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RESEARCH PAPER

Embryo growth, testa permeability, and endosperm weakening are major targets for the environmentally regulated inhibition of *Lepidium sativum* seed germination by myrigalone A

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Abstract

Myrigalone A (MyA) is a rare flavonoid in fruit leachates of *Myrica gale*, a deciduous shrub adapted to flood-prone habitats. As a putative allelochemical it inhibits seed germination and seedling growth. Using *Lepidium sativum* as a model target species, experiments were conducted to investigate how environmental cues modulate MyA's interference with key processes of seed germination. Time course analyses of *L. sativum* testa and endosperm rupture under different light conditions and water potentials were combined with quantifying testa permeability, endosperm weakening, tissue-specific gibberellin (GA) and abscisic acid (ABA) contents, as well as embryo growth and apoplastic superoxide production important for cell expansion growth. *Lepidium sativum* testa permeability and early water uptake by imbibition is enhanced by MyA. During late germination, MyA inhibits endosperm weakening and embryo growth, both processes required for endosperm rupture. Inhibition of embryo cell expansion by MyA depends on environmental cues, which is evident from the light-modulated severity of the MyA-mediated inhibition of apoplastic superoxide accumulation. Several important key weakening and growth processes during early and late germination are targets for MyA. These effects are modulated by light conditions and ambient water potential. It is speculated that MyA is a soil seed bank-destroying allelochemical that secures the persistence of *M. gale* in its flood-prone environment.

Key words: Allelochemical, apoplastic superoxide, embryo growth, endosperm cap weakening, gibberellins (GAs), imbibition, *Lepidium sativum*, myrigalone A (MyA), seed germination, testa permeability

Introduction

Seed shedding combined with appropriate seed germination responses to environmental cues, including ambient light conditions (e.g. darkness in the soil seed bank), soil water status, or allelochemicals produced by competing plants, control the persistence of a species in the local habitat, as well as long-distance dispersal important for expanding the species range (Donohue

et al., 2005; Batlla and Benech-Arnold, 2010). Germination timing is under extremely strong natural selection and influences phenotypic expression of and selection on post-germination traits as it is a major determinant of successful seedling establishment, survival, and colonization of a habitat (Donohue *et al.*, 2010). Phenological adaptation to environmental changes studied with sets of *Arabidopsis thaliana* recombinant inbred lines demonstrated that natural variation in germination responses to environmental factors is associated with differences in seed-related mechanisms involving hormones and the envelopes covering the embryo (Huang *et al.*, 2010; Barua *et al.*, 2011). While gibberellin (GA) sensitivity was associated with germination responses to multiple environmental factors, abscisic acid (ABA) sensitivity and testa permeability were associated with specific germination responses. Environmental effects on the mechanical and chemical properties of the testa were proposed to constitute major mechanisms for maternal effects on coat dormancy and germination traits (Roach and Wulff, 1987; Debeaujon and Koornneef, 2000; Debeaujon *et al.*, 2000; Donohue, 2009).

In the non-dormant seed state, germination starts with the uptake of water by imbibition of the dry seed, followed by embryo expansion and seed swelling (Holdsworth *et al.*, 2008; Weitbrecht *et al.*, 2011; Linkies and Leubner-Metzger, 2012). The uptake of water is triphasic, with a rapid initial uptake (phase I, i.e. imbibition) followed by a plateau phase (II), and finally phase III water uptake. The completion of germination by radicle protrusion through all seed envelopes depends on the increasing growth potential of the embryo and on the weakening of the restraint of the various seed envelopes. Embryo growth is promoted by GA and inhibited by ABA, and light-promoted *A. thaliana* seed germination is associated with light-increased GA/ABA ratios (Holdsworth *et al*., 2008). *Arabidopsis thaliana* embryo elongation is achieved by cell expansion in specific regions of the radicle–hypocotyl axis (Sliwinska *et al.*, 2009). The envelopes of most angiosperms are made up of the testa (seed coat) and the endosperm, which is retained to a variable degree in mature seeds (Linkies *et al.*, 2010). *Arabidopsis thaliana* testa mutants show reduced dormancy that is caused by alterations of the testa characteristics including increased permeability (Debeaujon and Koornneef, 2000; Koornneef *et al.*, 2002). The GA requirement for *A. thaliana* seed germination is determined by testa characteristics, embryonic growth potential, and embryonic ABA content. In many endospermic seeds, germination progresses in two visible steps, with testa rupture and endosperm rupture being two sequential events (Leubner-Metzger, 2002; Petruzzelli *et al.*, 2003; Linkies and Leubner-Metzger, 2012). Mature seeds with a thin endosperm layer include the Brassicaceae species *A. thaliana*, *Lepidium sativum* (garden cress), and *Sisymbrium officinale* (Müller *et al.*, 2006; Holdsworth *et al.*, 2008; Iglesias-Fernandez and Matilla, 2010). *Lepidium sativum* endosperm rupture is preceded by weakening of the micropylar endosperm (CAP) that covers the radicle. Embryo growth and endosperm weakening depend on hormonally regulated cell wall remodelling mechanisms mediated by hydrolytic enzymes, expansins, and reactive oxygen species (ROS) in the apoplast (Ni and Bradford, 1993; Toorop *et al.*, 2000; Petruzzelli *et al.*, 2003; Da Silva *et al.*, 2008; Müller *et al.*, 2009; Morris *et al.*, 2011; Voegele *et al.*, 2011).

Myrica gale ('sweet gale', 'bog myrtle', Myricaceae) is a deciduous shrub native to Northern and Western Europe and Canada, adapted to flood-prone habitats (Skene *et al.*, 2000). It grows in acidic peat bogs and at the intertidal zone of lakes and rivers which are often flooded by frequent rises and falls in water level. *Myrica gale* fruits and leaves secrete resin droplets containing essential oils. *Myrica gale* fruit exudates contain rare flavonoids, with myrigalone A (MyA) being the major C-methylated dihydrochalcone (Mathiesen *et al.*, 1995; Popovici *et al.*, 2011). Several biological activities have been reported for MyA, including radical scavenging and the inhibition of endoreduplication in radicles of *L. sativum* under continuous light conditions (Mathiesen *et al.*, 1997; Oracz *et al.*, 2012). MyA has also been shown to interfere with GA metabolism by inhibiting GA3-oxidase reactions and with GID1-type GA signalling pathways to inhibit endosperm rupture of *L. sativum* imbibed in the light and to inhibit shoot and root growth of dark-grown eudicot (cress, mustard, and knotweed) and monocot (sorghum) seedlings (Popovici *et al.*, 2011; Voegele *et al.*, 2011; Oracz *et al.*, 2012). The MyA inhibition of seedling growth is therefore evident for a wide range of weed and crop species, as well as for invasive knotweed (*Fallopia*×*bohemia*) for which the protective effect of *M. gale* against invasion is currently being investigated in the ecosystem.

It has been proposed that MyA is an allelochemical with a novel mode of action, but very little is known about the way in which MyA inhibits seed germination. Oracz *et al.* (2012) found that MyA interferes with GA biosynthesis, apoplastic ROS production, and endosperm cap weakening during the late phase of *L. sativum* seed germination in continuous light and at optimal conditions for water uptake. Here, in complementary work, investigations are carried out to determine how environmental cues (darkness and restrictive ambient water potential) affect MyA inhibition in order to elucidate its complex and developmentally regulated mode of action. It is shown that MyA interferes with several key mechanisms throughout the whole process of seed germination and that its inhibitory effect is under strong environmental control. It was found that the effects of MyA on seed germination are strongly intensified in darkness compared with light. Furthermore, it is shown that the very early processes of initial embryo imbibition, as well as testa permeability, are a major target for MyA during the early phase of germination. During the late phase of germination, it is demonstrated here by quantitative analysis that embryo growth and molecular mechanisms related to cell expansion growth of the radicle–hypocotyl axis, as well as endosperm cap weakening are affected by MyA. The severe impact of environmental cues such as water deficiency stress and light conditions on the effects of MyA presented here is placed into a speculative model to explain the complex interactions in connection with the ecophysiological roles of MyA as an allelochemical of *M. gale* important for the adaptation to flood-prone habitats.

Materials and methods

Germination conditions and puncture-force measurements

MyA (purity >99%), extracted and purified as described by Popovici *et al.* (2011), was generously provided by Professor G. Comte, CESN, University and CNRS, Lyon, France, and used as a methanol stock,

corresponding to 0.35% (v/v) methanol in the medium if 5×10^{-4} M MyA was used. Germination of after-ripened seeds of *L. sativum* L. FR14 ('Keimsprossen', Juliwa) was analysed using three replicates of 50 seeds per 9 cm Petri dish containing two layers of filter paper and 6 ml of sterile, distilled water with 0.35% (v/v) methanol ('CON medium') (Oracz *et al.*, 2012). Seeds were incubated at 18 °C in a Sanyo Versatile Environmental Test Chamber (MLR-350) in continuous white light (~75 μmol s^{-1} m^{-2}). A binocular stereo microscope was used to score the percentages of testa and endosperm rupture of the seed populations. Puncture-force measurements (Müller *et al.*, 2006) were used for quantifying endosperm cap weakening. For organ- or tissue-specific analyses, whole embryos, RAD (lower one-third of the hypocotyl–radicle axis) or CAP (micropylar endosperm) tissues were used from seeds displaying testa rupture but not endosperm rupture.

Embryo size measurements

Fifty seeds for each data point were imbibed with different concentrations of polyethylene glycol (PEG) 6000 (Carl Roth, Karlsruhe, Germany) and embryos carefully extracted at the times indicated. Embryos were transferred onto a black ceramic plate and images were taken with a binocular stereo microscope (Leica MZ 125) connected to a digital camera (Leica DFC480) at 20-fold magnification using Leica IM1000 software (V4.0 release 132). Twenty-four-bit RGB images at a resolution of 2560 × 1920 pixel (pixel aspect ratio=1) were saved in TIFF format. Image analysis was performed using the ImageJ distribution Fiji (http://fiji.sc/wiki/index.php/Fiji) with the ImageJ kernel version 1.45r. Following conversion to 8-bit images, thresholding was performed using the Auto Threshold plugin v1.14 (http://fiji.sc/wiki/index.php/Auto_Threshold) implemented in Fiji. The Auto Threshold plugin can binarize 8-bit images using various global (histogram-derived) thresholding algorithms. The 'Default' algorithm was selected for thresholding, which is a variation of the IsoData method for image thresholding (Ridler and Calvard, 1978). Options controlling the thresholding algorithm were set to: ignore white=true; ignore black=true; white objects on black ground=true. The resulting binarized images (showing a white embryo on a black background) were used for measuring the actual embryo sizes. Embryo mask areas were determined using ImageJ's 'Analyze Particles' command. Options for measuring the object were set to: size (pixel^2)=100 000–infinity (i.e. no objects smaller than this are going to be measured, thus excluding image artefacts resulting from the thresholding); circularity: 0.00–1.00 (i.e. allowing irregular shapes of the object); include holes=true; show=masks. This operation provides a pixel value for the measured area as well as an image showing the filled outline of the measured area; that is, a black embryo shape on a white background. These embryo mask images were used in the figures to illustrate the area quantification process. Each embryo mask was checked manually to control that the embryo shape was identified properly and without measuring artefacts. Using the described settings for image acquisition and analysis results in each pixel of the measured area representing a real physical embryo area of 7.4 μm^2.

Testa permeability assay using tetrazolium staining

Entire *L. sativum* seeds were incubated for 3 h or 15 h in continuous light at 18 °C with tetrazolium staining solution (Graeber *et al.*, 2010) containing 0.35% methanol (CON) or 5×10^{-4} M MyA. The embryos were subsequently extracted, classified according to their staining intensity and patterns, and photographed. Enhanced staining intensity indicates enhanced testa permeability (Debeaujon *et al.*, 2000).

Quantification of hormone and superoxide contents

A total of 200 RADs and 200 CAPs were isolated from *L. sativum* seeds imbibed for 15 h, immediately frozen in liquid nitrogen, and used for quantification of ABA and GA metabolites as previously described (Oracz *et al.*, 2012). To quantify apoplastic superoxide ($O_2^{\cdot -}$) contents, the XTT assay was used with 10 RADs or 20 CAPs as described by measuring the absorbance of four biological replicates at 470 nm (Müller *et al.*, 2009; Oracz *et al.*, 2012). Apoplastic $O_2^{\cdot -}$ was localized *in situ* by nitroblue tetrazolium (NBT) histostaining (dark-blue colour of insoluble formazan compounds) (Oracz *et al.*, 2007).

Results

The MyA-mediated inhibition of endosperm weakening and rupture during L. sativum *seed germination is more severe in darkness compared with light*

Lepidium sativum two-step seed germination was investigated by scoring testa (seed coat) rupture over time, as well as subsequent endosperm rupture which is connected to visible radicle protrusion through the weakened micropylar endosperm (CAP) and marks the completion of germination. Figure 1 shows that testa and endosperm rupture kinetics of *L. sativum* seeds imbibed in continuous light or in darkness are highly similar (CON). It was shown earlier (Oracz *et al.*, 2012) that the putative allelochemical MyA delays endosperm weakening and rupture of *L. sativum* seeds imbibed in continuous light. Interestingly, and in contrast to CON, when seeds were incubated in the presence of MyA, the inhibiting effect of MyA on endosperm rupture was more severe in darkness compared with light. While the time to reach 50% endosperm rupture ($ER_{50\%}$) was ~16 h for light- or dark-imbibed CON seeds, treatment with 5×10^{-4} M MyA caused an $ER_{50\%}$ of ~33 h in light and ~223 h in darkness (Fig. 1b). A concentration of 5×10^{-4} M MyA was chosen as a biologically relevant concentration as it results in a close to saturating response and causes the same delay in endosperm rupture as fruit leachates of *M. gale* on *L. sativum* seeds imbibed in continuous light (Oracz *et al.*, 2012). As in light, the MyA effect on endosperm rupture was dose dependent in darkness, and testa rupture was not affected (Fig. 1a; Supplementary Fig. S1a available at *JXB* online). The MyA inhibition in darkness was associated with a high incidence (~60%) of seeds in the population with atypical endosperm rupture, in which the CAP is torn off at its base instead of being penetrated by the radicle (Supplementary Fig. S1c). The stronger effect of MyA in darkness compared with light was mediated, at least in part, by prolonged inhibition of endosperm weakening in darkness (Fig. 1b). Whereas in the light MyA allowed 100% germination and acted phytotoxically on the growing seedling (Popovici *et al.*, 2011; Oracz *et al.*, 2012), in darkness MyA was already phytotoxic prior to the completion of germination. It decreased embryo viability and caused embryo death during the prolonged incubation (Supplementary Fig. S1b). To exclude that MyA is chemically inactivated upon light irradiation, the effect of MyA pre-incubation (light versus darkness) was compared prior to the addition of seeds (Fig. 1c). Light- or dark-pre-incubated MyA inhibited *L. sativum* endosperm rupture equally, showing that MyA's effect is persistent under the ambient incubation conditions used here. Endosperm weakening is clearly a major target of MyA, but it has not been investigated so far whether MyA acts in addition on *L. sativum* embryo growth as another major target.

Development of an image analysis method to quantify L. sativum *embryo growth*

To quantify *L. sativum* embryo sizes and thereby embryo expansion growth during the process of germination, embryos were

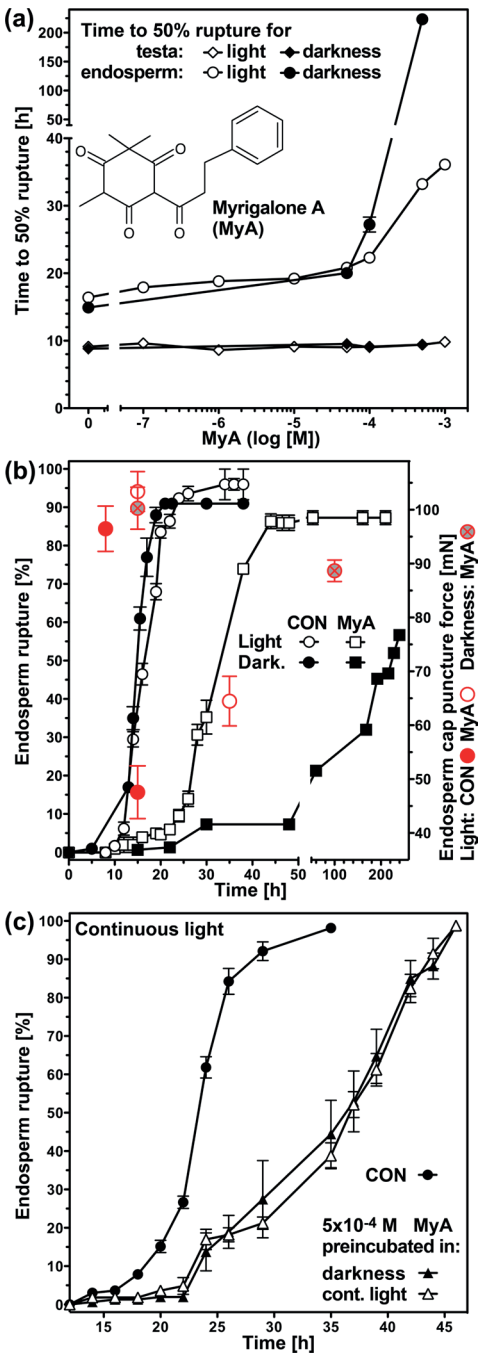

Fig. 1. The effect of myrigalone A (MyA) on *Lepidium sativum* seed germination in continuous light and in darkness. (a) The effect of different concentrations of MyA on the time required for germinating seed populations to reach 50% testa rupture ($TR_{50\%}$) or endosperm rupture ($ER_{50\%}$) displayed as dose–responses. (b) Endosperm cap puncture-force (red circles) and endosperm rupture kinetics during germination without (CON) or with 5×10^{-4} M MyA added. (c) Endosperm rupture kinetics of germinating seeds, incubated in continuous white light without (CON) and with 5×10^{-4} M MyA pre-incubated for 35 h in darkness or continuous light. Mean values ±SE of ≥3 replicates of 50 seeds each are shown for testa and endosperm rupture; mean values ±SE of 10–50 seeds are shown for the puncture-force measurements. (This figure is available in colour at *JXB* online.)

extracted from their seed envelopes at the indicated times, examined under a stereo microscope, and photographed thereafter. These raw images were analysed using the ImageJ software distribution Fiji using the workflow presented in Fig. 2a and described in detail in the Materials and methods. The size/shape detection process of this image analysis method worked accurately for different stages such as embryos extracted from fully imbibed seeds (e.g. 3 h CON+0 mM PEG) with a semi-opened hypocotyl hook, as well as for only partially imbibed seeds in the presence of osmotica (e.g. 3 h CON+30 mM PEG) with a closed hypocotyl hook (Fig. 2a). The CON+30 mM PEG incubation solution corresponded to an ambient water potential (Ψ_{medium}) of –0.58 MPa and caused a 1.6-fold smaller embryo size compared with CON (Ψ_{medium}=0 MPa) at 3 h (Fig. 2b).

MyA accelerates early water uptake by imbibition of L. sativum *seeds and inhibits embryo growth during the late germination phase*

The method developed to quantify *L. sativum* embryo sizes (Fig. 2a) was applied to embryos extracted at the indicated times from seeds incubated in 0, 10, 20, and 30 mM PEG. These PEG concentrations correspond to ambient water potentials (Ψ_{medium}) of 0, –0.05, –0.25, and –0.58 MPa, respectively, for which the embryo sizes in 3 h-imbibed seeds are presented as mean values ± SE (Fig. 2b). In contrast to higher Ψ_{medium} during the early germination phase, at low Ψ_{medium} (–0.58 MPa) MyA enhances water uptake by imbibition, leading to a MyA-mediated ~1.4-fold larger embryo size at 3 h. To verify with an independent method that this MyA-mediated larger embryo size at –0.58 MPa is indeed due to increased water uptake, the embryo masses were compared by weighing, and it was found that the 3 h MyA-imbibed embryos are heavier compared with the 3 h CON embryos, but that their weight is equal after drying (Supplementary Fig. S2 at *JXB* online). In Fig. 3, the data distribution of the embryo sizes over time is presented as box–whisker plots for the different treatments. Figure 3a shows that without MyA (CON series) the embryo size increased over time at all Ψ_{medium}, but also that the early (3 h) water uptake by imbibition is severely hampered by –0.58 MPa (30 mM PEG; Figs 2b, 3a; Supplementary Fig. S3a). At 22 h without osmoticum (CON+0 mM PEG); that is, at $ER_{50\%}$, a 1.2-fold growth in embryo size (compared with 3 h) to 5.4 ± 0.1 mm^2 and an opened hypocotyl hook was evident (Fig. 3a). In contrast to this, the embryo size at low Ψ_{medium} (CON+30 mM PEG) had reached only 4.3 ± 0.1 mm^2 at 22 h, no endosperm rupture had occurred, and the hypocotyl hook did not fully open (Fig. 3a). Although an ~1.5-fold increase in embryo size was evident from 3 h to 22 h in CON+30 mM PEG, the absolute embryo size at 22 h remained below the absolute embryo size in CON+0 mM PEG at 3 h (4.6 ± 0.1 mm^2). The –0.58 MPa decrease in Ψ_{medium} therefore severely inhibited early water uptake by imbibition (3 h) and later embryo growth to a size associated with $ER_{50\%}$ (22 h).

In contrast to the CON series, for the early phase of water uptake by imbibition (3 h, phase I water uptake), treatment of seeds with MyA completely abolished the severe inhibition of the embryo size increase by low Ψ_{medium} (MyA+30 mM PEG)

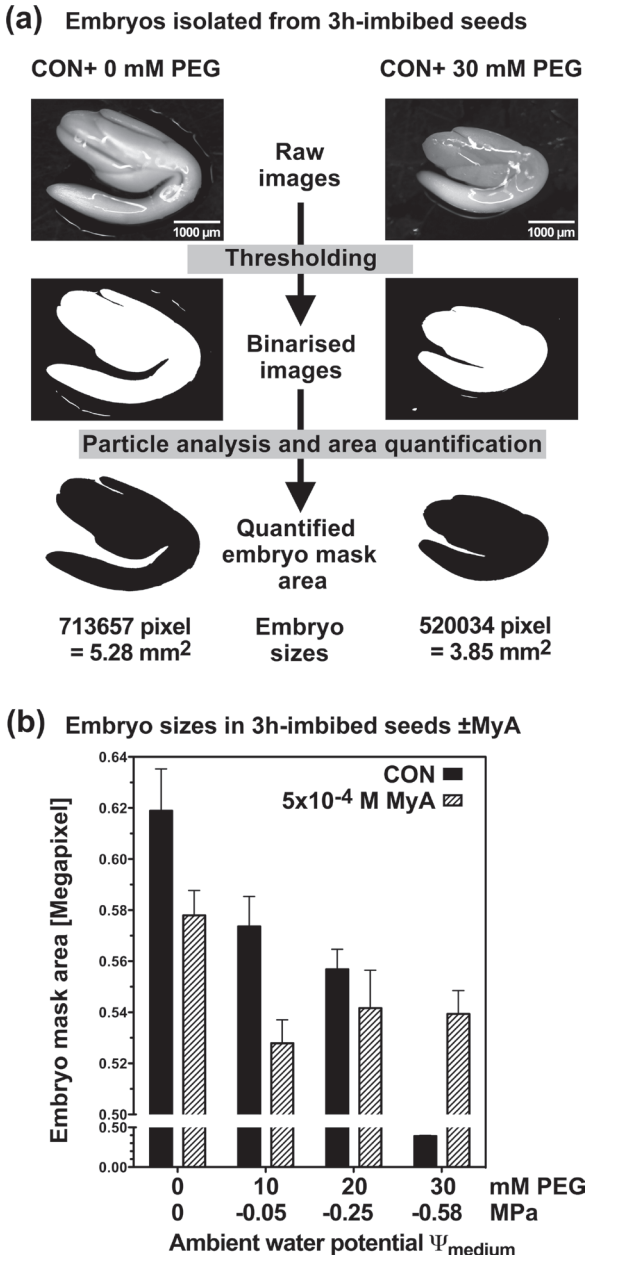

Fig. 2. A computer-based image analysis method to quantify *L. sativum* embryo sizes. (a) Workflow illustrating quantification of embryo sizes. After seed incubation for a specified time and condition, embryos were carefully extracted and photographed. Raw images were thresholded and image particle analysis was used to create embryo masks. The embryo mask areas were quantified using the Fiji distribution of the image analysis software ImageJ as described in detail in the Materials and methods. Using this workflow, 1 pixel of the embryo mask area equals 7.4 μm^2 real embryo area. Example raw images and mask areas are presented for embryos extracted from 3 h-imbibed seeds with or without 30 mM PEG added; pixel and mm^2 values correspond to the presented embryo mask areas. (b) The effect of decreasing ambient water potential (Ψ_{medium}) on embryo sizes from seeds incubated for 3 h in the light without (CON) and with 5×10^{-4} M MyA and the osmoticum PEG, as indicated. Mean values ±SE calculated from ≥50 extracted embryos are presented.

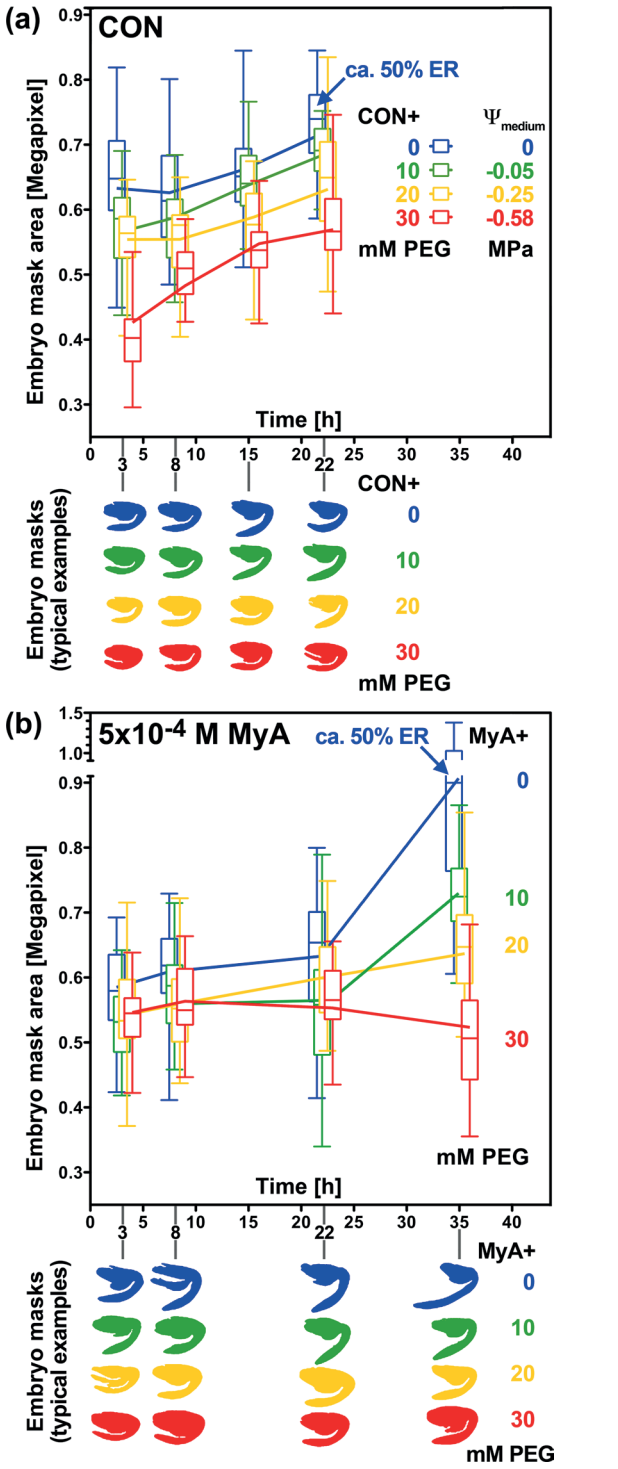

Fig. 3. The effect of MyA on the embryo mask areas during seed germination of *L. sativum.* Time course of mask areas of embryos extracted from seeds at different times during incubation (a) without (CON) and (b) with 5×10^{-4} M MyA, and 0, 10, 20, or 30 mM PEG in continuous light. Typical examples of embryo masks from each seed population at different times are shown below the graphs. Box–whisker plots each representing data from 50 embryos are shown, with the whiskers representing the minimum and maximum of the data distribution, the bottom and top of the boxes the 25th and 75th percentiles, and the horizontal bar the median.

when compared with MyA+0 mM PEG (Fig. 3b). After this initial swelling at 3 h to 4.0 ± 0.1 mm^2, the embryo size did not increase further over time in MyA+30 mM PEG (Fig. 3b; Supplementary Fig. S3a at *JXB* online). At 35 h, the MyA-treated seed population without osmoticum had reached $ER_{50\%}$ and the embryos had grown in size to 6.7 ± 0.2 mm^2, while the embryos of the MyA+30 mM PEG series remained at 3.8 ± 0.1 mm^2, without a fully opened hypocotyl hook and with no endosperm rupture (Fig. 3; Supplementary Fig. S3b). By plotting the endosperm rupture responses against the embryo sizes at the various Ψ_{medium} (Fig. 4a), it was found that the embryo size associated with the onset of endosperm rupture is roughly equal for CON and MyA (~4.6–4.7 mm^2). In contrast to this, the progression of endosperm rupture required larger embryo sizes in the presence of MyA. Regarding their endosperm rupture responses, the 22 h CON and the 35 h MyA seed populations are comparable. The $ER_{50\%}$ times (without osmoticum) were associated with embryo sizes of 5.4 ± 0.1 mm^2 (CON) and 6.8 ± 0.2 mm^2 (MyA); that is, 1.3-fold larger embryos upon MyA treatment (Fig. 4). Therefore, and in agreement with the inhibition of endosperm weakening by MyA (Fig. 1a), further embryo growth is required in the presence of MyA to achieve the same endosperm rupture response (Figs 3, 4; Supplementary Fig. S3).

To analyse further the effect of MyA on embryo cell expansion growth during late germination [i.e. well separated from the phase I water uptake (imbibition)], the phase II/III water uptake periods were analysed. When all Ψ_{medium} are taken into consideration, they correspond to incubation times >15 h (CON) and >8 h (MyA) (Fig. 3). Figure 4b shows that at 22 h the embryo sizes in the MyA series are smaller compared with the CON series at any Ψ_{medium}. MyA therefore inhibits embryos from growing above a critical size required for the onset of endosperm rupture (grey-shaded area, Fig. 4b, upper panel) until 22 h. In agreement with this, MyA inhibited the embryo growth rates (growth/time) between 15 h and 22 h (Fig. 4b, lower panel). This inhibition is, however, transient, as later at 35 h in the MyA series when the embryos are larger (Fig 4b, upper panel), the embryo growth rate is higher (Fig. 4b, lower panel) at high Ψ_{medium} (0 to –0.05 MPa) compared with the physiologically similar (in terms of endosperm rupture) 22 h CON series. At these physiologically comparable times, namely 22 h CON and 35 h MyA, the transition zone for passing the critical embryo size required for the onset of endosperm rupture (grey-shaded area, Fig. 4b, upper panel) seems to be about –0.3 MPa for both CON and MyA. When the MyA-mediated transient inhibition of embryo growth is overcome during the very late phase of germination, the MyA series embryos need to grow to a larger sizes to yield $ER_{50\%}$ compared with CON. This is obviously due to the inhibitory effect of MyA on endosperm weakening.

Embryo growth is mainly due to radicle–hypocotyl axis (RAD) elongation, and an excess in RAD growth is required to accomplish endosperm rupture in the presence of MyA

To answer the question of which part of the embryo contributes most to the measured embryo growth, the embryo was digitally

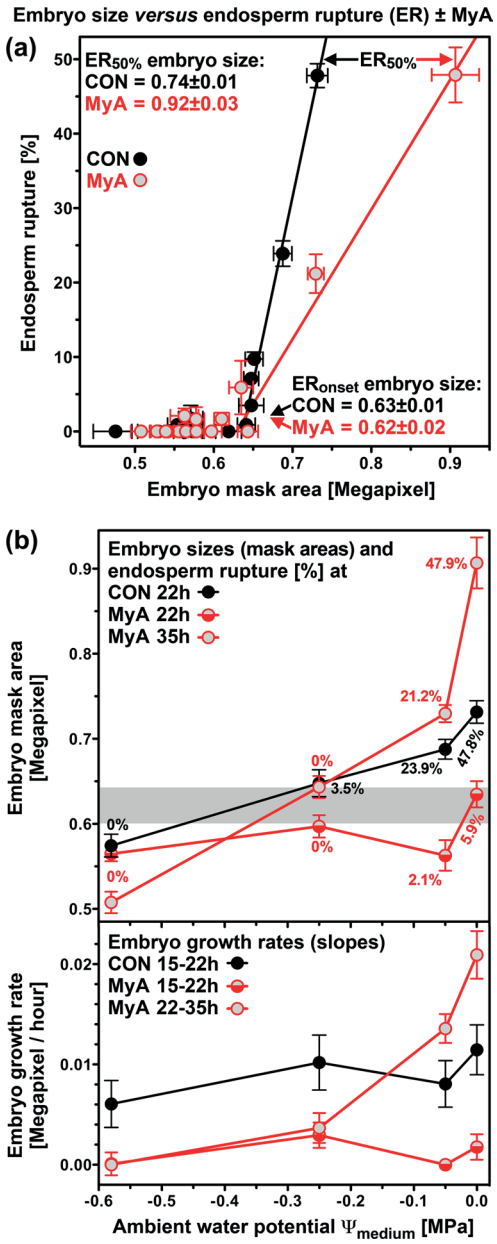

Fig. 4. The effect of MyA on the relationship between embryo size, growth, and endosperm rupture at different ambient water potentials (Ψ_{medium}). (a) The percentages of endosperm rupture at different times during the seed germination of *L. sativum* at various Ψ_{medium} without (CON) and with 5×10^{-4} M MyA (Fig. 3) are plotted against the corresponding embryo mask areas. Note that while the onset of endosperm rupture was associated with an equal embryo size of 4.6–4.7 mm^2, the progression of endosperm rupture required larger embryos in the MyA series compared with CON; the embryo sizes required for 50% endosperm rupture ($ER_{50\%}$) were 5.4 ± 0.1 mm^2 (CON) and 6.8 ± 0.2 mm^2 (MyA), and therefore increased 1.3-fold upon MyA treatment. (b) Embryo sizes and endosperm rupture (upper panel), and embryo growth rates (lower panel; slopes of the curves in Fig. 3) at different Ψ_{medium} during late germination. Mean values ±SE are presented from data shown in Fig. 3, and Supplementary Fig. S3b at *JXB* online. (This figure is available in colour at *JXB* online.)

dissected into two parts: the cotyledons (COT) and the radicle–hypocotyl axis (RAD), with the apical meristem being the cutting site (Fig. 5a). Figure 5b shows that the increases in RAD sizes contribute most to embryo growth. The relative RAD size increases are 4- to 5-fold higher compared with those of the COT, for both the CON and the MyA series. The RAD is therefore the major organ that contributes to embryo growth during *L. sativum* seed germination. An ~3-fold higher RAD size increase is observed in the presence of MyA to achieve $ER_{50\%}$ at 35 h (MyA) in comparison with CON ($ER_{50\%}$ at 22 h) (Fig. 5b). Since RAD is the main embryo part contributing to overall growth, the transient inhibition of embryo growth by MyA mentioned in the previous section is due to inhibition of RAD growth. Therefore, in the presence of MyA, the embryos need longer times to grow to larger sizes required for rupture of the less weakened endosperm compared with CON treatment (Figs 3–5).

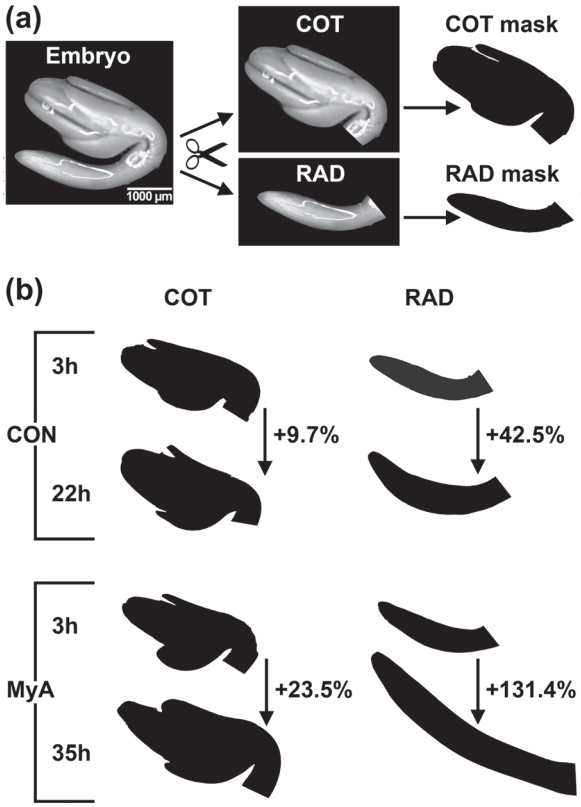

Fig. 5. The contribution of different embryo parts to the increase in embryo mask area during *L. sativum* seed germination. (a) Workflow for quantification of COT and RAD mask areas. The raw images were digitally dissected at the apical meristem with a 90 ° angle towards the stem into a 'cotyledon' (COT) part and a 'radicle–hypocotyl axis' (RAD) part. COT and RAD masks were created and their areas quantified as shown in Fig. 2a. (b) Increase in COT and RAD mask areas of embryos extracted from seeds incubated without (CON) or with 5×10^{-4} M MyA. The relative increase of the COT and RAD sizes from 3 h to the time for $ER_{50\%}$ for CON (22 h) and MyA (35 h) was calculated from 50 embryo raw images taken from seeds incubated in continuous light for each indicated time point. Mean values are presented; SE <1% for CON and MyA-COT, 7% for MyA-RAD.

MyA enhances L. sativum *testa permeability during imbibition, but does not affect the kinetics of testa rupture at different water potentials*

Figure 6a shows that decreasing water potentials (Ψ_{medium}) generated by increasing osmoticum concentrations inhibited testa rupture in a dose-dependent manner, with 50% testa rupture ($TR_{50\%}$) values of ~11 h and 40 h for 0 and 30 mM PEG, respectively. As during early germination at 3 h in the case of 30 mM PEG the embryo was 1.3-fold larger for MyA compared with CON (Fig. 2b), it was speculated that the MyA-mediated early facilitation of water uptake may be due to promotion of testa rupture. However, it was found that MyA did not affect the kinetics of testa rupture at any PEG concentration tested (Fig. 6a). As there was no significant effect of MyA on testa rupture, it was investigated whether the MyA-mediated early facilitation of water uptake by the embryo was associated with increased testa permeability. To test for testa permeability, tetrazolium assays were conducted with whole seeds, a method that is known from work with *A. thaliana* testa mutants to detect differences in testa permeability (Debeaujon *et al.*, 2000). *Lepidium sativum* seeds were imbibed in tetrazolium salt solution in the absence (CON) or the presence of MyA, and the embryos were extracted from the seeds at 3 h or 15 h (Fig. 6b–d). Figure 6b shows for embryos from 3 h-imbibed seeds that for 0 mM PEG there is no obvious difference in the weakly orange embryo staining between CON and MyA. In contrast to this, for seeds imbibed in tetrazolium salt solution with 30 mM PEG, the histochemical staining of the 3 h-imbibed embryos of the MyA series appeared to be more intense compared with CON (Fig. 6b). This difference was further enhanced for embryos from seeds imbibed in tetrazolium salt solution for 15 h with 30 mM PEG (Fig. 6d). Here the CON series seeds delivered 15 h embryos with only background staining intensity, suggesting that the testa layers of CON seeds have lower permeability. In contrast to this, the MyA-treated seeds delivered approximately one-third orange-stained and two-thirds red-stained embryos (Fig. 6d), demonstrating that MyA increased testa permeability during early seed germination. This increased testa permeability suggests that MyA causes testa weakening, which in turn may require a lower embryo force for its rupture. The finding that the embryo sizes at the onset of testa rupture and at $TR_{50\%}$ are smaller in MyA-treated seeds compared with CON (Fig. 6e) is in agreement with this hypothesis. This indicates that MyA-enhanced testa permeability is another major target by which MyA exerts its effects on seed germination.

MyA inhibits apoplastic superoxide accumulation in the embryo required for cell elongation growth and this effect is more severe in darkness compared with the light

It was shown earlier (Oracz *et al.*, 2012) that MyA inhibits the production of apoplastic $O_2^{\cdot -}$ in the growth zones of the RAD of embryos extracted from seeds imbibed in continuous light. It is shown here that MyA also inhibits the production

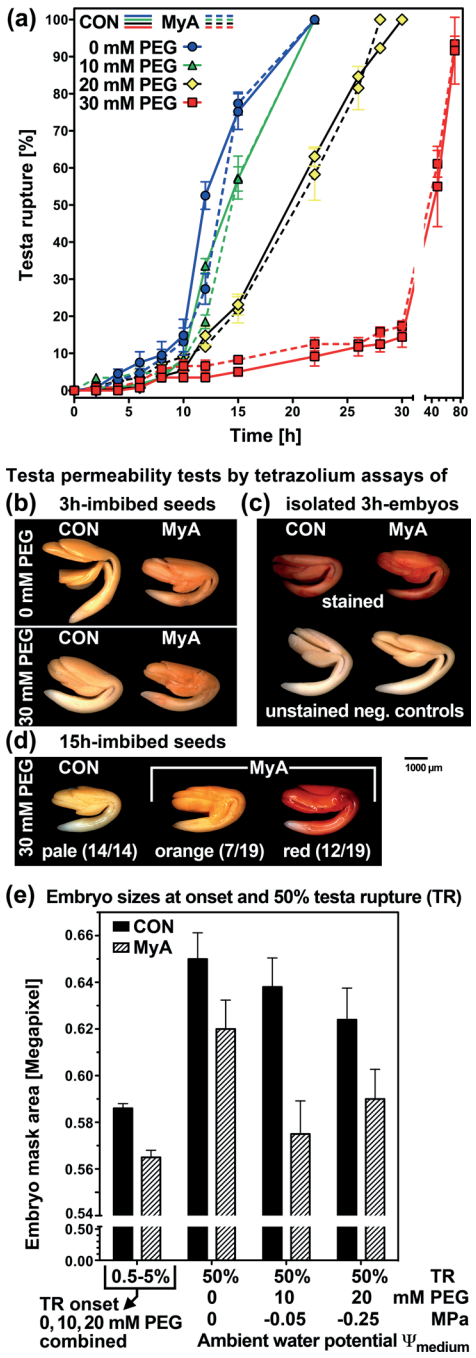

Fig. 6. The effect of MyA on testa characteristics. (a) Kinetics of testa rupture of *L. sativum* during seed germination without (CON) and with 5×10^{-4} M MyA added, and incubated in continuous light with 0, 10, 20, and 30 mM PEG. (b–d) Testa permeability test using the tetrazolium assay. Entire seeds were incubated in tetrazolium staining solution for 3 h (b) and 15 h (d) without (CON) and with 5×10^{-4} M MyA together with 0 and 30 mM PEG; embryos were extracted, and staining was scored. (c) Embryos extracted from 3 h CON and MyA-treated seeds were stained as positive controls. (e) The relationship between embryo sizes and testa rupture (onset and $TR_{50\%}$) for CON and MyA at different PEG concentrations. Mean values ±SE of 50 embryos.

of apoplastic $O_2^{\cdot-}$ in the growth zones of the RAD of embryos extracted from seeds imbibed in darkness (Fig. 7). The NBT histostains demonstrate that MyA inhibits apoplastic $O_2^{\cdot-}$ production in the RAD of embryos extracted from seeds imbibed for different times in darkness (Fig. 7a). In agreement with a stronger inhibition of endosperm rupture by MyA in darkness (Fig. 1), the quantification of apoplastic $O_2^{\cdot-}$ in the RAD by the XTT assay showed that an ~2-fold stronger reduction in apoplastic $O_2^{\cdot-}$ in the RAD was evident in darkness compared with the light (Fig. 7b). In agreement with a stronger inhibition of endosperm weakening by MyA in darkness (Fig. 1a), the XTT quantification showed that a stronger reduction of apoplastic $O_2^{\cdot-}$ in the CAP was evident in darkness compared with the light (Fig. 7c). The stronger inhibition of apoplastic $O_2^{\cdot-}$ production at 22 h by MyA in darkness compared with the light could be due either to directly lowered MyA contents resulting in a decreased radical scavenger effect or to an indirect effect by altered GA/ABA levels upon light irradiation.

MyA alters gibberellin metabolism during L. sativum *seed germination in a tissue-specific and light-dependent manner*

MyA severely interfered with GA metabolism and signalling of *L. sativum* seeds imbibed in continuous light (Oracz *et al.*, 2012). Supplementary Fig. S4 at *JXB* online shows that MyA did not appreciably affect the ABA contents in the RAD, but severely interfered with GA metabolism in the RAD in a similar manner in darkness and light. In the RAD, MyA caused an ~3-fold decrease in the (bioactive) GA_4 contents, while the inactive precursor GA_9 accumulated ~400- (darkness) and 200-fold (light), demonstrating that MyA inhibits important steps catalysed by GA3-oxidase and thereby may act by decreasing embryo growth (Supplementary Fig. S4b). In contrast to the inhibitory effects of MyA on the GA_4 contents in the RAD in light and darkness, there was no appreciable inhibitory effect on GA_1 in the RAD, but a differential effect of light/darkness on the GA_{13} contents (Supplementary Fig. S4c, d). MyA also affected the GA metabolite contents of the CAP (Supplementary Table S1), but there was no change that could explain the stronger inhibition of endosperm weakening (Fig. 1a) by MyA in darkness compared with the light.

Discussion

MyA enhances testa permeability and early embryo water uptake by imbibition

Early water uptake by imbibition is driven by the difference in water potential $\Delta\Psi$ between the ambient incubation medium (Ψ_{medium}) and the embryo. For the embryo water potential Ψ_{embryo}, the matric potential is primarily responsible for imbibition, whereas the osmotic potential becomes more important later during germination (Woodstock, 1988; Schopfer, 2006; Weitbrecht *et al.*, 2011). Final values reached for *L. sativum* embryo water uptake by imbibition at low Ψ_{medium} (–0.58 MPa) were equal without and with MyA treatment, but MyA caused them to be reached much earlier, already at 3 h. It is concluded

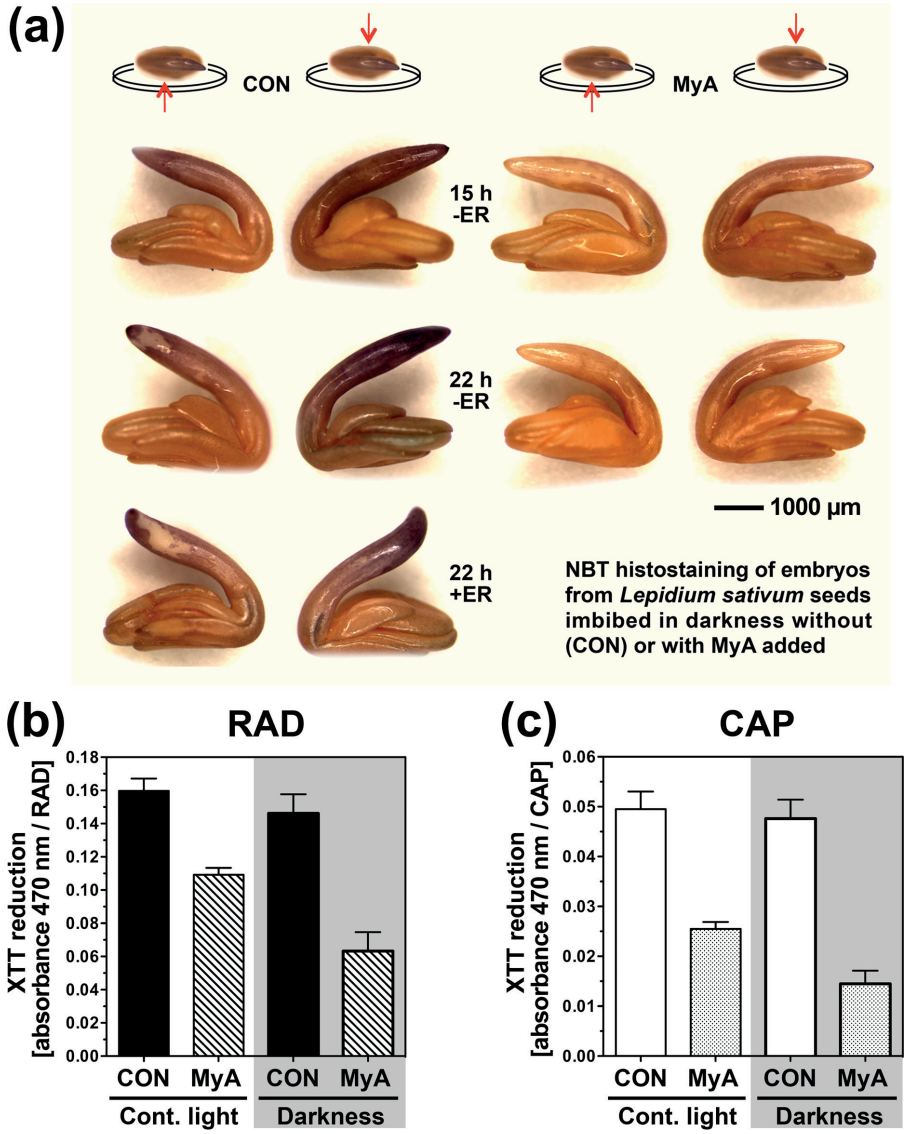

Fig. 7. Accumulation of apoplastic superoxide ($O_2^{\cdot-}$) in *L. sativum* embryos extracted from seeds incubated without (CON) or with 5×10^{-4} M MyA in darkness. (a) *In situ* localization of the superoxide radical $O_2^{\cdot-}$ by NBT histostaining of embryos isolated from CON- and MyA-treated seeds incubated in darkness for 15 h and 22 h. Seeds without endosperm rupture (–ER) are presented for CON and MyA-treated seeds at 15 h and 22 h of incubation and with completed endosperm rupture (+ER) for CON at 22 h. Left images show the lower, and right images show the upper side of the embryos as positioned in the seed during incubation in the Petri dish. Quantification of apoplastic $O_2^{\cdot-}$ production using the XTT assay in (b) RAD (radicle+lower hypocotyl) and (c) CAP (micropylar endosperm) isolated from CON and MyA seeds incubated for 22 h in darkness (this work) and for comparison in continuous light (Oracz *et al.*, 2012). Mean values ±SE of four biological replicates.

that MyA does not alter Ψ_{embryo}, but increases the permeability of the seed envelopes to allow faster water uptake. In agreement with this, it was found that MyA increases testa permeability for tetrazolium salts. Several *A. thaliana* mutants as well as genetic variants of *Brassica* spp. have increased testa permeability for tetrazolium salts linked to altered testa structure and/or pigmentation (Debeaujon and Koornneef, 2000; Debeaujon *et al.*, 2000; Lepiniec *et al.*, 2006). For other Brassicaceae with heterogeneous seed populations (e.g. *Brassica rapa* and *Sisymbrium officinale*), higher water uptake rates and lower dormancy were associated with altered hormone sensitivities in lighter coloured seeds (Matilla *et al.*, 2005; Iglesias-Fernandez *et al.*, 2007). The MyA-mediated increase in the water uptake rate (enhanced water flow-through) and testa tetrazolium salt permeability during *L. sativum* seed imbibition therefore seem to be caused by MyA-induced alterations of the testa properties. Reduced testa pigmentation is associated with enhanced inhibitor leakage during *Sinapis arvensis* imbibition (Duran and Retamal, 1989). In Fabaceae species such as soybean, lighter coloured seeds often imbibe too rapidly and this may cause imbibitional damage

[i.e. higher solute leakage and decreased vigour and viability (Powell *et al.*, 1986; Chachalis and Smith, 2000; Norikazu and Setsuko, 2008)]. Micro-magnetic resonance imaging of soybean seed imbibition demonstrated that the testa regulates water uptake and minimizes imbibitional injury by ensuring optimal reconstitution of cellular structures (Koizumi *et al.*, 2008). Besides soybean seed testa colour, structural aspects, such as a relatively loose adherence of the testa to the embryo, are known to be associated with an enhanced imbibitional water uptake (Norikazu and Setsuko, 2008). Although MyA did not alter the kinetics of *L. sativum* testa rupture, the onset and the $TR_{50\%}$ cardinal points of testa rupture were associated with a smaller embryo size when compared with CON (Fig. 6). This finding is in agreement with a MyA-mediated loose adherence of the testa, increased testa permeability, and, in turn, a weaker testa leading to easier rupture already by a less swollen embryo. Increased testa permeability also allows facilitated uptake of inhibitors and phytotoxins from the ambient medium into the embryo, as is known from the genetic weakening for *A. thaliana* testa mutants (Debeaujon and Koornneef, 2000; Debeaujon *et al.*, 2000) and as can be expected from the biochemical weakening caused by MyA (this work). In agreement with a negative impact of the MyA-induced faster *L. sativum* embryo imbibition due to increased testa permeability, MyA treatment lowered seed viability in darkness (this work) and prevented seedling establishment by acting as a phytotoxin (Popovici *et al.*, 2011; Oracz *et al.*, 2012).

MyA inhibits endosperm cap weakening and this inhibition is more severe in darkness compared with the light

Prior to radicle protrusion and endosperm rupture of *A. thaliana* and *L. sativum*, the embryo RAD elongates due to cell expansion growth (Sliwinska *et al.*, 2009; this work) and the CAP weakens (Müller *et al.*, 2006; Bethke *et al.*, 2007; Linkies *et al.*, 2009; Oracz *et al.*, 2012; this work). An early embryo signal, which can be replaced by GA, initiates endosperm cap weakening which further proceeds in an organ-autonomous manner and is promoted by GA and ethylene, and inhibited by ABA in *L. sativum* (Müller *et al.*, 2006; Linkies *et al.*, 2009; Graeber *et al.*, 2010; Morris *et al.*, 2011). Molecular mechanisms for endosperm cap weakening depend on environmentally and hormonally regulated expression of cell wall remodelling proteins and/or ROS (e.g. Ni and Bradford, 1993; Bewley, 1997; Petruzzelli *et al.*, 2003; Müller *et al.*, 2009; Voegele *et al.*, 2011). *Datura ferox* endosperm weakening is promoted by light and inhibited by darkness combined with low ambient water potential (Sanchez *et al.*, 2002). The germination of *L. sativum* seeds is, however, not inhibited by darkness, but the inhibitory effect of MyA on endosperm rupture was much stronger in darkness compared with continuous light and this was associated with prolonged inhibition of endosperm cap weakening in darkness (Fig. 1b). Light alleviates the inhibitory effects of MyA on endosperm weakening and rupture by a mechanism which does not seem to play a role under CON germination conditions in which light or darkness do not influence germination of *L. sativum*. Altered ABA or GA contents were not found in the CAP or RAD that might explain the more severe MyA inhibition in darkness. Scarification (removal or pricking of the seed envelopes) experiments with *A. thaliana* mutant seeds highlight the permeability and constraint weakening of the testa and/or endosperm being major determinants for adaptation to environmental change (Barua *et al.*, 2011). Based on the mechanistic model of two opposing forces during seed germination (Ni and Bradford, 1993; Linkies and Leubner-Metzger, 2012), environmental modulation of the endosperm weakening inhibition combined with increased testa permeability and decreased embryo growth seems to alter the degree of MyA inhibition of seed germination.

The MyA-mediated inhibition of embryo growth and molecular mechanisms for radicle–hypocotyl cell elongation growth are regulated developmentally and by environmental cues

Cell expansion growth required for radicle protrusion and endosperm rupture depends on environmentally and hormonally regulated cell wall-loosening mechanisms mediated by proteins such as xyloglucan endotransglycosylases/hydrolases (XTHs) and expansins (Cosgrove, 2005; Knox, 2008; Voegele *et al.*, 2011) and/or by apoplastic ROS (Renew *et al.*, 2005; Schopfer, 2006; Müller *et al.*, 2009). Specific zones in the *A. thaliana* and *L. sativum* embryo hypocotyl–radicle axis exhibit endoreduplication-associated cell expansion growth, and in *L. sativum* are also associated with apoplastic ROS production as a molecular mechanism for this cell expansion growth (Müller *et al.*, 2009; Sliwinska *et al.*, 2009; Graeber *et al.*, 2010; Oracz *et al.*, 2012). MyA inhibits endoreduplication and apoplastic ROS production in the RAD of *L. sativum* seeds. In the present study, embryo sizes of *L. sativum* seeds were quantified and it was demonstrated that MyA inhibits embryo growth in terms of size and its rate during late germination (15–22 h). While the embryo sizes at the onset of endosperm rupture were equal between the CON and the MyA series, the embryo size required for $ER_{50\%}$ was 1.4-fold larger for MyA ($ER_{50\%}$ at ~35 h) compared with CON ($ER_{50\%}$ at ~22 h). A larger embryo due to prolonged RAD growth to achieve this response later in time is in agreement with the MyA-mediated inhibition of endosperm cap weakening.

ABA inhibits embryo cell expansion growth and decreases the embryo growth potential of coffee, tomato, and *B. napus* (Schopfer and Plachy, 1985; Toorop *et al.*, 2000; Da Silva *et al.*, 2004, 2008). ABA also inhibits the production of apoplastic ROS in the *L. sativum* RAD, whereas GA and ethylene promote it (Müller *et al.*, 2006; Graeber *et al.*, 2010). Apoplastic ·OH is produced from apoplastic H_2O_2 and $O_2^{\cdot-}$, and plasma membrane-localized NADPH oxidase has been proposed to produce the apoplastic $O_2^{\cdot-}$; and this apoplastic ROS causes cell wall loosening required for cell expansion growth, as has been demonstrated for seed germination, and embryo and seedling growth (Renew *et al.*, 2005; Schopfer, 2006; Müller *et al.*, 2009). MyA was characterized as an effective C-methylated dihydrochalcone radical scavenger (Mathiesen *et al.*, 1995, 1997) and it was demonstrated here that MyA reduced the apoplastic $O_2^{\cdot-}$ production in the *L. sativum* RAD from seeds imbibed in continuous light or darkness between 15 h and 22 h (Oracz *et al.*, 2012; this work). The ~2-fold lower contents of apoplastic $O_2^{\cdot-}$ in the RAD in the

dark compared with the light (Fig. 7) explains, at least in part, why the MyA-mediated inhibition of *L. sativum* seed germination is stronger in darkness. As it was shown that light itself does not chemically inactivate MyA, it is speculated that a light-induced accumulation of an unknown MyA detoxification enzyme may be an explanation for the difference between light and darkness; light-induced detoxification is known in other systems (e.g. Kucera *et al.*, 2003; Barrero *et al.*, 2012). In agreement with a requirement for apoplastic $O_2^{\cdot-}$ production for cell expansion growth, light is known to target NADPH oxidase to the plasma membrane and nucleus of wheat coleoptiles (Schopfer, 2006; Chandrakuntal *et al.*, 2010). It is concluded that MyA, due to its radical-scavenging properties, interferes with the ROS-mediated cell expansion growth required for embryo elongation and seedling growth. Many other allelochemicals, such as sorgoleone and (–)-catechin, inhibit seedling establishment in the ecosystem by causing enhanced ROS production and cell death (Inderjit and Duke, 2003; Weir *et al.*, 2004; Oracz *et al.*, 2007). They therefore act phytotoxically by increasing ROS production, whereas MyA acts phytotoxically by decreasing ROS production in germinating seeds and seedlings.

MyA as a soil seed bank-destroying allelochemical: a speculative ecophysiological working model based on its multiple targets and mechanisms

Ambient temperature and light conditions together with the soil water status are the key abiotic environmental factors that determine seasonal germination pattern and modulate persistence and dormancy of soil seed banks (Walck *et al.*, 2005; Batlla and Benech-Arnold, 2010; Footitt *et al.*, 2011). Light intensity and spectral composition vary for seeds located at different depths in the soil bank and for germinated seeds and growing seedlings depending on shading by their plant neighbours. The soil water status affects the persistence and dormancy of soil seed banks as the fluctuations in the ambient water potential affect the seed hydration levels causing altered hormone and light sensitivities (e.g. Batlla and Benech-Arnold, 2006, 2010; Footitt *et al.*, 2011). Flooding, for example, can cause seed and seedling death of unadapted species, thus opening gaps in the vegetation and facilitating the establishment of flooding-tolerant species (Benech-Arnold *et al.*, 2000). It was found that the phytotoxic effects of the putative allelochemical MyA of *M. gale* are achieved by targeting several key steps of seed germination and seedling establishment, and that these effects are modulated in a complex manner by the ambient light conditions and water potentials.

Myrica gale is found in environments such as the wet and flooded areas around lakes, along rivers, or in peat bogs, and has a wide distribution in Northern and Western Europe and on the American continent (Skene *et al.*, 2000). It is a shrub, scattered with many plants clustering close together at a growing site, and is described as 'light loving' and as a wet site indicator. The fruits of *M. gale* have fleshy bracts that aid the dispersal of seeds by flotation, keeping them afloat when they fall into the water of their often flooded habitat. Germination of *M. gale* requires cold stratification and extended exposure to light, which has been suggested to provide a mechanism to ensure that germination only occurs when seeds are located on relatively stable substrate following transport by water. *Myrica gale* and *Myrica rubra* dormancy can be released by GA, and for *M. rubra* it was demonstrated that the endogenous GA and ABA contents in the endocarp and seed coat are regulating coat dormancy and germination (Schwintzer and Ostrofsky, 1989; Chen *et al.*, 2008). *Myrica gale* fruit exudates are known to contain MyA as the major C-methylated dihydrochalcone and putative allelochemical that inhibits seed germination and seedling growth (Svoboda *et al.*, 1998; Popovici *et al.*, 2011; Oracz *et al.*, 2012). That MyA is indeed an allelochemical of *M. gale* in its habitat has so far not been proven by ecosystem competition experiments, but such experiments have been conducted for three other (invasive) *Myrica* species (Vitousek *et al.*, 1987; Walker and Vitousek, 1991). Their leaf litter inhibits target species germination by the release of unknown allelochemicals, and for *M. cerifera* and *M. pensylvanica* this inhibition is pronounced under low light conditions (Collins and Quinn, 1982; Tolliver *et al.*, 1995; Shumway, 2000). The complex interaction of shading and allelopathy is in agreement with the finding that darkness and low light conditions cause a stronger MyA-mediated inhibition of seed germination and seedling growth (Popovici *et al.*, 2011; this work). It is speculated that MyA is a soil seed bank-destroying allelochemical that secures the persistence of *M. gale* in its flood-prone environment.

The speculative ecophysiological working model is based on the finding that MyA acts via multiple inhibitory mechanisms that have important key processes of seed germination and seedling establishment of competitor species as targets. (i) During early germination, MyA enhances testa permeability and thereby facilitates too rapid water uptake during embryo imbibition. This may cause imbibitional injury and untimely loss of embryo desiccation tolerance of competitor species, and may also cause enhanced uptake of phytotoxins like MyA itself, as well as leakage of metabolites including ABA. With their protection mechanisms deactivated by MyA, seeds of these competitor species may be easily prone to decay processes. (iii) During late germination, MyA inhibits endosperm weakening and interferes with embryo growth; and the finding that this is more pronounced in darkness is in agreement with the proposal that MyA is an allelochemical that acts under low light conditions. Seeds of competitor species of the soil bank buried next to *M. gale* plants or fruits would germinate, but, due to the enhanced phytotoxicity of MyA in darkness, would have reduced viability leading to slow growth and fast decay. (iii) During late germination and subsequent seedling growth, MyA acts as a scavenger of apoplastic ROS required for cell wall loosening and cell expansion growth. MyA thereby targets seed germination and seedling growth mechanisms that are important for a wide range of species.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. The effect of myrigalone A (MyA) on the germination and viability of *Lepidium sativum* seeds imbibed in darkness.

Figure S2. The effect of myrigalone A (MyA) on embryo water uptake by imbibition during the early phase of *L. sativum* seeds.

Figure S3. The effect of MyA and the ambient water potential (Ψ_{medium}) on embryo imbibition, embryo size, and endosperm rupture during the germination of *L. sativum* seeds.

Figure S4. The tissue-specific effect of MyA on gibberellin (GA) and abscisic acid (ABA) metabolism during the germination of *L. sativum* seeds incubated in continuous light or darkness without (CON) or with 5×10^{-4} M MyA added.

Table S1. Contents of active and inactive gibberellins (GAs) and abscisic acid (ABA) in RAD and CAP isolated from *L. sativum* seeds.

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