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Myrigalone A Inhibits *Lepidium sativum* Seed Germination by Interference with Gibberellin Metabolism and Apoplastic Superoxide Production Required for Embryo Extension Growth and Endosperm Rupture

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***Myrica gale* L. (sweet gale) fruit leachate contains myrigalone A (MyA), a rare C-methylated dihydrochalcone and putative allelochemical, which is known to be a phytotoxin impeding seedling growth. We found that MyA inhibited *Lepidium sativum* L. seed germination in a dose-dependent manner. MyA did not affect testa rupture, but inhibited endosperm rupture and the transition to subsequent seedling growth. MyA inhibited micropylar endosperm cap (CAP) weakening and the increase in the growth potential of the radical/hypocotyl region (RAD) of the embryo, both being key processes required for endosperm rupture. We compared the contents of abscisic acid (ABA) and gibberellins in the tissues and found that the major bioactive forms of gibberellin in *L. sativum* seed tissues were GA_4 and GA_6, while GA_8 and GA_{13} were abundant inactive metabolites. MyA did not appreciably affect the ABA contents, but severely interfered with gibberellin metabolism and signaling by inhibiting important steps catalyzed by GA3 oxidase, as well as by interfering with the GID1-type gibberellin signaling pathway. The hormonally and developmentally regulated formation of apoplastic superoxide radicals is important for embryo growth. Specific zones within the RAD were associated with accumulation of apoplastic superoxide radicals and endoreduplication indicative of embryo cell extension. MyA negatively affected both of these processes and acted as a scavenger of apoplastic reactive oxygen species. We propose that MyA is an allelochemical with a novel mode of action on seed germination.**

Keywords: Embryo cell extension growth • Endoreduplication • Endosperm rupture • Gibberellin metabolism • *Lepidium sativum* • *Myrica gale* • Phytotoxicity • Reactive oxygen species.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; CAP, micropylar endosperm cap; CON, control; FLU, fluridone; GA3ox, GA3 oxidase; GID1, GIBBERLLIN INSENSITIVE DWARF 1; HPPD, *p*-hydroxyphenylpyruvate dioxygenase; MRM, multiple-reaction monitoring; MyA, myrigalone A; NBT, nitroblue tetrazolium; $O_2^{\cdot-}$, superoxide radical; qRT–PCR, quantitative reverse transcription–PCR; RAD, lower radicle/hypocotyl axis; ROS, reactive oxygen species; XTT, 3′-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis[4-methoxy-6-nitro] benzenesulfonic acid hydrate.

Introduction

Compounds of natural origin, such as phytotoxins, produced by plants hold great prospects for finding alternative strategies for weed management including the use of plant secondary metabolites as natural herbicides to promote sustainable agriculture (Weston and Duke 2003). Due to their origin from natural sources, these phytotoxic compounds usually are biodegradable and considered as more 'environmentallly friendly' compared with many traditional herbicides. Several of the plant-derived phytotoxins seem to be allelochemicals that affect germination and growth of surrounding 'target' plants through leaching into the rhizosphere (Inderjit and Duke 2003, Weir et al. 2004, Blair et al. 2008). Examples of such 'donor' plants with allelopathic phytotoxic potential on surrounding 'target' plants include not only the native species *Juglans nigra*

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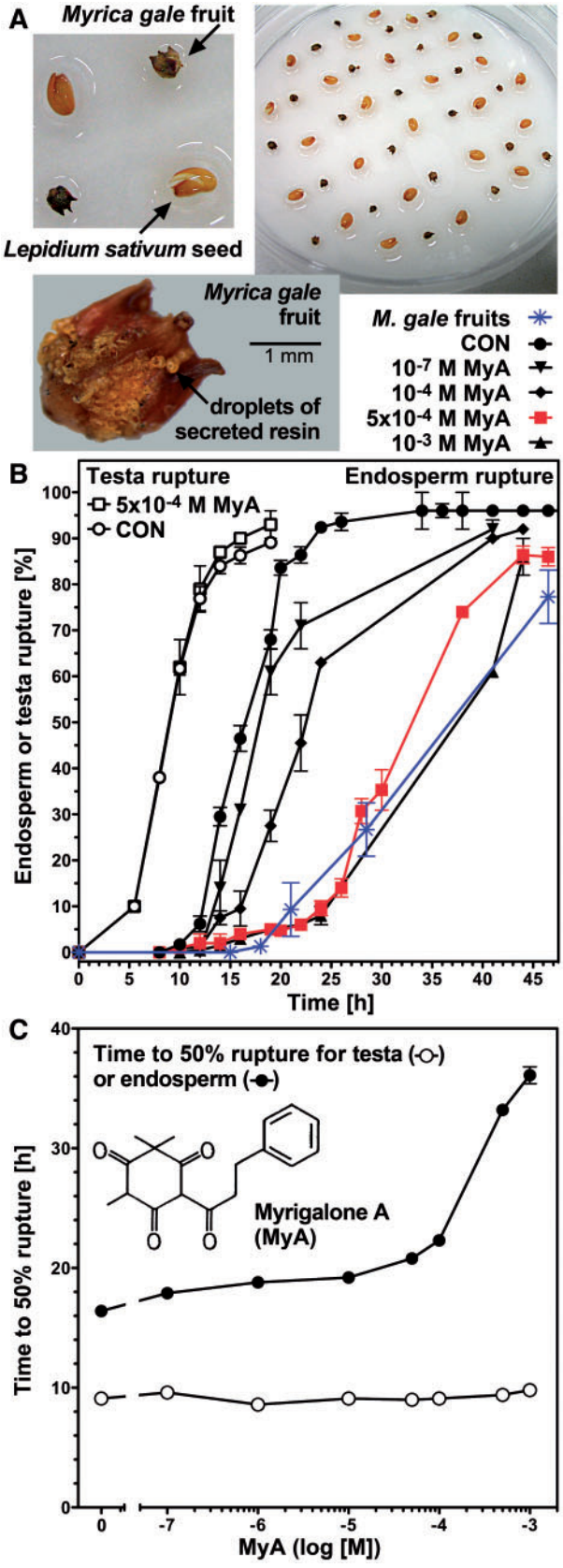

Fig. 1 The effect of *Myrica gale* fruit leachate and myrigalone A (MyA) on germination of *Lepidium sativum* seeds incubated in continuous white light. (A) *Lepidium sativum* seeds germinating with *M. gale* fruits in the light. *Myrica gale* fruit was covered with droplets of secreted resin containing essential oils. (B) The effect of different concentrations of MyA (as indicated above the graph) on the kinetics of testa and endosperm rupture of germinating *L. sativum* seeds in the light, and (C) on the time to obtain $TR_{50\%}$ and $ER_{50\%}$ of seed populations (dose–response curve). Mean values ± SE of ≥3 plates with 50 *L. sativum* seeds or 25 *L. sativum* seeds and 25 *M. gale* fruits for the co-incubation experiment.

(Weston and Duke 2003) and invasive species such as *Centaurea maculosa* (Bais et al. 2003), *Myrica faya* (Vitousek et al. 1987) or the knotweed *Fallopia*×*bohemica* (Murrell et al. 2011), but also crop plants such as *Sorghum bicolor* (Weir et al. 2004), *Oryza sativa* (Weston and Duke 2003) and *Helianthus annuus* (Oracz et al. 2007a). Fruit and leaf exudates of *Myrica gale* ('sweet gale' or 'bog myrtle', Myricaceae) exhibit phytotoxic activity on seedling growth of *Fallopia*×*bohemica* and other species (Popovici et al. 2011). *Myrica gale* is a shrub 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). The fruits (**Fig. 1A**) and leaves of *M. gale* are covered with droplets of secreted resin containing essential oils in which the major terpenes have been identified as α-pinene, 1,8-cineole, germacrone, *p*-cymene, α-phellandrene and limonene (Svoboda et al. 1998, Popovici et al. 2008). *Myrica gale* fruit exudates are also known to contain rare flavonoids, with myrigalone A (MyA) as the major C-methylated dihydrochalcone (Anthonsen et al. 1971, Mathiesen et al. 1995, Popovici et al. 2010). Several biological activities, including antioxidant and radical-scavenging properties, have been reported for these C-methylated dihydrochalcones (Mathiesen et al. 1995, Malterud et al. 1996, Mathiesen et al. 1996, Mathiesen et al. 1997).

Seedling growth and seed germination assays of *Lepidium sativum* ('garden cress', Brassicaceae) as 'target species' provide a classical test system in phytotoxicity and allelopathy research (e.g. Hoekstra et al. 2002, Terzi and Kocacaliskan 2009, De Martino et al. 2010). Seed germination is completed by visible radicle protrusion through all covering layers, and is followed by seedling establishment. The mature seeds of most angiosperms, including the Brassicaceae *L. sativum*, *Arabidopsis thaliana* and *Sisymbrium officinale*, consist of the embryo surrounded by the testa (seed coat) and the triploid endosperm as distinct covering layers (Holdsworth et al. 2008, Iglesias-Fernandez and Matilla 2010, Linkies et al. 2010). Rupture of the testa and the endosperm are two visible events during the process of germination of many seeds, including these endospermic Brassicaceae species. The completion of germination by endosperm rupture and radicle protrusion depends on the interaction between the weakening of the micropylar endosperm cap (CAP) surrounding the radicle and the increase in the embryo growth potential (Nonogaki 2006, Holdsworth et al. 2008, Linkies et al. 2009). Embryo cells elongate prior to radicle protrusion, which indicates the completion of germination. However, cell division is

not evident during this process in *A. thaliana* and other species (Sliwinska et al. 2009, Weitbrecht et al. 2011). Sliwinska et al. (2009) demonstrated that endoreduplication is associated with cell extension within the lower hypocotyl and the hypocotyl/radicle transition zone of the *A. thaliana* embryo. An increase of embryo growth potential followed by cell extension is promoted by gibberellins and inhibited by ABA (Nonogaki 2006, Da Silva et al. 2008, Holdsworth et al. 2008). Studies on *A. thaliana* seeds demonstrated that the late steps of gibberellin biosynthesis, catalyzed by GA3 oxidases (GA3oxs) which convert inactive GA_9 into bioactive GA_4, localize to the cortex and endodermis of the hypocotyl (Yamaguchi et al. 2001, Ogawa et al. 2003). Expression of gibberellin-responsive genes is, however, not restricted to these sites of the synthesis of bioactive gibberellin, and therefore movement of gibberellin itself or gibberellin-derived signals across tissues seems to be important for seed germination. An early embryo signal is required to induce CAP weakening of *L. sativum* seeds, which is an organ-autonomous process after its induction. Treatment of the CAP with gibberellin can replace the embryo signal (Müller et al. 2006, Morris et al. 2011). The direct biomechanical measurement of the weakening of the CAP of germinating *L. sativum* seeds showed that this process is promoted by gibberellin and ethylene, and inhibited by ABA (Müller et al. 2006, Linkies et al. 2009). While ABA contents decrease upon imbibition of non-dormant seeds, the synthesis of bioactive gibberellin increases and gibberellin signaling via the soluble GID1-type gibberellin receptors mediates downstream processes that confer embryo extension and endosperm weakening (e.g. Holdsworth et al. 2008, Yamaguchi 2008, Seo et al. 2009, Voegele et al. 2011).

Plant cell extension is driven by water uptake and restricted by the cell wall (collectively called the apoplast). Cell wall loosening, which affects cell extension and tissue weakening, is an important process in all stages of plant development (Schopfer 2006), including endosperm weakening and embryo growth required for the endosperm rupture. Cell wall-remodeling proteins (Leubner-Metzger 2002, Holdsworth et al. 2008, Weitbrecht et al. 2011) and apoplastic reactive oxygen species (ROS) play important roles in cell wall loosening and cell extension (Fry et al. 2001, Renew et al. 2005, Schopfer 2006, Müller et al. 2009b). ROS comprise very short-lived molecules such as superoxide ($O_2^{\cdot-}$) and hydroxyl radicals (·OH) that can only act close to the site where they are produced, as well as hydrogen peroxide (H_2O_2) that can easily diffuse between cells and tissues. A direct mechanism of apoplastic ·OH is scission of cell wall polysaccharides essential for the cell wall loosening and cell extension during seed germination and seedling growth. The apoplastic ·OH is produced from apoplastic H_2O_2 and $O_2^{\cdot-}$. Plasma membrane-localized NADPH oxidase has been proposed to produce the apoplastic $O_2^{\cdot-}$ (Schopfer et al. 2001). Müller et al. (2009b) used the ^3H fingerprinting technique and provided direct evidence for in vivo cell wall polysaccharide attack by apoplastic ·OH in RAD (the lower one-third of the radicle/hypocotyl axis) and CAP tissues of germinating *L. sativum* seeds. An important conclusion from this study is that in vivo production of apoplastic $O_2^{\cdot-}$ and ·OH is developmentally and hormonally regulated. The accumulation of ROS in RAD and CAP tissues is inhibited by ABA, which also inhibits endosperm rupture. Furthermore, gibberellin and ACC (1-aminocyclopropane-1-carboxylic acid, the direct biosynthetic precursor of ethylene) counteract the ABA inhibition on the apoplastic ·OH production in the RAD (Graeber et al. 2010), which is in agreement with their roles as ABA antagonists in terms of seed germination control. In this study, we demonstrate that MyA affects *L. sativum* seed germination by interfering with gibberellin metabolism and signaling, as well as apoplastic $O_2^{\cdot-}$ production required for embryo growth and endosperm rupture.

Results

Myrica gale fruit exudates and MyA inhibit endosperm rupture of germinating *L. sativum* seeds in a dose-dependent manner

Fig. 1 shows that when *M. gale* fruits were used in a classical 'donor–target' assay (Blair et al. 2008) they leached compounds that inhibited the germination of *L. sativum* seeds. Visible radicle protrusion through all seed covering layers (**Fig. 2A, B**) was considered as the completion of germination and was scored. While the time to reach 50% endosperm rupture ($ER_{50\%}$) was 16.4 h for the control (CON), co-imbibition with *M. gale* fruits delayed the $ER_{50\%}$ time to 37.6 h (**Fig. 1B**, **Table 1**). *Myrica gale* fruits are covered with droplets of secreted resin (**Fig. 1A**) of essential oils which include the major terpenes. *Myrica gale* fruit exudates contain MyA as the major C-methylated dihydrochalcone (Mathiesen et al. 1995, Svoboda et al. 1998, Popovici et al. 2008). We therefore tested if these terpenes and/or MyA can account for the inhibitory effect of the *M. gale* fruit leachate on *L. sativum* seed germination.

The addition of MyA delayed endosperm rupture of *L. sativum* seeds, but did not affect testa rupture (**Fig. 1B**). The inhibitory effect was dose dependent, with 5×10^{-4} M MyA as an optimal concentration close to a saturating response ($ER_{50\%}$ time: 33.2 h), and 10^{-3} M MyA causing the same delay of approximately 20 h as the *M. gale* fruit leachate (**Fig. 1**, **Table 1**). In contrast to MyA, none of the terpenes tested caused a delay in endosperm rupture comparable with that caused by MyA (**Supplementary Fig. S1**, **Table 1**). 1,8-Cineol alone or all terpenes combined caused a small delay of approximately 4 h and, when combined with MyA, the inhibitory effects were additive. This result also suggests that the targets of terpenes and MyA are distinct. MyA shares structural features with the commercial herbicide sulcotrione, known to act by inhibiting HPPD (*p*-hydroxyphenylpyruvate dioxygenase), which is important for tocopherol biosynthesis (Dayan et al. 2009). In contrast to MyA, sulcotrione did not inhibit *L. sativum* seed germination (**Supplementary Fig. S1**, **Table 1**), and

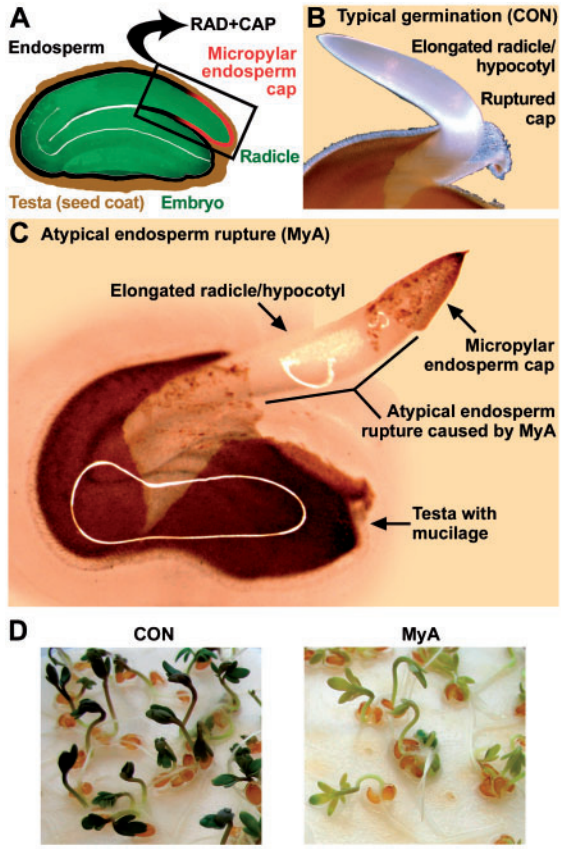

Fig. 2 The effect of myrigalone A (MyA) on seeds and seedlings of *Lepidium sativum* incubated in continuous white light. (A) Seed structure of *L. sativum*. The RAD (lower one-third of the radicle/hypocotyl axis) and CAP (micropylar endosperm) tissues are indicated. (B) Typical endosperm rupture and radicle protrusion of a germinated *L. sativum* seed (CON, control). (C) Atypical endosperm rupture of a MyA-treated seed. (D) *Lepidium sativum* seedlings grown from seeds without (CON) and with treatment with 5×10^{-4} M MyA (with bleached cotyledons).

therefore HPPD is not the molecular target of MyA in germinating seeds. In addition to seed germination, MyA also delayed greening of seedlings (**Fig. 2D**). Based on our results, MyA appears to be the major compound of *M. gale* fruit leachate that inhibits endosperm rupture of *L. sativum* seeds.

MyA synergistically enhances the inhibitory effect of ABA on endosperm weakening and rupture, and counteracts the gibberellin- and ethylene-mediated promotion of *L. sativum* seed germination

Endosperm rupture of *L. sativum* is preceded by CAP weakening, a process that is inhibited by ABA, and promoted by gibberellin and ethylene/ACC (Müller et al. 2006, Linkies et al. 2009). Typical endosperm rupture is achieved by protrusion of the radicle through the weakened CAP (**Fig. 2B**). MyA not only delayed endosperm rupture, but also led to atypical endosperm rupture in about 60% of the seeds in which the CAP was torn off at its base instead of being penetrated by the radicle (**Fig. 2C**). Measurements of the tissue resistance (puncture force) of the CAP showed that CAP weakening was inhibited in MyA-treated seeds (**Fig. 3B**). In both ABA- and MyA-treated seeds, the CAP puncture force values were not decreased at 15 h, while it was about half in CON seeds at that time ($ER_{50\%}$ for CON). At the $ER_{50\%}$ time for MyA-treated seeds the CAP puncture force was considerably decreased but still significantly higher compared with CON, and a high frequency of atypical endosperm rupture was observed. Treatment of seeds with 'MyA + ABA' caused a synergistic inhibitory effect on endosperm weakening and rupture (**Fig. 3A, B**). The ABA biosynthesis inhibitor fluridone (FLU) did not cause reversion of the inhibitory effect of MyA (**Fig. 3A**), suggesting that MyA does not exert its effect through enhanced ABA biosynthesis. Co-application of ACC or gibberellin with MyA did not result in complete reversion of the MyA-mediated delay of endosperm rupture, while they caused partial reversion (**Supplementary Fig. S2, Table 1**). Taken together, these results suggests that MyA acts, at least in part, by interfering with processes that may be regulated by gibberellin and ethylene metabolism and/or signaling.

MyA interferes with the expression of ABA- and gibberellin-related genes during *L. sativum* seed germination and alters ABA and gibberellin metabolism in a tissue-specific manner

To determine transcript abundances by quantitative RT–PCR (qRT–PCR), we used superior cross-species reference genes for Brassicaceae seed germination (Graeber et al. 2011); and to determine ABA and gibberellin metabolites we used optimized methods that allow their quantification in minute amounts of specific seed tissues (Hradecká et al. 2007, Turečková et al. 2009, Urbanová et al. 2011) as described in detail in the Materials and Methods. To obtain tissue-specific profiles of hormone contents during *L. sativum* germination, we excised the CAP and RAD from seeds with an unruptured CAP at 15 h ($ER_{50\%}$ time for CON seeds) imbibed without (CON) or with 5×10^{-4} M MyA. **Supplementary Fig. S3** shows that the CAP has higher ABA contents compared with the RAD. MyA caused an approximately 1.5- and 2-fold increase of the ABA contents in the RAD and CAP, respectively. However, these changes in the ABA contents are relatively small and do not fully explain the inhibitory action of MyA. This is also in agreement with the synergistic action of MyA and ABA, as well as with the inability of FLU to revert the MyA response (**Fig. 3**). Taken together, ABA metabolism does not appear to be a major target of MyA.

Fig. 4 shows that gibberellin metabolism is one of the targets of MyA; only the most important changes are presented in the figure (**Fig. 4B–F**), and all detailed data are compiled in **Supplementary Table S1**. We detected mainly GA_4 and GA_6, approximately 10-fold less GA_1, and traces of GA_3 as bioactive

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Table 1 The effect of *Myrica gale* fruit leachate, myrigalone A (MyA), phytohormones (ABA, ACC and gibberellin) and an inhibitor of ABA synthesis (FLU, fluridone) on the time required to obtain 50% of testa rupture ($TR_{50\%}$) and endosperm rupture ($ER_{50\%}$) during germination of *Lepidium sativum* seeds incubated in continuous white light

	Time to 50% rupture (h)[a]	
	Testa	Endosperm
Experiment 1		
CON[b]	9.1 ± 0.1	16.4 ± 0.2
Myrica gale fruits[c]	11.7 ± 0.3	37.4 ± 1.1
10^{-7} M MyA	9.6 ± 0.5	17.9 ± 0.4
10^{-6} M MyA	8.6 ± 0.1	18.8 ± 0.3
10^{-5} M MyA	9.1 ± 0.0	20.8 ± 0.3
5×10^{-5} M MyA	9.0 ± 0.1	19.2 ± 0.1
10^{-4} M MyA	9.1 ± 0.2	22.3 ± 0.4
5×10^{-4} M MyA	9.4 ± 0.5	33.2 ± 0.5
10^{-3} M MyA	9.8 ± 0.2	36.1 ± 0.7
1 mM ACC[b]	8.9 ± 0.2	15.4 ± 0.1
ACC + MyA	9.2 ± 0.0	27.1 ± 0.6
10 μM Gibberellin[b]	9.0 ± 0.2	14.9 ± 0.1
Gibberellin + MyA	9.0 ± 0.3	29.8 ± 0.2
10 μM Fluridone	8.9 ± 0.2	16.4 ± 0.1
Fluridone + MyA	9.3 ± 0.3	35.5 ± 0.4
3 μM ABA[b]	9.3 ± 0.1	77.9 ± 7.6
ABA + MyA	9.8 ± 0.2	288.0 ± 25.0
1 mM Sulcotrione	~9–10	16.4 ± 0.3
Experiment 2		
CON[b]	9.7 ± 0.4	17.2 ± 0.3
1 mM 1,8-Cineole	10.8 ± 0.2	21.2 ± 1.5
1 mM 1,4-Cineole	10.1 ± 0.2	23.9 ± 0.6
1 mM α-Phellandrene	~9–10	19.3 ± 0.1
1 mM α-Pinene	~9–10	19.8 ± 0.1
1 mM Limonene	~9–10	19.1 ± 0.0
1 mM *p*-Cymene	~9–10	20.4 ± 0.2
1 mM Terpenes[d]	~9–10	22.0 ± 0.3
5×10^{-4} MyA	~9–10	29.1 ± 1.1
MyA + 1 mM 1,8-cineole	~9–10	34.7 ± 3.7
MyA + Terpenes	~9–10	34.1 ± 2.1

[a] The percentages of testa and endosperm rupture were scored over time in populations of 50 seeds with 3–6 plates. Mean ± SE for the times to reach 50% rupture were obtained. For the germination kinetics see **Figs. 1**, **3**, and **Supplementary Figs. S1**, **S2**.

[b] CON (control), 0.35% (v/v) methanol or 0.5% (v/v) dimethylsulfoxide (DMSO; for terpenes); both additions did not appreciably affect the kinetics of seed germination; ACC, 1-aminocyclopropane-1-carboxylic acid; gibberellin, GA_{4+7}; MyA, 5×10^{-5} M myrigalone A if no other concentration is indicated.

[c] Twenty-five *M. gale* fruits and 25 *L. sativum* seeds were co-incubated (**Fig. 1A**).

[d] Terpenes = 1 mM of each of the terpenes also used in single treatments. Terpenes were dissolved as higher concentrated stocks in DMSO or methanol; the final concentration of the solvents was 0.35% (v/v) methanol or 0.5% (v/v) DMSO.

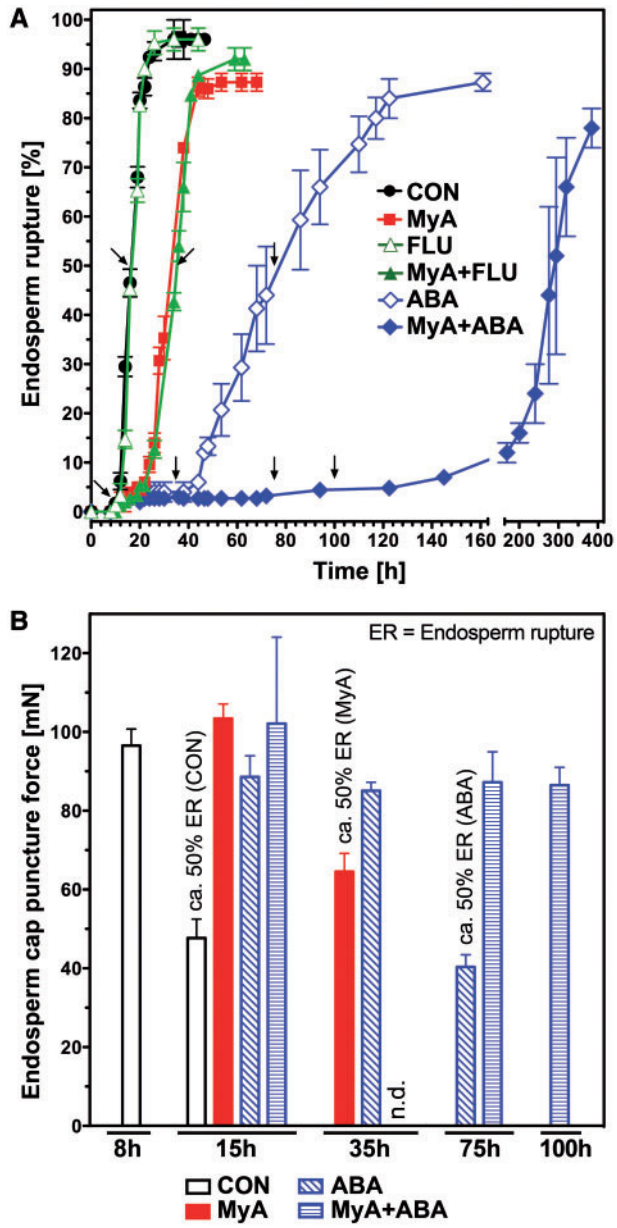

Fig. 3 The effect of myrigalone A (MyA) and ABA on the endosperm weakening and rupture of germinating *Lepidium sativum* seeds incubated in continuous white light. (A) The effect of MyA, ABA and FLU (fluridone, an inhibitor of ABA synthesis) on the endosperm rupture of *L. sativum* seeds. (B) The effect of MyA and ABA on the endosperm cap weakening quantified by puncture force measurements at different times of incubation. Mean values ± SE of ≥3 plates with 50 *L. sativum* seeds; Puncture force: mean ± SE of 10–50 seeds.

gibberellins in the RAD and CAP (**Fig. 4**, **Supplementary Table S1**). Therefore, the 13-non-hydroxylated and the 13-hydroxylated gibberellin biosynthesis pathways (Pimenta Lange and Lange 2006, Yamaguchi 2008) are active in germinating *L. sativum* seeds (**Fig. 4A**). The 13-non-hydroxylated pathway leads to synthesis of bioactive GA_4 in CON seeds for which the contents in the RAD are about 2-fold higher compared with

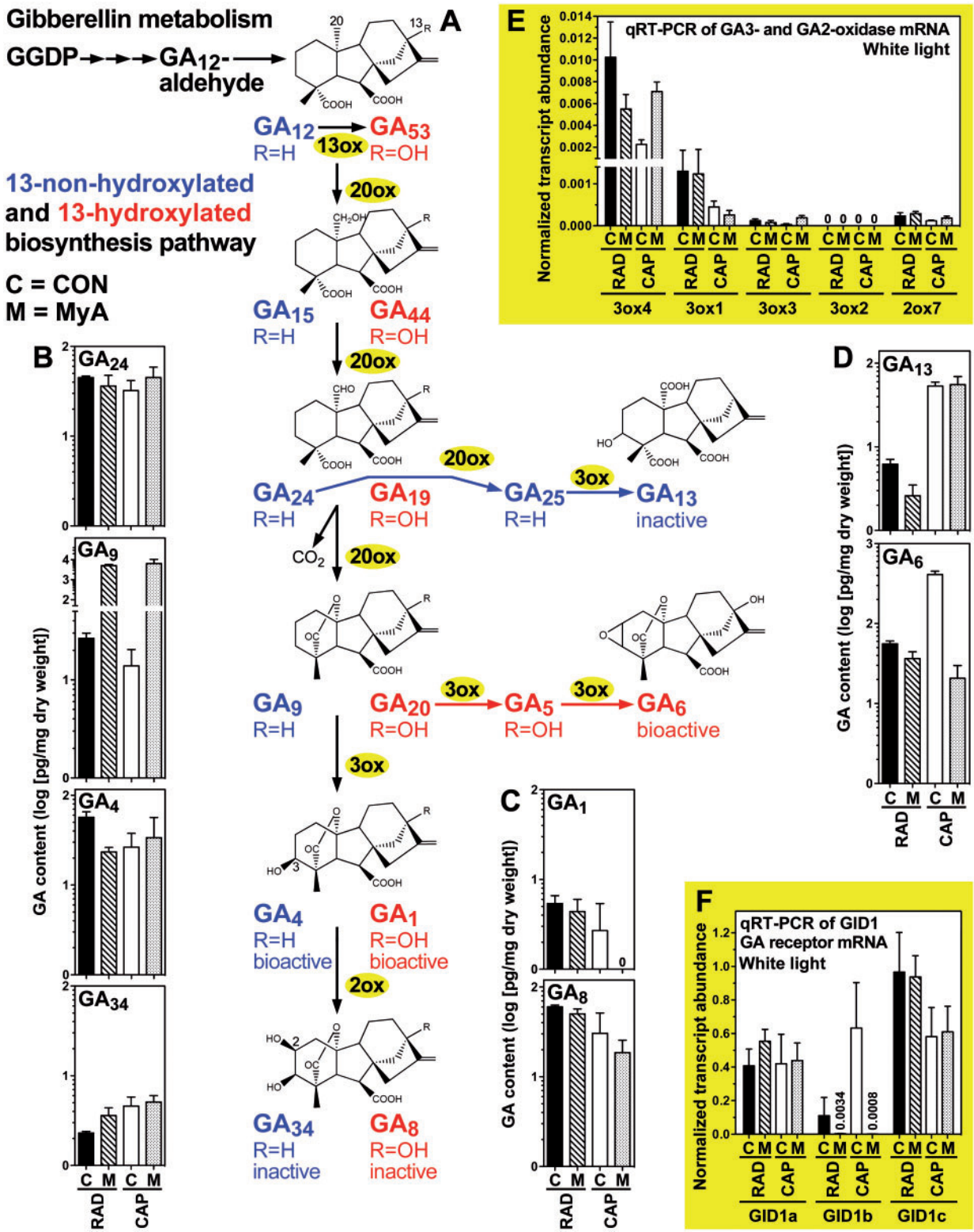

Fig. 4 The tissue-specific (RAD, CAP) effect of myrigalone A (MyA) on gibberellin metabolism and gibberellin-related transcript abundance during germination of *Lepidium sativum* seeds incubated in continuous white light. (A) The 13-non-hydroxylated (in blue) and 13-hydroxylated (in red) gibberellin biosynthesis and inactivation pathways and important metabolites detected in *L. sativum* seeds. (B–D) Contents of bioactive gibberellin such as GA_1, GA_4 and GA_6, and inactive forms such as GA_{24}, GA_9, GA_{34}, GA_8 and GA_{13} quantified in RADs and CAPs excised from control (CON) and MyA-treated seeds incubated for 15 h. (E) Normalized transcript abundance quantified by qRT–PCR in the RAD and CAP of CON and MyA-treated seeds incubated for 15 h for *GA3 oxidase* (*GA3ox1*, *GA3ox2*, *GA3ox3* and *GA3ox4*) and *GA2 oxidase* (*GA2ox7*) genes, whose products catalyze activation and inactivation steps of bioactive gibberellin as indicated in A. (F) Normalized transcript abundance for gibberellin receptors of the *GID1ac* (*LesaGID1a* and *LesaGID1c*) and *GID1b* (*LesaGID1b*) groups. Mean values ± SE of four biological replicates.

the CAP. MyA caused an approximately 3-fold decrease in the GA_4 contents in the RAD, but did not appreciably affect the GA_4 contents in the CAP. The most striking effect of MyA was, however, the accumulation of GA_9, the direct biosynthetic precursor of GA_4. MyA treatment resulted in an approximately 200- and 450-fold accumulation of GA_9 in the RAD and CAP, respectively (**Fig. 4B**, **Supplementary Table S1**). GA_9 accumulation to this extent can be explained by a blockage of the GA3ox-mediated conversion of GA_9 to GA_4, as well as a blockage or absence of other metabolic pathways that use GA_9 as a substrate, e.g. the 13-hydroxylation to GA_{20}. Four GA3ox genes are known in *A. thaliana*, and transcripts of the putative orthologs were detected at 15 h in *L. sativum* seed tissues with different abundances (**Fig. 4E**): *GA3ox4* > *GA3ox1* > *GA3ox3* (*GA3ox2* transcripts were not detected at 15 h, but were detected by our qRT–PCR assay in dry seeds). MyA caused an approximately 2-fold decrease in the transcript abundance of *GA3ox4* in the RAD and of *GA3ox1* in the CAP, and an approximately 3-fold increase of *GA3ox4* transcript in the CAP. The dramatic accumulation of GA_9 in all tissues and conditions must be due to MyA-mediated inhibition of the GA3ox reaction, which includes down-regulation of *GA3ox4* expression in the RAD.

The 13-hydroxylated pathway in *L. sativum* seeds leads mainly to the synthesis of bioactive GA_6 which contains a stabilizing 2,3-epoxide group, while the GA_1 contents are approximately 10-fold lower (**Fig. 4C, D**). GA_6 appears to be important in the endosperm as its contents in the CAP compared with the RAD were 7.4-fold higher. In the CAP tissue MyA caused a 20-fold decrease of the GA_6 contents, while its decrease in the RAD was only 1.5-fold. In *L. sativum* seeds GA_6 production and GA_1 inactivation by GA2 oxidase (GA2ox, GA_8 production) seem to be major reasons for the low GA_1 contents derived from the 13-hydroxylation pathway. In contrast, GA_4 appears to be less prone to GA2 oxidation (GA_{34} production, **Fig. 4B**) in the 13-non-hydroxylation pathway. We did not find evidence that MyA affects GA_2 oxidation or *GA2ox7* transcript abundance (**Fig. 4E**). In addition to GA_9 accumulation and GA_4 production, the 13-non-hydroxylated pathway in *L. sativum* seeds also leads to the production of inactive GA_{13} (**Fig. 4D**). GA_{13} contents were 9-fold higher in the CAP compared with the RAD. MyA treatment in the light caused an approximately 2.4-fold decrease of GA_{13} contents in the RAD, but did not affect the CAP. Taken together, the CAP of CON seed contains higher contents of GA_6 (bioactive) and GA_{13} (inactive), but similar contents of GA_4 (bioactive) compared with the RAD.

The two distinct, GID1-type gibberellin receptor-mediated signaling pathways, the GID1ac (LesaGID1a and LesaGID1c receptors) and GID1b (LesaGID1b receptor) pathways, were proposed to operate during *L. sativum* seed germination (Voegele et al. 2011). Interestingly, while MyA did not affect the transcript abundance of the GID1ac-type receptors, it caused a severe down-regulation of the *LesaGID1b* transcript accumulation in the RAD and CAP (**Fig. 4F**). We conclude that interference with gibberellin metabolism and signaling is a major mechanism by which MyA inhibits the downstream processes that lead to *L. sativum* endosperm rupture.

MyA inhibits hormonally regulated apoplastic superoxide accumulation in the embryo essential for cell extension in distinct elongation zones

Endosperm rupture of *L. sativum* depends on the embryo cell extension by apoplastic ROS, namely ·OH formed from $O_2^{\cdot-}$; and it is known that apoplastic ·OH accumulation in the embryo is inhibited by ABA and promoted by gibberellin and ethylene/ACC (Müller et al. 2009b, Graeber et al. 2010). To investigate if MyA affects the accumulation of apoplastic $O_2^{\cdot-}$, we used a combination of biochemical quantification {XTT [3′-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis[4-methoxy-6-nitro] benzenesulfonic acid hydrate] assay} and histochemical detection [NBT (nitroblue tetrazolium) staining] of the embryos, as well as the RAD and CAP from *L. sativum* seeds (**Fig. 5**). **Fig. 5A** shows NBT staining of an embryo just after endosperm rupture, in which $O_2^{\cdot-}$ accumulated preferentially in the cells in the upper side of the RAD region, i.e. the cells that undergo extension due to the gravitropic response of the RAD. Quantification by the XTT assay (**Fig. 5B**) showed that MyA treatment decreased the $O_2^{\cdot-}$ contents in the RAD and CAP excised from imbibed CON seeds just prior to endosperm rupture (22 h) approximately 1.5- and 2-fold, respectively. These results of biochemical $O_2^{\cdot-}$ quantifications by the XTT assay are in agreement with the results of NBT histostaining which suggested (i) that apoplastic $O_2^{\cdot-}$ accumulates over time and in association with radicle elongation; and (ii) that treatment of *L. sativum* seeds with MyA reduced $O_2^{\cdot-}$ accumulation in the RAD (**Fig. 5C**). In agreement with promotion of endosperm rupture and enhancement of apoplastic ·OH accumulation in the embryo by ACC treatment (Graeber et al. 2010), NBT staining also showed enhanced apoplastic $O_2^{\cdot-}$ accumulation in the RAD upon ACC treatment (data not shown). Taken together, the radical-scavenging property of MyA on apoplastic $O_2^{\cdot-}$ in the RAD, and the hormonal regulation of this process support the view that apoplastic ROS production is an important mechanism for cell expansion in the embryo.

Interestingly, the MyA treatment also revealed three distinct zones of NBT staining within the lower RAD region (**Fig. 6A**). MyA especially reduced the NBT staining in zone 2. This suggests that zone 2 is associated with the strongest inhibitory effect of MyA on apoplastic $O_2^{\cdot-}$ accumulation, which is important for cell extension growth of *L. sativum* embryos. The same effect was evident in the ABA-treated embryos, in which the NBT staining was reduced specifically in zone 2 of the lower RAD region (data not shown). Sliwinska et al. (2009) demonstrated that endoreduplication was accompanied by regions of cell extension within the *A. thaliana* embryo. To determine if MyA affects DNA synthesis and endoreduplication during *L. sativum* seed germination and early seedling growth, flow cytometric analysis was performed on nuclei isolated from

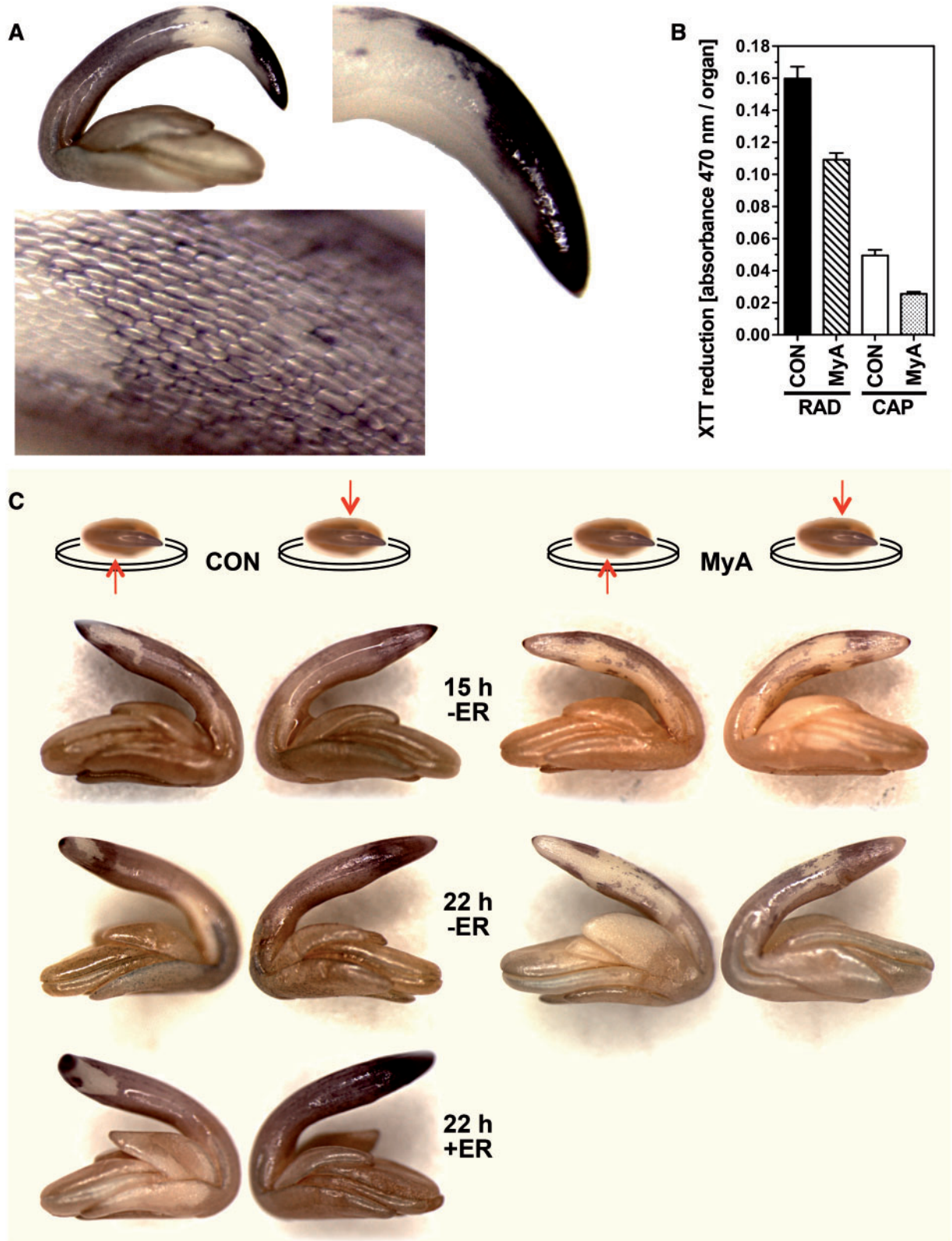

Fig. 5 Accumulation of apoplastic superoxide ($O_2^{\cdot-}$) in the embryos from *Lepidium sativum* seeds imbibed in continuous white light. (A) NBT (nitroblue tetrazolium) histostaining of apoplastic $O_2^{\cdot-}$ in the embryo isolated from a seed just after endosperm rupture at 22 h. Enlarged views of the RAD and the tissue-level localization of apoplastic $O_2^{\cdot-}$ in the elongating lower radicle/hypocotyl axis are shown. Note that apoplastic $O_2^{\cdot-}$ accumulated at the upper side of the RAD exhibiting a gravitropic response. (B) XTT {3′-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis[4-methoxy-6-nitro] benzenesulfonic acid hydrate} quantification of apoplastic $O_2^{\cdot-}$ release by the RAD and CAP isolated from *L. sativum*

Continued

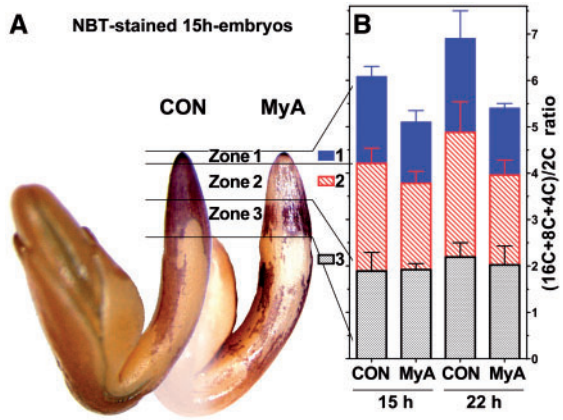

Fig. 6 Effect of MyA on the endoreduplication in distinct zones within the RAD during the germination of *Lepidium sativum* seeds incubated in continuous white light. (A) A uniformly NBT-histostained RAD of control embryo (left) and three distinct visible zones of apoplastic superoxide ($O_2^{\cdot-}$) accumulation within the RAD region of a MyA-treated embryo (right) at 22 h. (B) Effect of MyA on the (4C + 8C + 16C)/2C ratios in three distinct zones of the RAD isolated from seeds imbibed for 15 and 22 h. For complete results and statistics see **Supplementary Table S2**.

the three different zones of the RAD. Only 2C and 4C nuclei were present in zone 1 of the radicle of a dry seed, while endoreduplicated 8C nuclei also occurred in the other two zones (**Supplementary Fig. S4**, **Supplementary Table S2**). In all three zones of CON seeds the nuclei underwent endoreduplication resulting in 8C and 16C nuclei. Eight-C nuclei were detected in zone 1, and 8C plus 16C nuclei were detected in zones 2 and 3 of the RAD excised from CON seeds imbibed for 22 h. However, when the seeds were treated with MyA, no 16C nuclei occurred and also the proportion of 8C nuclei was not as high as in CON embryos at the same time point (**Fig. 6**, **Supplementary Fig. S4**, **Supplementary Table S2**). The inhibition of endoreduplication by MyA was even more evident at 30 h. The mean C-value and the (4C + 8C + 16C)/2C ratio increased with time in embryos and seedlings imbibed under CON conditions. However, these parameters remained at, or were slightly below, those of the CON embryos at 15 h, when MyA was applied. Taken together, these results demonstrate that MyA inhibits *L. sativum* endosperm rupture by decreasing apoplastic superoxide accumulation, with the strongest effect in zone 2 of the elongating RAD, and by inhibiting endoreduplication associated with cell extension.

Discussion

Myrica gale as a 'donor' plant of the natural phytotoxin MyA, which inhibits seed germination and early seedling growth of 'target' plants

Popovici et al. (2011, 2010) proposed that the rare flavonoid MyA of *M. gale* could be used as a natural herbicide against invasive knotweed, as it has strong phytotoxic activity on seedling growth. The ongoing, ecosystem competition experiments to demonstrate that MyA is an allelochemical are discussed in Popovici et al. (2011). Some of the experiments have already shown that *M. gale* fruit and leaf exudates and MyA itself inhibit shoot and root growth of dark-grown eudicot (cress, mustard and knotweed) and monocot (sorghum) seedlings (Popovici et al. 2011). However, how these phytotoxic effects are achieved was not investigated. The physiological and molecular mechanisms by which putative allelochemicals with phytotoxic effects act include inhibition of cell expansion (juglone) and division (1,8-cineol), photosynthesis (sorgoleone), respiration [(−)-catechin] or enzymes such as asparagine synthetase (1,4-cineole) and HPPD (sorgoleone), and the promotion of cell death of root meristems due to elevated ROS production [(−)-catechin] (Inderjit and Duke 2003, Weir et al. 2004). We demonstrate here that *M. gale* fruit leachates and MyA itself inhibit *L. sativum* seed germination and the transition to subsequent seedling growth. We found that MyA, but not the fruit terpenes tested, can fully account for the inhibitory effects of *M. gale* fruit leachates that target endosperm rupture (**Fig. 1**).

MyA still allowed germination; however, those seeds exhibited atypical endosperm rupture (**Fig. 2**), a phenomenon that also occurs when endosperm weakening was artificially inhibited by 10 mM H_2O_2, most probably through cell wall-tightening reactions by cross-linking extraprotoplasmatic polymers (Müller et al. 2009b). The typical endosperm rupture can be prevented by the inhibition of proteolysis in isolated *L. sativum* CAP (Morris et al. 2011). The phenomenon of atypical endosperm rupture is also known from *Syringa reflexa* seeds for which chilling or gibberellin treatment normally causes endosperm weakening, but atypical endosperm rupture can be observed when embryo growth potential is increased without endosperm weakening (Juntilla 1973). The phytotoxic action of MyA on *L. sativum* seeds included interference with gibberellin- and ROS-mediated cell extension in the RAD region. We provided novel molecular and cellular mechanisms for the phytotoxic action of a plant secondary metabolite. We propose that the remarkable developmental specificity of the MyA phytotoxin to the very early growth phase qualifies it as a putative allelochemical of *M. gale*.

Fig. 5 Continued
seeds with unruptured endosperms incubated for 22 h. Mean values ± SE of four biological replicates. (C) NBT histostaining of apoplastic $O_2^{\cdot-}$ in the embryos isolated from control (CON) and MyA-treated seeds with unruptured (−ER) and ruptured (+ER) endosperms incubated for 15 and 22 h. Photographs on the left and right show the lower (the part of the seed touching the Petri dish) and the upper sides of the embryos, respectively, as they had been positioned in the seed (a schematic representation with a red arrow is shown above each column of photographs to indicate the side of the embryo shown). Note the difference in NBT histostaining between the lower and upper sides of the RAD.

MyA specifically interferes with gibberellin-regulated processes important for endosperm weakening and embryo growth

We detected that both the 13-non-hydroxylated and the 13-hydroxylated pathways operate in the RAD and CAP of *L. sativum* seeds (**Fig. 4**). These pathways produced bioactive GA_4 and approximately 10-fold less GA_1, respectively. GA_4 is far more abundant compared with GA_1 also in germinating *A. thaliana* seeds (Ogawa et al. 2003, Yamauchi et al. 2004, Seo et al. 2009). MyA caused an approximately 3-fold decrease of the GA_4 contents in the RAD, but did not affect the GA_4 contents in the CAP. This suggests that the inhibition of endosperm rupture by MyA is mediated, at least in part, by gibberellin-promoted mechanism(s) that affect the embryo growth potential. It is possible that the gibberellin promotes production of apoplastic ROS which are required for cell wall loosening and cell extension of the embryo. The MyA-mediated inhibition of GA_4 production in the RAD was accompanied by a 200-fold accumulation of its precursor GA_9. Therefore, the major target of the MyA-mediated blockage of gibberellin biosynthesis may be the conversion of GA_9 to GA_4 by GA3ox. In agreement with this, the *GA3ox4* transcript abundance in the RAD decreased about 2-fold. We assume that MyA inhibits the GA3ox reaction leading to reduced levels of bioactive GA_4 in the RAD, but does not affect it in the CAP (the increased GA_9 contents in the CAP may be due to diffusion from the RAD), and does not affect the preceding GA20 oxidase (GA20ox) reactions.

GA3ox is also required for the production of GA_6 and GA_{13} detected in the RAD and CAP (**Fig. 4D**). These steps are inhibited by MyA treatment in the RAD, 1.5- and 2.4-fold for GA_6 and GA_{13}, respectively. MyA treatment caused an approximately 20-fold reduction in bioactive GA_6, and did not affect the contents of GA_{13} in the CAP. The MyA-mediated inhibition of the GA3ox reaction is therefore not a general effect on these enzymes, but has some specificity regarding RAD/CAP and substrate/product for which the molecular mechanisms remain unknown. Four GA3ox genes are known from *A. thaliana* and all encode active enzymes, but only *GA3ox2* and *GA3ox1* transcripts are abundant during *A. thaliana* seed germination and regulated by environmental cues (e.g. Yamaguchi et al. 2001, Ogawa et al. 2003, Yamauchi et al. 2004, Seo et al. 2006, Seo et al. 2009). In contrast, we did not detect *GA3ox2* transcripts and found that *GA3ox4* transcripts were most abundant in RAD and CAP tissues from imbibed *L. sativum* seeds (**Fig. 4**). In agreement with our results, Hu et al. (2008) found that the *GA3ox4* promoter was active in the micropylar region and that *GA3ox4* transcripts are more abundant compared with three other *GA3ox* transcripts in *A. thaliana* seeds imbibed for 6 h. It is therefore possible that *GA3ox4* expression is highly confined to the micropylar end (RAD and CAP) of germinating *L. sativum* and *A. thaliana* seeds.

In the present study, we utilized an optimized method for gibberellin metabolite measurement (Urbanová et al. 2011) that detected GA_6 and GA_{13} as major compounds with 4- to 40-fold higher contents in the CAP compared with the RAD (**Fig. 4**). Interestingly, the major bioactive gibberellin of *L. sativum* seeds produced by the 13-hydroxylated pathway is not GA_1 but GA_6. The bioactivity of GA_6 and its precursor GA_5 is known from work on the grass *Lolium temutentum* where they serve as major floral inducers, but show weak stem extension activity (King et al. 2003, Mutasa-Göttgens and Hedden 2009). GA3ox has been shown to have 2,3-epoxidase activity to produce GA_6 from GA_5 also in seeds (Kwak et al. 1988, Zhou et al. 2004, Appleford et al. 2006). The 2,3-epoxide group of GA_6 confers its resistance to inactivation by GA2ox. GA_6 may also serve for transport or accumulation, as it is a stable bioactive gibberellin (Pimenta Lange and Lange 2006, Yamaguchi 2008). As GA_6 specifically accumulated in the CAP of *L. sativum* seeds, we propose that it contributes, together with GA_4, to CAP weakening. In agreement with this, MyA delayed CAP weakening and caused a 20-fold decrease in the GA_6 contents in the CAP. GA_{13} was another major compound that accumulated in the CAP of *L. sativum* seeds. GA_{13} is typically found in developing seeds, although it is inactive and its function is unknown (Pimenta Lange and Lange 2006). A study on developing pumpkin seeds demonstrated that a GA3ox that converts GA_9 to GA_4 also converts GA_{25} to GA_{13} (Frisse et al. 2003). In support of this, GA_{13} contents were reduced in seedlings of the *A. thaliana ga3ox1* mutant (Talon et al. 1990). As GA_{13} can bind to GA2oxs, its accumulation in *L. sativum* CAP may serve as an inhibitor of GA2ox and thereby prevent GA_4 inactivation. Taken together, the inhibitory effect of MyA on endosperm rupture can be explained in part by an inhibition of the biosynthesis of bioactive gibberellin (GA_4 in the RAD and GA_6 in the CAP). From our tissue-specific measurements, the combined bioactive GA_4 and GA_6 concentrations can be estimated as approximately 20 nM in the RAD and approximately 125 nM in the CAP. These concentrations are reduced by MyA approximately 2- and 8-fold in the RAD and the CAP, respectively, which in turn may cause impaired gibberellin signaling as an additional mechanism.

Gibberellin signaling, via the soluble GID1-type GA receptors, mediates downstream processes that confer embryo extension and endosperm weakening (e.g. Holdsworth et al. 2008, Piskurewicz et al. 2009, Seo et al. 2009, Voegele et al. 2011). Voegele et al. (2011) found that eudicot GID1s clustered into two eudicot groups (GID1ac and GID1b) and proposed that the two groups mediate different downstream processes required for the completion of germination by endosperm rupture. Gibberellin signaling via the GID1ac receptors is required for *A. thaliana* seed germination, as GID1b cannot compensate the impaired germination of the *gid1agid1c* mutant. A gibberellin-triggered, negative feedback loop in the CAP and RAD of germinating *L. sativum* seeds was evident only for the *GID1ac* transcripts, but not for *GID1b*, and this was associated with a similar regulatory pattern of downstream genes. Nakajima et al. (2006) shows that the binding affinity for GA_4 of GID1b was approximately 10-fold higher compared with

the GID1ac proteins (K_D approximately 30 nM for GID1b and approximately 300 nM for GID1ac). We found that MyA specifically down-regulated the *GID1b* transcripts (**Fig. 4F**) for which the encoded GID1b receptor proteins have a higher GA_4 binding affinity (K_D approximately 30 nM). If we assume that gibberellin signaling is then mediated only by the GID1ac receptor that has a lower GA_4 binding affinity (K_D approximately 300 nM), the low concentrations of bioactive GA_4 and GA_6 would not allow optimal gibberellin signaling. Taken together, these results support the view that gibberellin signaling via both GID1 pathways is required for the endosperm rupture of Brassicaceae seeds, and that MyA acts by interfering with gibberellin metabolism and signaling important for downstream cell wall-loosening factors such as xyloglucan endotransglycosylase/hydrolase (XTH) and expansins (Voegele et al. 2011) and/or apoplastic ROS (Müller et al. 2009b).

The phytotoxic effects of MyA are caused by its radical-scavenging properties that interfere with the ROS-mediated cell extension of the embryo

Enhanced ROS production is usually associated with biotic and abiotic stress that is deleterious to cells, which is also true for the mode of action of many phytotoxins (Inderjit and Duke 2003, Weir et al. 2004, Oracz et al. 2007a). Controlled ROS production is also important for the biology of seeds. ROS act either indirectly as a key component of signaling networks together with plant hormones to regulate some processes such as dormancy release, after-ripening and germination (Bailly 2004, Oracz et al. 2007b, El-Maarouf-Bouteau and Bailly 2008, Oracz et al. 2008, Müller et al. 2009a, Oracz et al. 2009, Liu et al. 2010, Bahin et al. 2011), or directly in the apoplast by mediating cell wall loosening and weakening required for germination (Schopfer 2006, Müller et al. 2009b, Graeber et al. 2010). We demonstrated that apoplastic $O_2^{\cdot-}$ is generated in the RAD region of the expanding *L. sativum* embryo. The spatial distribution of $O_2^{\cdot-}$ accumulation in the RAD was asymmetric to the gravitropic stimulus; and this apoplastic $O_2^{\cdot-}$ is therefore indicative of cell extension; the faster growing side of the RAD was more strongly stained (**Fig. 5**). This finding is in agreement with a direct mode of action of apoplastic ROS in mediating cell wall loosening, which is required for cell extension (Renew et al. 2005, Schopfer 2006, Müller et al. 2009b). According to the model of Schopfer (2001), the apoplastic ·OH is produced from apoplastic H_2O_2 and $O_2^{\cdot-}$, and plasma membrane-localized NADPH oxidase produces the apoplastic $O_2^{\cdot-}$. The apoplastic ·OH causes in vivo scission of cell wall polysaccharides required for cell wall loosening and cell extension, as was demonstrated for *L. sativum* seed germination and seedling growth (Renew et al. 2005, Schopfer 2006, Müller et al. 2009b). The apoplastic ·OH production in the *L. sativum* RAD was inhibited by ABA and promoted by gibberellin and ethylene/ACC which is in agreement with the effect of these hormones on endosperm rupture and embryo growth (Linkies et al. 2009, Müller et al. 2009b, Graeber et al. 2010). Schopfer et al. (2001) also provided evidence that apoplastic ROS production of radish embryos is regulated by ABA and gibberellin. Our results for the apoplastic $O_2^{\cdot-}$ production in the RAD are in full agreement with these findings. The comparison of ABA and ACC, as well as MyA, which interferes with gibberellin metabolism and signaling, supports the important conclusion that $O_2^{\cdot-}$ and ·OH production in the apoplast of the RAD is developmentally and hormonally regulated and mediates embryo cell extension.

Embryo cells elongate prior to the completion of seed germination of *A. thaliana* and other species, and this cell extension growth is associated with endoreduplication (Sliwinska et al. 2009, Weitbrecht et al. 2011). Similarly to *A. thaliana* (Sliwinska et al. 2009), endoreduplication in *L. sativum* is associated with embryo axis elongation. MyA treatment revealed that the RAD region of the *L. sativum* embryo harbors three zones, and MyA inhibited endoreduplication and apoplastic $O_2^{\cdot-}$ production especially in zone 2 (**Fig. 6**). Taken together, we propose that MyA inhibits ROS-mediated cell extension in this region. The zone 2 of the radicle of *L. sativum* seed is therefore an elongation zone important for radicle protrusion during endosperm rupture. One way to interpret the MyA effects is that it interferes with gibberellin-mediated cell extension by its effects on gibberellin metabolism and signaling that then indirectly cause a reduction of in vivo $O_2^{\cdot-}$ and ·OH production in the apoplast. MyA could also act directly as a radical scavenger against apoplastic ROS production. MyA was characterized as one of the C-methylated dihydrochalcones with the highest radical-scavenging activity (Mathiesen et al. 1995, Mathieson et al. 1997) and has been reported to perturb the oxidative electron transfer in the respiratory chain of rat mitochondria and to inhibit ATP synthesis, indicating a substantial effect of those flavonoids on the membrane redox environment (Mathiesen et al. 1996). This mode of action would provide a very interesting and novel mechanism for a phytotoxin and putative allelochemical. The phytotoxicity of many quinones (e.g. sorgoleone) and phenols [e.g. (−)-catechin] can largely be attributed to enhanced formation of radicals that donate electrons to molecular oxygen, forming $O_2^{\cdot-}$ and causing cell death (Inderjit and Duke 2003, Weir et al. 2004). They therefore act as phytotoxins by increasing ROS production, while MyA acts as a phytotoxin by decreasing ROS production.

The remarkable developmental specificity of the MyA phytotoxin to the very early growth phase qualifies it as a putative allelochemical. Although a consensus for identifying a compound as an allelochemical is lacking, it is clear that to detect and accurately quantify natural phytotoxins released by the 'donor' species and characterize them in relation to the growth inhibition of natural 'target' species in the context of soil ecology is a requirement (Inderjit and Duke 2003, Weir et al. 2004, Blair et al. 2008). Ongoing, ecosystem competition experiments to identify MyA as an allelochemical in the natural environment are discussed by Popovici et al. (2011). Based on our results, the ecological experiments should aim to investigate MyA as an inhibitor of seed germination and seedling

establishment. This is especially important in the ecosystems that undergo environmental changes and between-species competition, as early events of the plant life cycle, such as seed germination and successful seedling growth, are among the first to exhibit adaptation to novel conditions (Donohue 2005). In summary, MyA is a putative allelochemical with a remarkable mode of action that directly affects cell extension required for completion of seed germination and seedling establishment.

Materials and Methods

Plant materials

Myrica gale L. fruits were collected in December 2004 on the shore of Biscarosse lake, Bordeaux, France (Popovici et al. 2011). MyA, extracted and purified as described by Popovici et al. (2011), was generously provided by Professor G. Comte, CESN, University and CNRS, Lyon, France. The purity of this MyA exceeded 99%; a methanol stock of the MyA was prepared and used throughout our work. After-ripened seeds of *L. sativum* L. FR14 ('Keimsprossen', Juliwa) were used (Graeber et al. 2010).

Germination assays, puncture force measurements and viability tests

Germination assays were performed with 50 *L. sativum* seeds placed in 9 cm Petri dishes (four replicates) containing two layers of filter paper, moistened with 6 ml of sterile distilled water containing a final concentration of 0.35% (v/v) methanol (CON). The 0.35 (v/v) methanol was required in the CON as the MyA stock was in methanol, but methanol did not affect the germination kinetics when compared with water. Terpenes or other chemicals were added as indicated (**Supplementary Table S3**). The pH of all solutions was adjusted to 7 with 5 M NaOH, and 0.1% (v/v) Plant Preservative Mixture (Plant Cell Technology) was added. Petri dishes were incubated at 24°C in a Sanyo Versatile Environmental Test Chamber (MLR-350) in continuous white light (approximately 75 $\mu mol\, s^{-1}\, m^{-2}$). The percentages of testa and endosperm rupture of the seed populations were scored over time using a binocular microscope (Leica DCF480), and CAP weakening was quantified by puncture force measurements as described (Müller et al. 2006). If not otherwise stated, whole embryos, RAD or CAP were excised from seeds with testa rupture, but unruptured endosperm.

In situ localization and quantification of superoxide radical ($O_2^{\cdot-}$) contents

For in situ localization of apoplastic $O_2^{\cdot-}$ by NBT histostaining (dark-blue colour of insoluble formazan compounds), isolated embryos were incubated in 6 mM NBT in 10 mM Tris–HCl buffer (pH 7) at room temperature for 3 min as described (Oracz et al. 2007b). For biochemical quantification of apoplastic $O_2^{\cdot-}$ contents, the XTT assay was used as described (Müller et al. 2009b). Ten RADs or 20 CAPs were excised from *L. sativum* seeds incubated for 22 h in continuous white light, and further incubated for 30 min in 2.5 ml of 0.5 mM XTT in 1 mM Tris–HCl buffer solution (pH 6) at 25°C, on a shaker. The changes in absorbance were measured at 470 nm. Mean values ± SE per organ of four biological replicates were calculated.

Quantification of ABA

To quantify ABA and related metabolites, 200 RADs and 200 CAPs were isolated from *L. sativum* CON and MyA-treated seeds incubated for 15 h in continuous white light, and immediately frozen in liquid nitrogen. Internal standard mixtures, containing 20 pM of each of (−)-7′,7′,7′-[$^2H_3$]phaseic acid, (−)-7′,7′,7′-[$^2H_3$]dihydrophaseic acid, (−)-8′,8′,8′-[$^2H_3$]neophaseic acid, (+)-4,5,8′,8′,8′-[$^2H_5$]ABAGE, (−)-5,8′,8′,8′-[$^2H_4$]7′-OH-ABA and (+)-3′,5′,5′,7′,7′,7′-[$^2H_6$]ABA and 1 ml of cold methanol : water : acetic acid (80 : 19 : 1, v/v) were added to previously ground and freeze-dried tissues. After 24 h of shaking in the dark at 4°C, the homogenates were centrifuged (20,000 r.p.m., 5 min, 4°C) and the pellets were then re-extracted in 0.5 ml of extraction solvent for 60 min. The supernatants were dried under vacuum, then were dissolved in 100 µl of 99% methanol : 1% acetic acid (v/v) topped up to 1 ml with 99% water : 1% acetic acid (v/v), purified by solid-phase extraction on an Oasis® HLB cartridge (60 mg, 3 ml, Waters) and evaporated to dryness in a Speed-Vac (UniEquip). Subsequently the evaporated samples were methylated and analyzed by ultra-performance liquid chromatography–electrospray tandem mass spectrometry [UPLC-ESI(+)-MS/MS] (Turečková et al. 2009).

Quantification of gibberellins

To quantify gibberellin metabolites, 50 RADs and 150 CAPs were isolated from *L. sativum* CON and MyA-treated seeds incubated for 15 h in continuous white light, and immediately frozen in liquid nitrogen. Quantification of gibberellins was performed according to the method of Urbanová et al. (2011). Aliquots of 2 mg of lyophilized material were homogenized using an MM301 Vibration Mill for 10 min at 30 Hz (Retsch Technology GmbH) in 1 ml of 80% acetonitrile containing 5% formic acid with addition of gibberellin internal standards ([$^2H_2$]GA_1, [$^2H_2$]GA_3, [$^2H_2$]GA_4, [$^2H_2$]GA_5, [$^2H_2$]GA_7, [$^2H_2$]GA_8, [$^2H_2$]GA_9, [$^2H_2$]GA_{12}, [$^2H_2$]GA_{12ald}, [$^2H_2$]GA_{15}, [$^2H_2$]GA_{19}, [$^2H_2$]GA_{20}, [$^2H_2$]GA_{24}, [$^2H_2$]GA_{29}, [$^2H_2$]GA_{34}, [$^2H_2$]GA_{44}, [$^2H_2$]GA_{51} and [$^2H_2$]GA_{53}; purchased from Professor Lewis Mander, Australia). After centrifugation at 14,000 r.p.m. for 10 min, the supernatant was loaded onto a pre-equilibrated MCX cartridge coupled to a HLB cartridge (both Waters). After an elution and evaporation in a stream of nitrogen, the extract was finally purified using MAX cartridges (Waters). Dried eluates were re-solubilized in the mobile phase and analyzed. Compounds were separated by an Acquity UPLC™ System (Waters), which consisted of a binary solvent manager, sample manager and 2996 PDA detector. Retention times for

the analyzed compounds ranged from 4.5 to 18.3 min. The effluent was introduced into the tandem mass spectrometer Xevo^{TM} TQ MS (Waters MS Technologies). Gibberellins were detected using multiple-reaction monitoring (MRM) of the transition of the $[M-H]^-$ ion to the appropriate product ion. The second transition of the $[M-H]^-$ ion to another product ion was used to confirm endogenous occurrence and to calculate the MRM ratio. The data were then analyzed using Masslynx 4.1 software (Waters) and quantified by the standard isotope dilution method as described by Urbanová et al. (2011).

Flow cytometry

For flow cytometry analysis, *L. sativum* seeds were imbibed for 15, 22 and 30 h as described above. Samples were prepared as previously described (Sliwinska and Lukaszewska 2005) from the three zones (**Fig. 6**) of the isolated RAD of dry and imbibed seeds. Nuclei released from 10 dissected zones constituted a sample. For each sample, 2,000–4,000 nuclei were analyzed using a Partec CCA flow cytometer. Analyses were performed on four replicates, using a logarithmic scale. Histograms were analyzed using a DPAC v.2.2 computer program (Partec GmbH), and the percentage of nuclei with particular DNA contents, the (4C + 8C + 16C)/2C ratio and the mean C-value (Lemontey et al. 2000) were calculated. The results were analyzed using a one-way analysis of variance (ANOVA) and a Duncan's test (**Supplementary Table S2**).

qRT–PCR

Normalized transcript abundance was analyzed by qRT–PCR and by using superior reference genes as described by Graeber et al. (2011). In brief, total RNA was extracted from 50 RAD and 200 CAP tissues that were dissected from *L. sativum* seeds after 15 h of imbibition. Downstream applications were performed using four biological replicate RNA samples for each treatment. First-strand cDNAs were obtained using the Superscript III reverse transcriptase kit (Invitrogen) with 0.3 nmol random pentadecamers (R15) for 20 µl reverse transcription reactions of 5 µg of RNA. The qRT–PCRs and post-run data analysis were then conducted as described (Graeber et al. 2011). Transcript abundance was normalized against the geometric mean of the three validated reference genes LesaG17210 (HQ912755), LesaG04660 (HQ912754) and LesaG20000 (HQ912757) for each sample. **Supplementary Table S4** contains a list of analyzed genes and primers used in this study.

Supplementary data

Supplementary data are available at PCP online.

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