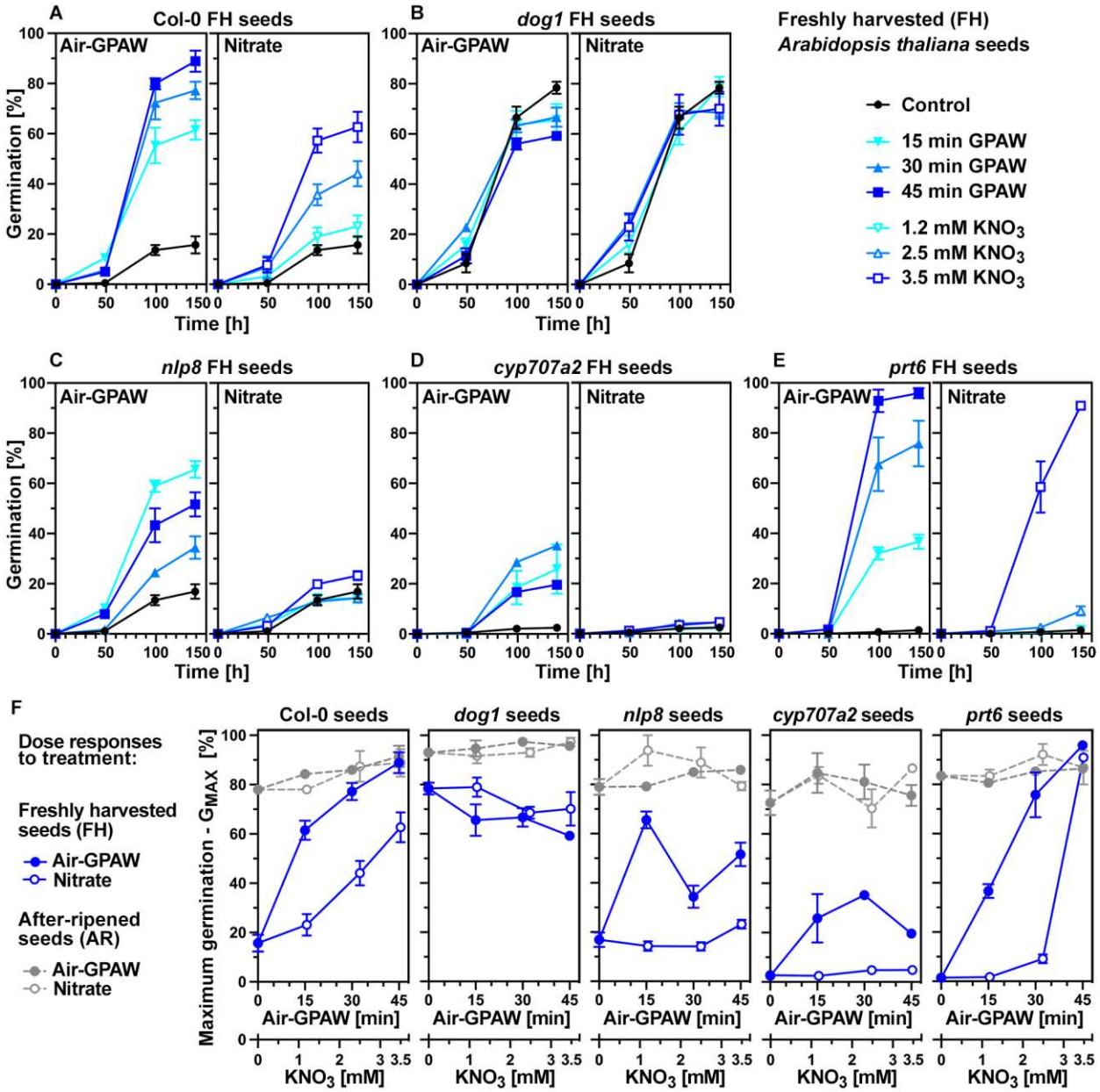
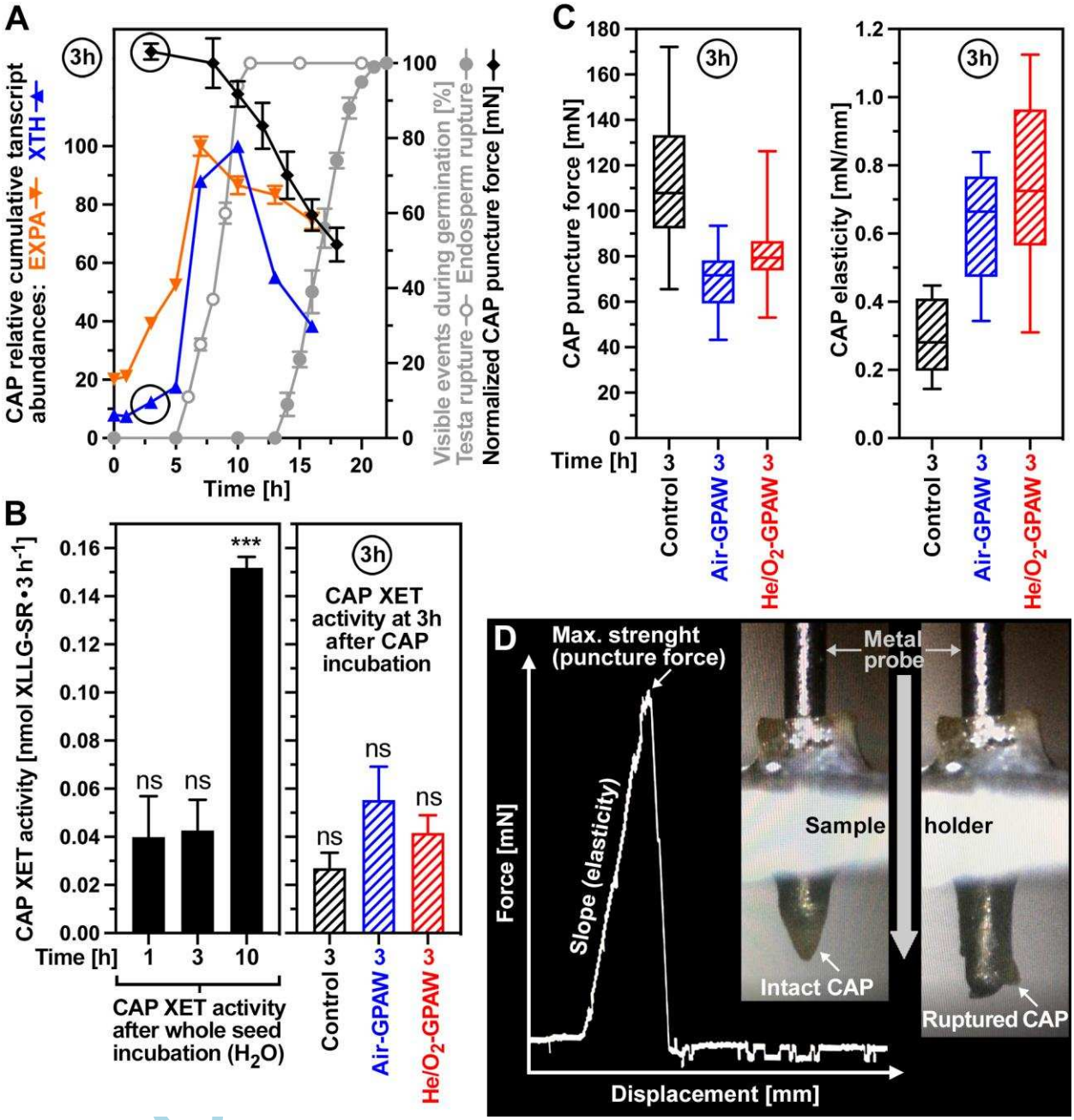


Figure 5



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

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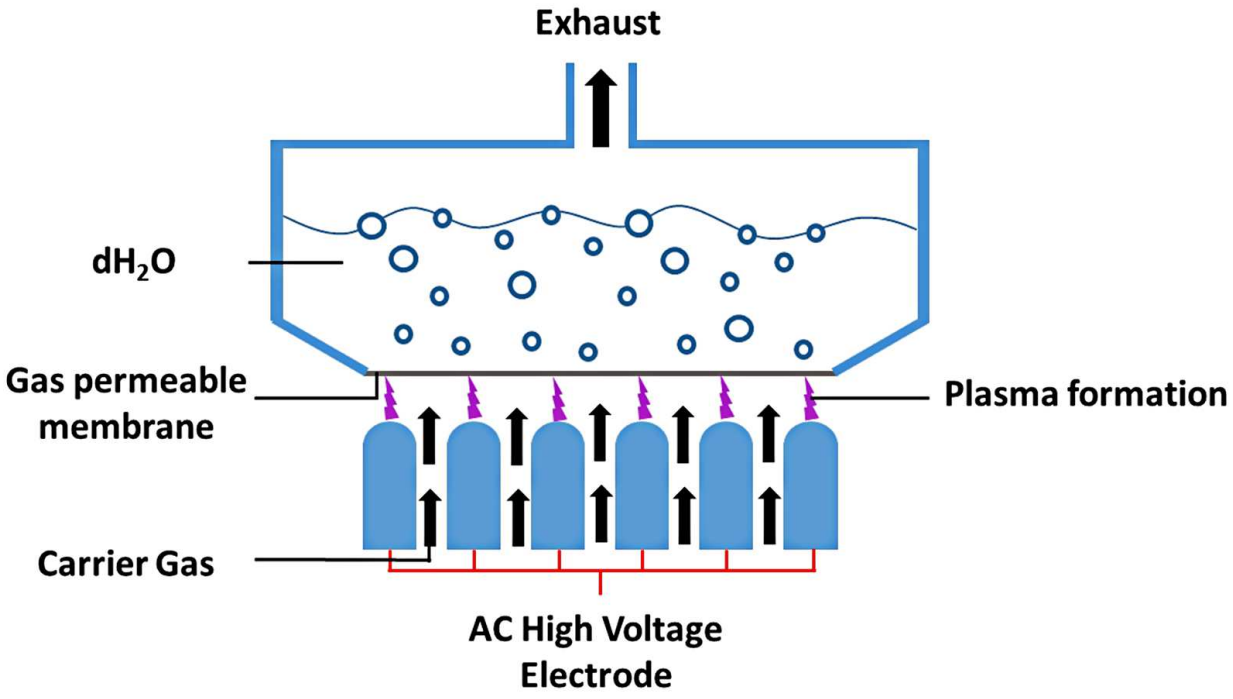
Supplementary data



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):

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

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

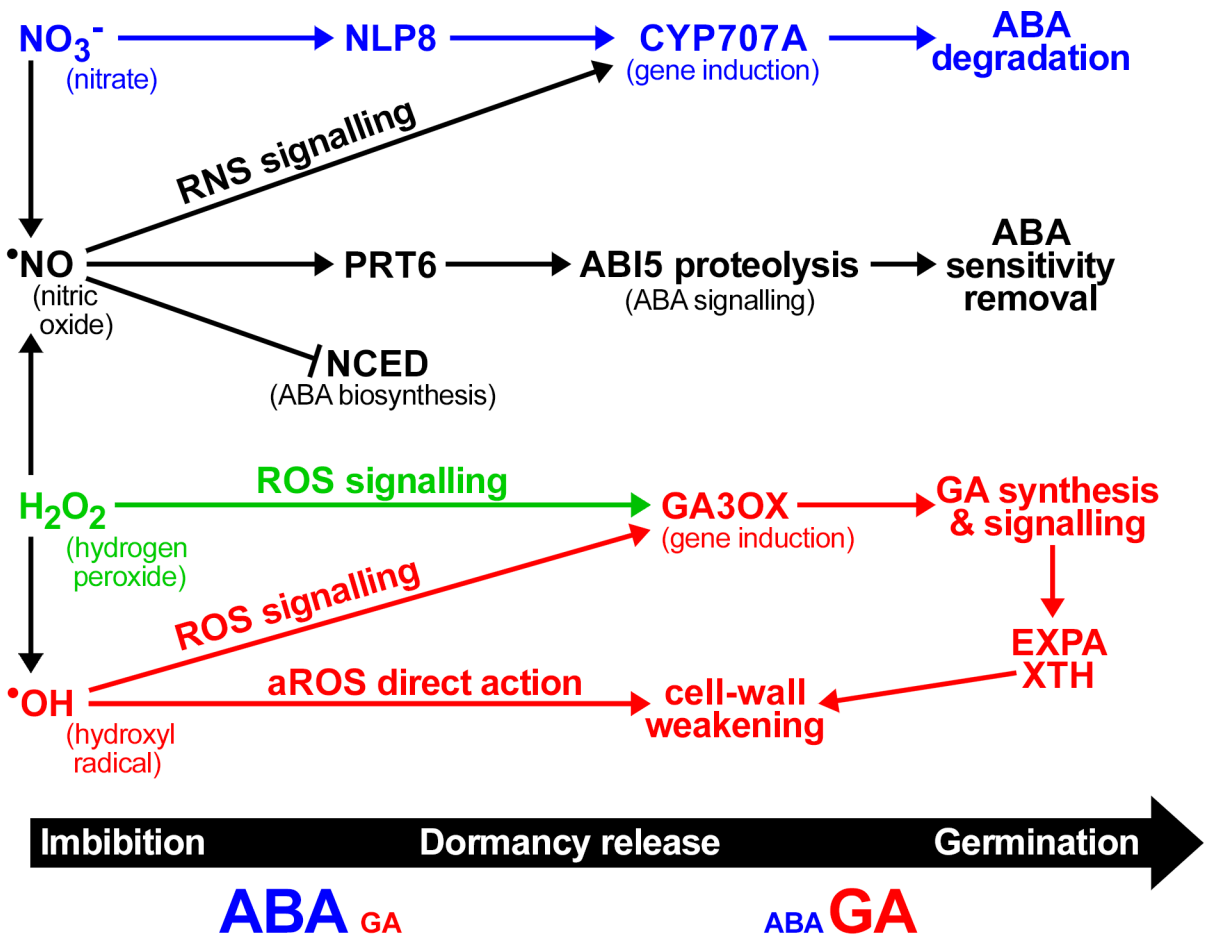
$${}^{\bullet}N + {}^{\bullet}OH \rightarrow {}^{\bullet}NO + {}^{\bullet}H \quad [13]$$

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):

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

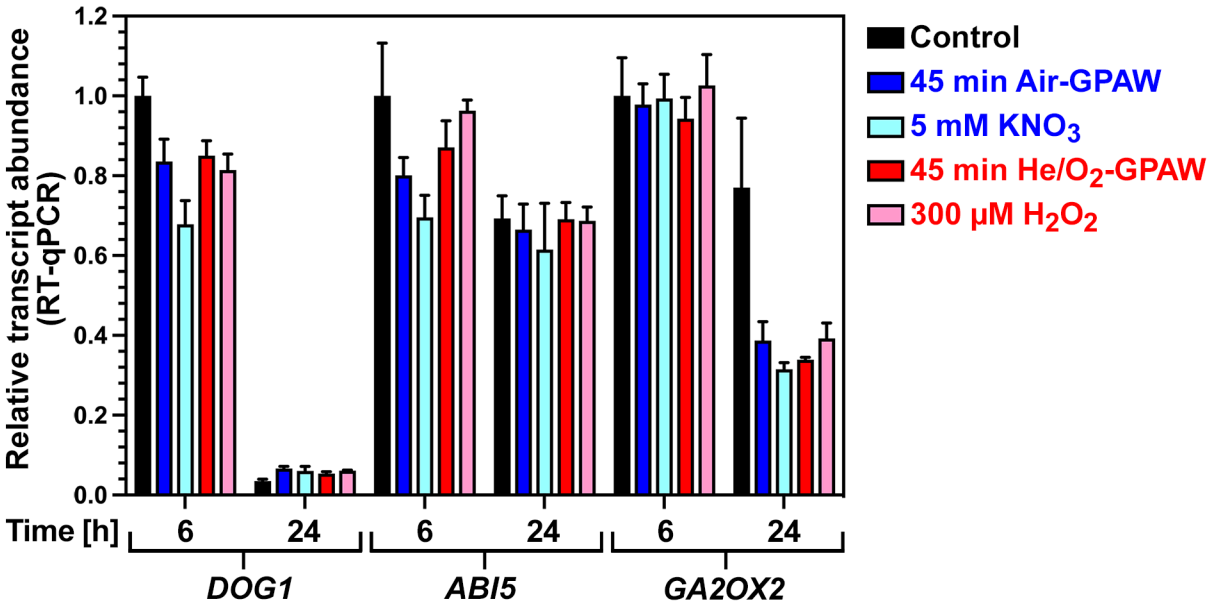
$${}^{\bullet}NO_2 + O_3 \rightarrow {}^{\bullet}NO + 2O_2 \quad [15]$$

Seed dormancy releasing pathways of major chemical species in GPAW

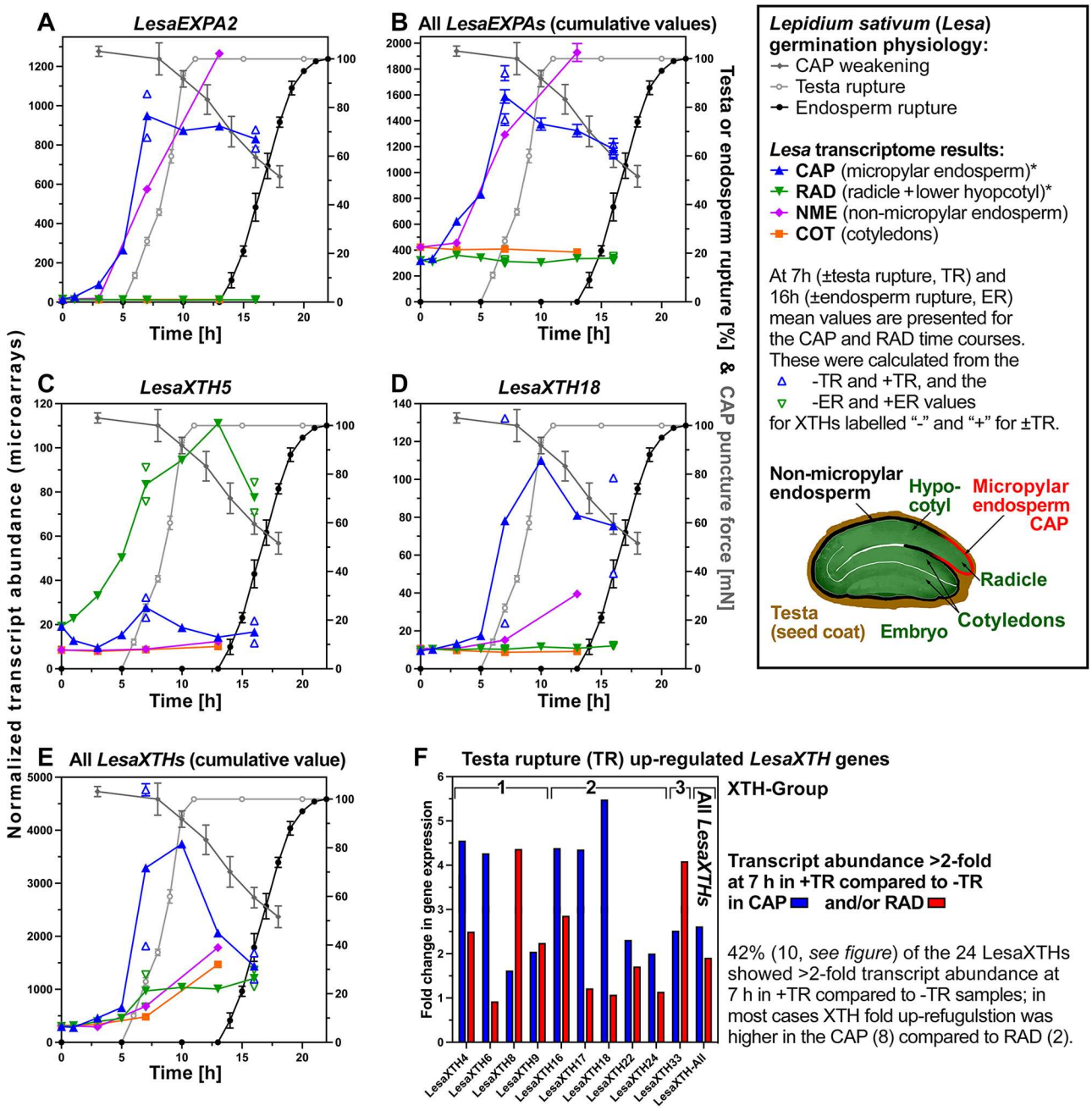


Supplementary Fig. S2 Simplified schematic presentation of ROS and RNS signalling pathways in plants. Major chemical species produced in GPAW include NO_3^-, $^{\bullet}NO$, H_2O_2 and $^{\bullet}OH$ (Figure 2, Supplementary Figure S1) which are also produced *in planta* and are known for their signalling 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

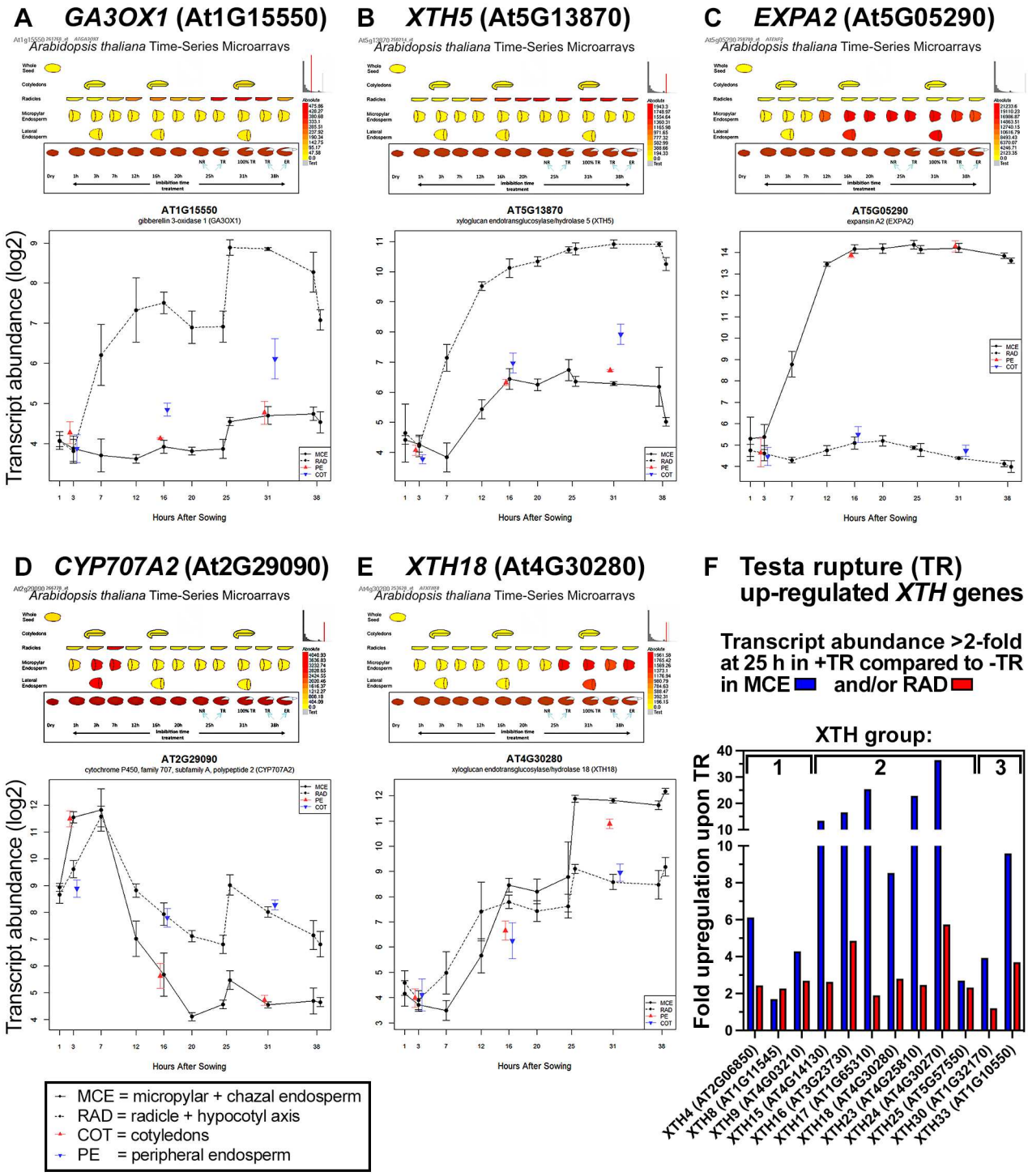
endo-transglycosylase enzyme activity (XET) enzyme activity are involved in promoting testa rupture and enhanced endosperm CAP weakening (Chen *et al.*, 2002; Graeber *et al.*, 2014; Steinbrecher and Leubner-Metzger, 2017; Voegele *et al.*, 2011). Due to the altered balance in GA and ABA metabolism and 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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