

Endosperm-limited Brassicaceae Seed Germination: Absciscic Acid Inhibits Embryo-induced Endosperm Weakening of *Lepidium sativum* (cress) and Endosperm Rupture of Cress and *Arabidopsis thaliana*

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The endosperm is a barrier for radicle protrusion of many angiosperm seeds. Rupture of the testa (seed coat) and rupture of the endosperm are two sequential events during the germination of *Lepidium sativum* L. and *Arabidopsis thaliana* (L.) Heyhn. Absciscic acid (ABA) specifically inhibits the endosperm rupture of these two closely related Brassicaceae species. *Lepidium* seeds are large enough to allow the direct measurement of endosperm weakening by the puncture force method. We found that the endosperm weakens prior to endosperm rupture and that ABA delays the onset and decreases the rate of this weakening process in a dose-dependent manner. An early embryo signal is required and sufficient to induce endosperm weakening, which afterwards appears to be an organ-autonomous process. Gibberellins can replace this embryo signal; de novo gibberellin biosynthesis occurs in the endosperm and weakening is regulated by the gibberellin/ABA ratio. Our results suggest that the control of radicle protrusion during the germination of Brassicaceae seeds is mediated, at least in part, by endosperm weakening. We propose that *Lepidium* is an emerging Brassicaceae model system for endosperm weakening and that the complementary advantages of *Lepidium* and *Arabidopsis* can be used in parallel experiments to investigate the molecular mechanisms of endosperm weakening.

Keywords: ABA — *Arabidopsis thaliana* — Embryo signal — Endosperm weakening — Gibberellin — *Lepidium sativum*.

Abbreviations: ABA, absciscic acid; FLU, flurprimidol; GA₄₊₇, gibberellin A₄₊₇; N, Newton; WT, wild type

Introduction

A major reason for the evolutionary success of the angiosperms is the 'invention' of seeds and double fertilization (Baskin and Baskin 1998; Friedman 1998). In a typical angiosperm seed, the embryo is surrounded

by two covering layers (Web: 'The Seed Biology Place', <http://www.seedbiology.de>): the endosperm (nutritive tissue, living cells) and the testa (seed coat; maternal tissue, dead cells). The mature seeds of most angiosperms are endospermic. They have a more or less abundant endosperm layer, though the evolutionary trend is towards cotyledon storage and endospermless seeds. This trend is also evident for the phylogenetically advanced Brassicaceae family (rosid clade): the mature seeds of species of the genera *Raphanus*, *Sinapis* and *Brassica* are endospermless (Schopfer and Plachy 1984, Bergfeld and Schopfer 1986, Schopfer et al. 2001); the single-celled aleurone layer of these species is diploid maternal tissue and is part of the inner testa. The mature seeds of *Arabidopsis thaliana*, *Lepidium* spp. and *Cleome* spp. have retained a thin endosperm layer, and the aleurone layer of these species has been described to be endosperm tissue (O'Brien and McCully 1969, Vaughan et al. 1971, Corner 1976, Ruiz and Escalé 1995, Nguyen et al. 2000). In the model species *A. thaliana* (*Arabidopsis*), this endosperm is a single cell layer (Pritchard et al. 2002, Nambara and Marion-Poll 2003, Liu et al. 2005). The endosperm development of *Arabidopsis* and *Lepidium* spp. is a cellularization process that begins in the micropylar region and later during seed development spreads to the central and chalazal regions (e.g. Brown et al. 1999, Debeaujon et al. 2000, Nguyen et al. 2000, Windsor et al. 2000). The important point related to the work presented here is that mature seeds of *Arabidopsis* and *Lepidium* spp. both have been described to contain cellular endosperm tissue between the embryo and the testa.

The process of seed germination begins with water uptake by the dry seed through imbibition and ends when the radicle has protruded through all covering layers (Bewley 1997b, Koornneef et al. 2002, Kucera et al. 2005). In order for a seed to complete germination, the growth potential of the radicle must overcome the tissue resistance of the seed covering layers. For many species, including *Arabidopsis*, testa rupture and endosperm rupture are two sequential steps during germination (e.g. Karssen 1976,

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Hepher and Roberts 1985, Leubner-Metzger et al. 1995, Krock et al. 2002, Petruzzelli et al. 2003, Leubner-Metzger 2003, Liu et al. 2005). The role of the testa as a germination constraint has been studied by using testa mutants of *Arabidopsis* (Debeaujon and Koornneef 2000, Koornneef et al. 2002). Also the role of the endosperm as a nutritive tissue has been studied thoroughly in many species. Far less is known about the role of the endosperm as a constraint to radicle protrusion during germination.

The original hypothesis that weakening of the micropylar endosperm covering the radicle tip by cell wall-modifying proteins is a prerequisite for the completion of germination was proposed based on pioneering work with lettuce and was published in *Plant and Cell Physiology* by Ikuma and Thimann (1963). Subsequent work with seeds of the asterid clade with either a thin (e.g. lettuce) or a thick (e.g. tomato, tobacco, coffee) endosperm layer strongly supports the view that the micropylar endosperm is a barrier for radicle protrusion (reviewed in Hilhorst 1995, Bewley 1997a, Leubner-Metzger 2003). The original work cited in these reviews provided evidence for the involvement of several cell wall-modifying proteins in endosperm weakening as well as for the importance of plant hormones in regulating the resistance of the seed covering layers. Although some progress has been made, no definitive conclusions have been reached regarding the molecular mechanisms that cause endosperm weakening. Further progress is, at least in part, hampered by the fact that in these classical asterid seed model systems, the direct measurement of the endosperm weakening by the puncture force method is often not possible (e.g. tobacco seeds are too small) or is biased by the inclusion of additional tissues (e.g. testa for tomato, embryo for lettuce).

Results produced using the different asterid seed model systems have led to the conclusion that inhibition of endosperm rupture by abscisic acid (ABA) appears to be an evolutionary widespread phenomenon (reviewed in Bewley 1997a, Leubner-Metzger 2003, Nambara and Marion-Poll 2003). It is not known whether ABA specifically inhibits the endosperm rupture of rosid seeds and whether the thin endosperm of the model species *Arabidopsis* and other Brassicaceae weakens and can act as a barrier for radicle protrusion. In the present work, we used seeds of *Arabidopsis* and the much bigger seeds of its close relative *Lepidium sativum* (Lepidium, garden cress). Both species belong to the Brassicoideae subfamily of the Brassicaceae (Hall et al. 2002, Koch et al. 2003) and are highly similar in seed structure and physiology. We found that ABA specifically inhibits endosperm weakening of *Lepidium* and endosperm rupture of *Lepidium* and *Arabidopsis*. The close relationship to *Arabidopsis*, the bigger seed size and additional advantages make *Lepidium*

an emerging seed model system for studying endosperm weakening.

Results

Testa rupture and endosperm rupture are separate events during the seed germination of Lepidium sativum and Arabidopsis thaliana

The mature seeds of *L. sativum* (*Lepidium*) contain a fully developed embryo with differentiated meristems, radicle and cotyledons (Fig. 1). Seeds of *Arabidopsis* and of its close relative *Lepidium* were used in our parallel experiments. Both species are highly similar in seed anatomy (Fig. 2B, G), but differ significantly in seed size (Fig. 2A). In mature seeds of *Arabidopsis*, the radicle is covered by a single cell layer of micropylar endosperm (Fig. 2G; Koornneef et al. 2002, Liu et al. 2005). We found that the embryo of *Lepidium* is enclosed by a thin endosperm, mostly consisting of a single cell layer (Fig. 1); however, the micropylar endosperm surrounding the radicle tip has up to two cell layers (Fig. 1C). *Lepidium* and *Arabidopsis* both exhibit a typical two-step germination: during testa rupture (Fig. 2H for *Arabidopsis*; Fig. 2C, D for *Lepidium*), the micropylar endosperm is exposed as a cap-like structure that covers the radicle tip. This stage is followed by endosperm rupture and radicle emergence (Fig. 2E, I), i.e. the completion of seed germination. Under our control conditions, 50% rupture of the *Lepidium* seed population was evident after 7 h for the testa and after 16 h for the endosperm (Fig. 3A, B), while 50% rupture of the *Arabidopsis* seed population was evident after 24 h for the testa and after 33 h for the endosperm (Fig. 3C, D).

ABA inhibits endosperm rupture of Lepidium and Arabidopsis, but does not appreciably affect testa rupture of after-ripened seeds

Addition of ABA to the medium does not appreciably affect testa rupture (Fig. 3A, C), but delays endosperm rupture of both species in a dose-dependent manner (Fig. 3B, D). Upon ABA treatment, the intact micropylar endosperm cap enclosing the radicle tip is visible (Fig. 2F, J) at incubation times when seedlings are already growing in control medium. The ABA concentrations used in these experiments are in the range of the endogenous concentrations known from seeds. For 10 μ M ABA, the delayed 50% endosperm rupture of *Lepidium* seed populations occurred at approximately 85 h (i.e. a 5-fold delay), while testa rupture was not affected (Fig. 3A, B). Fig. 3 shows an ABA dose-dependent inhibition of *Lepidium* endosperm rupture, e.g. for 5 μ M ABA the 50% value was approximately 65 h. At the same ABA concentration, delayed 50% endosperm

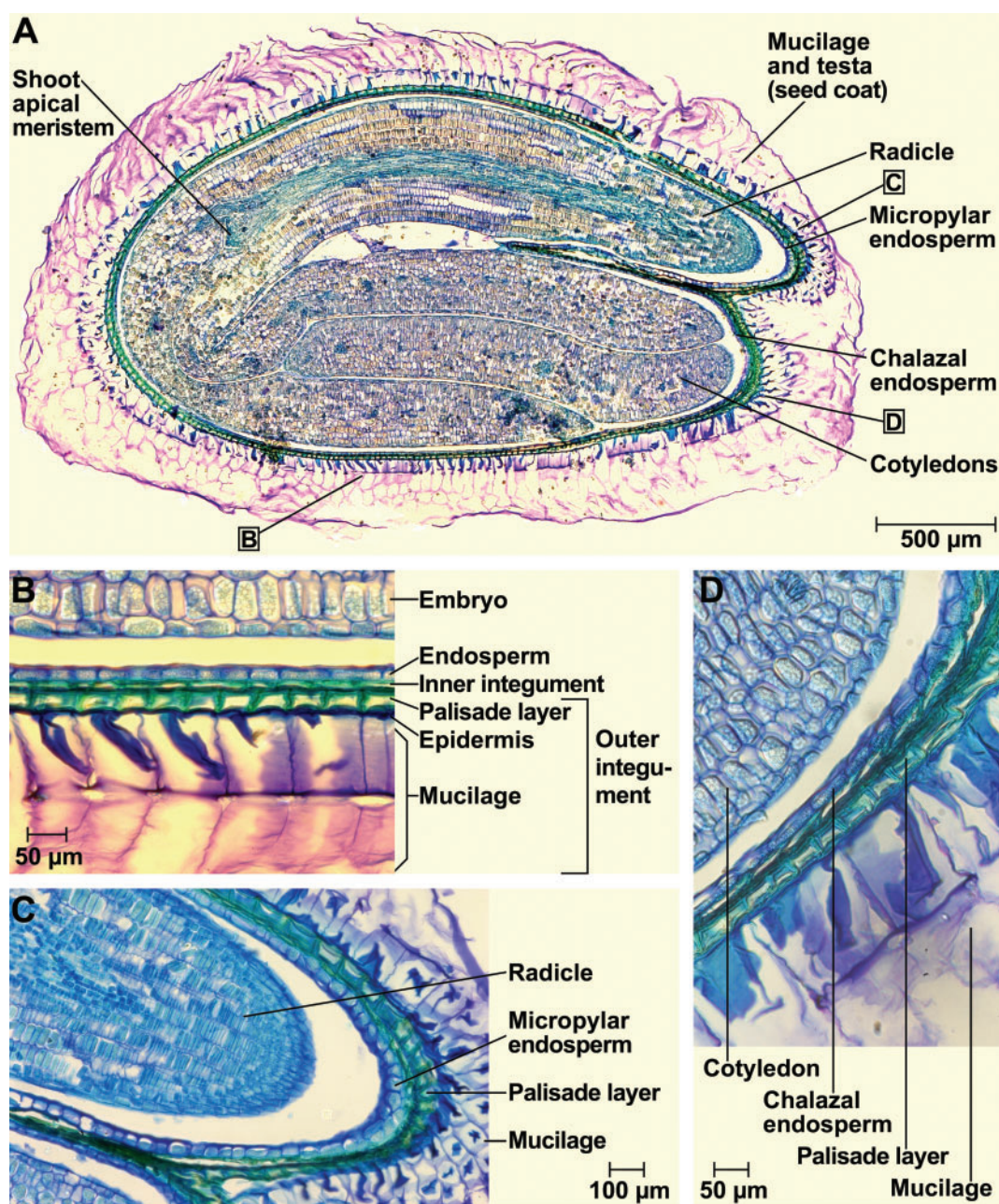


Fig. 1 Structure of a mature seed of *Lepidium sativum*. Bright field microscopy of longitudinal sections of seeds imbibed for 2–3 h stained with toluidine blue. (A) Entire seed, showing the mature and fully differentiated embryo, the endosperm and the testa (seed coat). The boxed letters refer to the positions of the close-up sections. (B) Structure of the seed-covering layers: endosperm, 1 cell layer; and testa (seed coat), composed of inner and outer integument. Note that the mucilage is generated from the outer testa upon imbibition. (C) Structure of the micropylar cap enclosing the radicle tip. The micropylar endosperm has 1–2 cell layers. (D) Structure of the chalazal seed region. Blue light filter (C, D); differential contrast (D). Size bars are given for each panel.

rupture of *Arabidopsis* seed populations occurred at approximately 390 h (i.e. a 12-fold delay), while testa rupture was only delayed by about 10 h (Fig. 3C, D). Thus, ABA delayed endosperm rupture of *Lepidium*

and *Arabidopsis* (Figs. 2, 3), but did not appreciably affect testa rupture.

For our experiments we used fully after-ripened *Lepidium* seeds without a stratification pre-treatment.

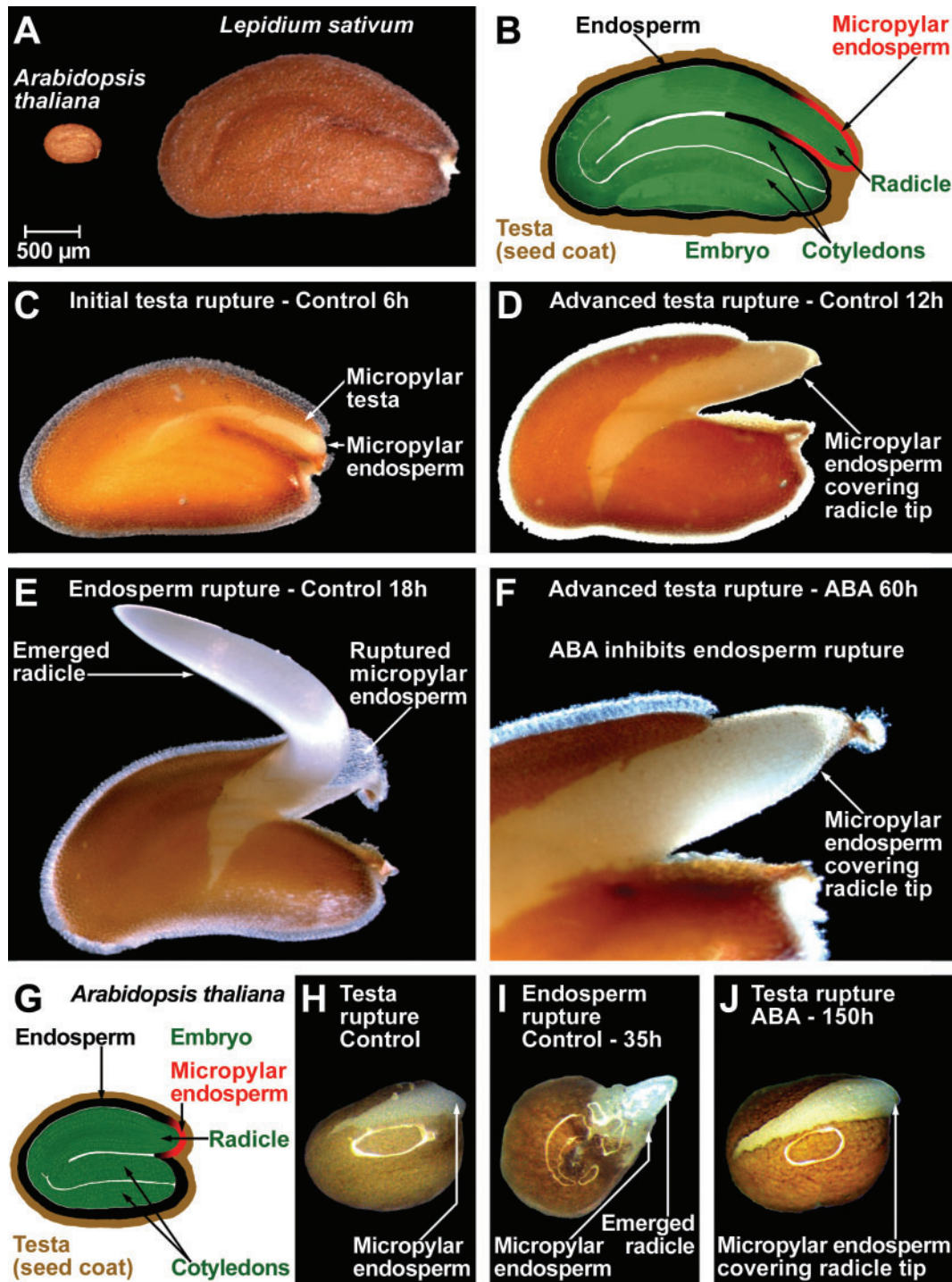


Fig. 2 Seeds of *Lepidium sativum* and *Arabidopsis thaliana* have similar seed anatomy and similar germination physiology. (A) The larger size of *Lepidium* seeds allows methods for which *Arabidopsis* seeds are too small, e.g. puncture force experiments. (B) Drawing of a mature *Lepidium* seed; the embryo is enclosed by the endosperm and the surrounding testa. (C–F) During the two-step germination of *Lepidium*, testa rupture (C, D) is followed by endosperm rupture, which occurs after 16 h under control conditions (E). Due to the microphotographic settings, the transparent outer mucilage layer is not visible. (F) ABA specifically inhibits endosperm rupture; the radicle remains covered by the micropylar endosperm even after 60 h incubation in the presence of ABA. (G) Drawing of a mature *Arabidopsis* seed; the seed anatomy is very similar to that of *Lepidium*. (H–J) *Arabidopsis* seeds also germinate with testa rupture (H) preceding endosperm rupture (I). Also, during the two-step germination of *Arabidopsis*, ABA specifically inhibits endosperm rupture (J). Seeds were incubated in continuous light without (control) or with 10 μ M ABA added to the medium.

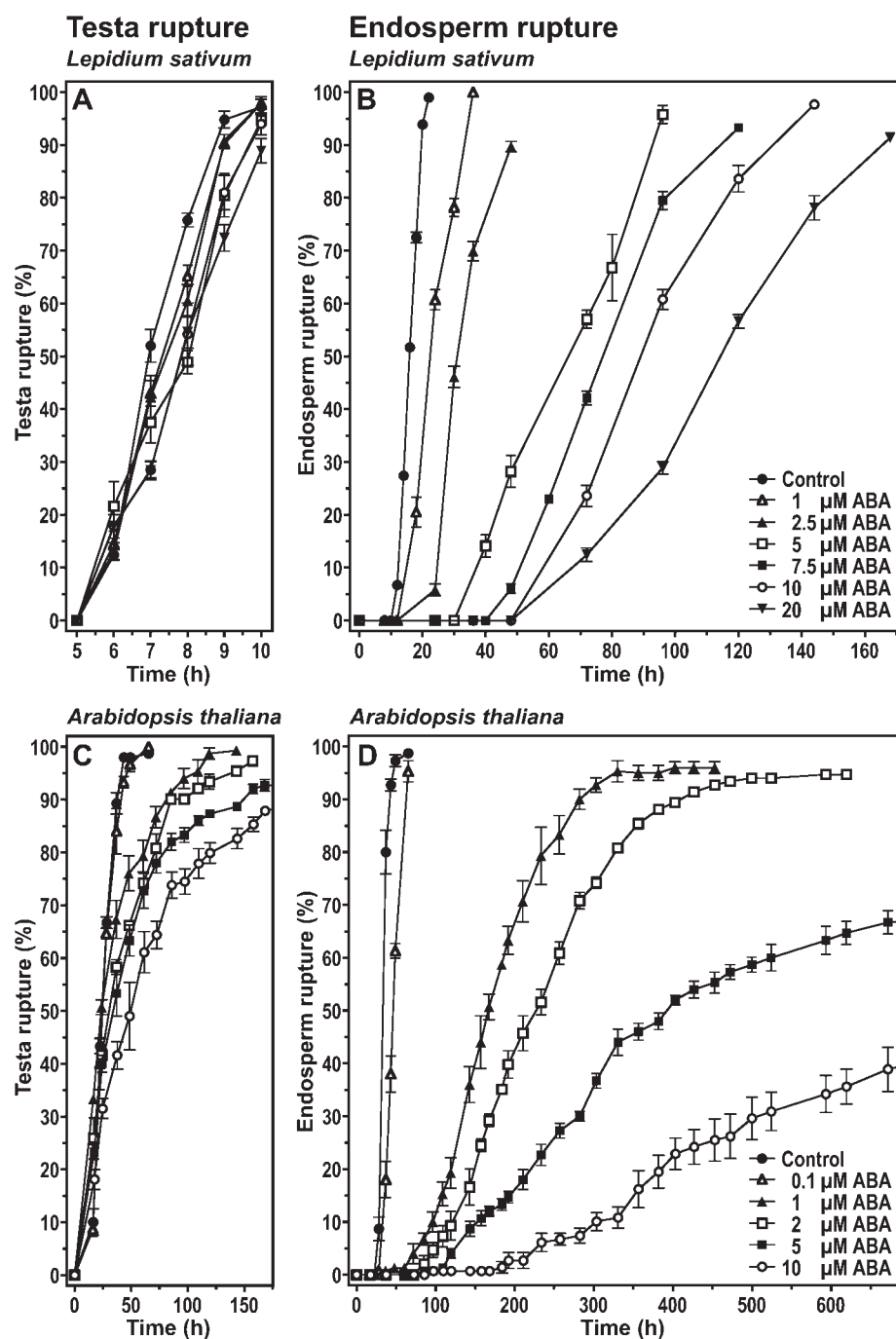


Fig. 3 The effect of ABA on the time courses of testa rupture and endosperm rupture of *Lepidium sativum* (A, B) and *Arabidopsis thaliana* (C, D). ABA inhibited endosperm rupture (B, D) of both species in a dose-dependent manner, but did not appreciably affect testa rupture (A, C). Note the different time scales. The incidence of testa and endosperm rupture was scored with time after the start of incubation in continuous light at 18°C (*Lepidium*) or 24°C (*Arabidopsis*). Mean values \pm SE from one experiment with at least three plates with 50 seeds each are presented.

In contrast, we used a 24 h stratification pre-treatment for our *Arabidopsis* experiments. Table 1 shows that the 24 h stratification treatment promoted the endosperm rupture of *Arabidopsis* seeds. Stratification promoted 50% endosperm rupture of a fully after-ripened *Arabidopsis* seed population by about 5 h. Testa rupture of fully after-ripened seeds was not affected by either stratification or ABA

treatment (Table 1). In contrast to after-ripened seeds, the testa rupture of conditionally dormant seeds was promoted by stratification (Table 1). The physiological status of the seed batch and not the stratification treatment per se is therefore the reason for the differences in the kinetics of testa rupture. The times for 50% testa rupture of the different wild type (WT, *A. thaliana* ecotype Col) seed

Table 1 Effect of stratification at 4°C on testa rupture and endosperm rupture of *Arabidopsis thaliana* ecotype *Col* seeds

		Testa rupture (%)			Endosperm rupture (%)
Experiment 1: fully after-ripened seeds ^a					
		23 h ^b	30 h	23 h	30 h
CON	No stratification	66.7 ± 1.4	99.3 ± 0.5	0.0 ± 0.0	39.3 ± 5.5
	24 h stratification	70.0 ± 1.9	96.0 ± 0.9	22.0 ± 7.5	73.3 ± 1.4
ABA	No stratification	67.3 ± 2.4	96.0 ± 1.1	0.0 ± 0.0	0.0 ± 0.0
	24 h stratification	57.3 ± 2.0	96.7 ± 2.0	0.0 ± 0.0	0.0 ± 0.0
Experiment 2: conditionally dormant seeds ^c					
		24 h	48 h	24 h	48 h
CON	No stratification	0.7 ± 0.5	47.3 ± 1.1	0.0 ± 0.0	13.3 ± 0.5
	24 h stratification	20.0 ± 2.5	100	0.7 ± 0.5	100
	48 h stratification	34.7 ± 6.4	100	7.3 ± 2.7	100
	72 h stratification	54.7 ± 3.9	100	14.7 ± 2.2	100
	96 h stratification	46.0 ± 3.3	100	14.7 ± 2.9	100

^a Fully after-ripened seeds (>9 months of dry storage) were stratified for 24 h at 4°C in darkness on agar medium without (control; CON) or with 1 µM ABA added (ABA).

^b The percentage of ruptured seeds scored in populations at the times indicated after the start of incubation at 24°C in continuous light. Mean ± SE values of three plates each with 50 seeds are presented.

^c Conditionally dormant, i.e. only partially after-ripened, seeds were stratified for the times indicated.

populations was about 33 h (Table 1, conditionally dormant seeds), about 24 h (Fig. 3, seeds after 2–3 months of after-ripening storage) and about 22 h (Table 1 and Fig. 4, seeds after 9 months of after-ripening storage). Thus, the *Arabidopsis* seed batch used in the experiment presented in Fig. 3 was not fully after-ripened, which is the reason for the minor effect of ABA on the testa rupture. This is further supported by the finding that the ABA sensitivity of the endosperm rupture of fully after-ripened WT seeds (Fig. 4) was slightly reduced compared with not fully after-ripened WT seeds (Fig. 3).

To test further the effects of ABA on testa rupture and endosperm rupture, we used after-ripened seeds of *Arabidopsis* ABA biosynthesis mutants. The ABA-deficient *aba1* mutant (Debeaujon and Koornneef 2000), WT, and the *cyp707a1* and *cyp707a2* mutants were compared. The ABA 8' hydroxylases encoded by the *CYP707A* genes are key enzymes for ABA degradation, and ABA breakdown is impaired in the *cyp707a1* and *cyp707a2* mutants (Kushiro et al. 2004). Endosperm rupture of the *aba1-5* mutant was slightly faster, while endosperm rupture of the *cyp707a1* and *cyp707a2* mutants was delayed compared to WT (Fig. 4). For the mutant seed populations with impaired ABA degradation, the addition of ABA to the medium caused a greater delay in endosperm rupture compared to WT: 50% endosperm rupture was at about 75, 95 and 135 h for WT, *cyp707a1* and *cyp707a2*, respectively (Fig. 4B). Testa rupture was not affected by either ABA added to the medium or the ABA biosynthesis mutations (Fig. 4A).

Thus, endosperm rupture, but not testa rupture, is the primary target for the inhibitory action of ABA on after-ripened *Arabidopsis* and *Lepidium* seeds.

Endosperm weakening occurs during Lepidium seed germination and is inhibited by ABA and promoted by gibberellin

As shown for other after-ripened seeds, addition of 10 µM GA₄₊₇ did not appreciably affect the kinetics of endosperm rupture of *Lepidium* seeds incubated in continuous light (data not shown). Flurprimidol (FLU) is known to be a highly specific inhibitor of gibberellin biosynthesis; the targets of FLU are cytochrome P450-dependent monooxygenases that catalyze the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Rademacher 2000). We used FLU in experiments and found that it inhibits endosperm rupture of *Lepidium* and *Arabidopsis* seeds (Fig. 5). FLU did not affect testa rupture of *Lepidium* seeds (Fig. 5B) and of a subpopulation of *Arabidopsis* seeds (Fig. 5A; 'lower' half), but FLU delayed testa rupture of the other subpopulation of *Arabidopsis* seeds (Fig. 5A; 'upper' half). These findings strongly suggest that, as for ABA, endosperm rupture is the primary target for the action of endogenous gibberellin on after-ripened endospermic seeds. An additional possibility is that FLU acts by inhibiting gibberellin biosynthesis plus ABA degradation. Some gibberellin biosynthesis inhibitors are also inhibitors of the ABA 8'-hydroxylase, i.e. they inhibit ABA degradation (Kushiro et al. 2004). Whether FLU inhibits gibberellin

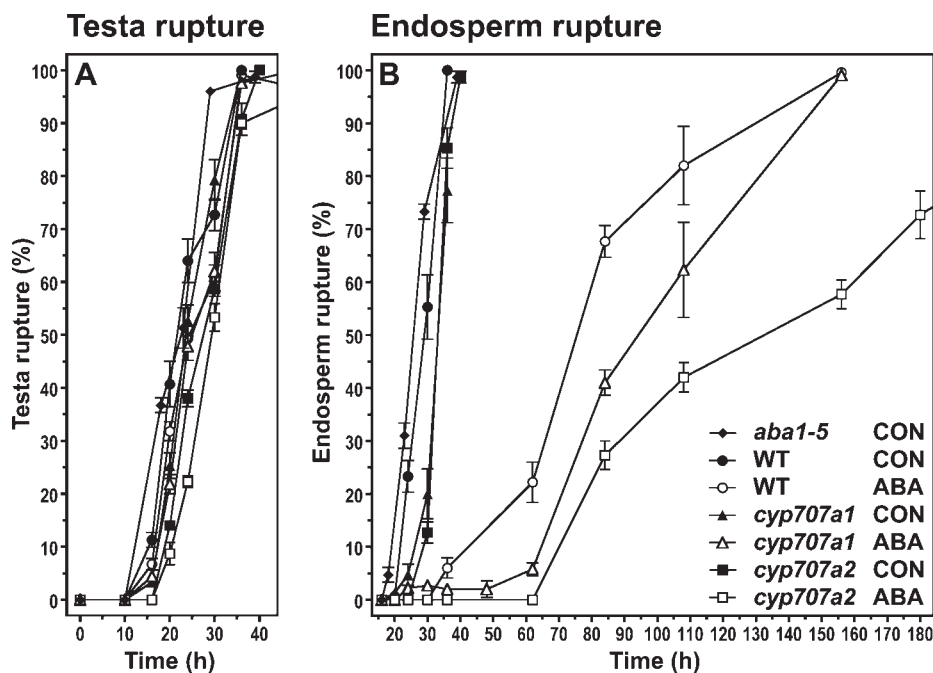


Fig. 4 The effect of ABA biosynthesis mutations on the time courses of testa rupture (A) and endosperm rupture (B) of *Arabidopsis thaliana*. Wild type (WT) seeds were compared with seeds of the ABA-deficient *aba1-5* mutant and of the *cyp707a1* and *cyp707a2* mutants, which are impaired in ABA degradation. The incidence of testa and endosperm rupture was scored with time after the start of incubation without (control; CON) or with 1 μ M ABA added to the medium. The incubation was in continuous light at 24°C. Mean values \pm SE from two independent experiments with at least three plates with 50 seeds each are presented.

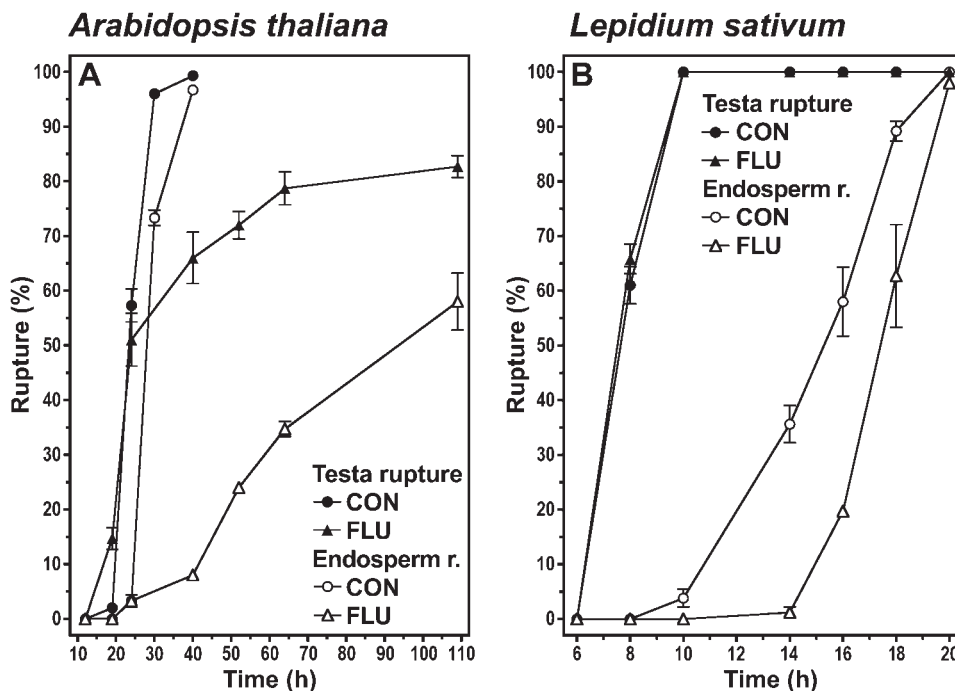


Fig. 5 The effect of gibberellin biosynthesis inhibition on the time courses of testa rupture and endosperm rupture of *Arabidopsis thaliana* (A) and *Lepidium sativum* (B). The incidence of testa and endosperm rupture was scored with time after the start of the incubation at 18°C (*Lepidium*) or 24°C (*Arabidopsis*) in continuous light. The medium was either without (control; CON) or with the gibberellin biosynthesis inhibitor flurprimidol (FLU; 10 μ M for *Arabidopsis*, 100 μ M for *Lepidium*) added. Mean values \pm SE from one experiment with at least three plates with 50 seeds each are presented.

biosynthesis only or gibberellin biosynthesis plus ABA degradation is not known. In either case, this will cause an increased ABA/gibberellin ratio that is in agreement with the observed delay of endosperm rupture.

For the direct measurement of endosperm weakening by the puncture force method we isolated micropylar endosperms from *Lepidium* seeds imbibed for defined periods of time without (control) and with hormones (ABA or gibberellin) added to the medium, as indicated (Fig. 6). While an average force of 37.8 ± 2.5 mN was necessary to puncture the endosperm at 8 h, which is just after testa rupture, only 19.5 ± 1.7 mN was necessary at 18 h when the radicle will protrude through the tissue within the next 2–4 h (control, Fig. 6). Thus, a 2-fold decrease in puncture force was evident during germination: the endosperm tissue weakened prior to its rupture by the radicle.

The addition of $10 \mu\text{M}$ ABA to the incubation medium inhibited endosperm rupture and endosperm weakening measured at 18 h (Fig. 6). At 120 h, i.e. just prior to the ABA-delayed endosperm rupture, endosperm weakening

was evident in the ABA-treated seeds and was comparable in value with the control at 18 h. This inhibition of endosperm weakening by ABA was dose-dependent, which is shown by the comparison of puncture force and endosperm rupture values for different ABA concentrations at 18 h (Fig. 6) and at 44 h (puncture force/endosperm rupture: 33.9 ± 1.0 mN/ $17.4 \pm 4.5\%$ for $5 \mu\text{M}$ ABA, 25.7 ± 2.8 mN/ 76.5% for $2.5 \mu\text{M}$ ABA, 100% endosperm rupture with $1 \mu\text{M}$ ABA). Thus, ABA inhibits endosperm weakening of *Lepidium* as well as endosperm rupture of *Lepidium* and *Arabidopsis* in a dose-dependent manner.

At 18 h, *Lepidium* seed populations without (control) and with gibberellin added to the medium had equal percentages of endosperm rupture (Fig. 6). At this time point endosperm weakening had caused a decrease in the puncture force by about 40 mN in the gibberellin-treated seeds compared with about 20 mN in the control. Essentially, gibberellin caused complete weakening to a near-zero puncture force value. Simultaneous treatment of *Lepidium* seeds with $10 \mu\text{M}$ GA₄₊₇ plus $10 \mu\text{M}$ ABA

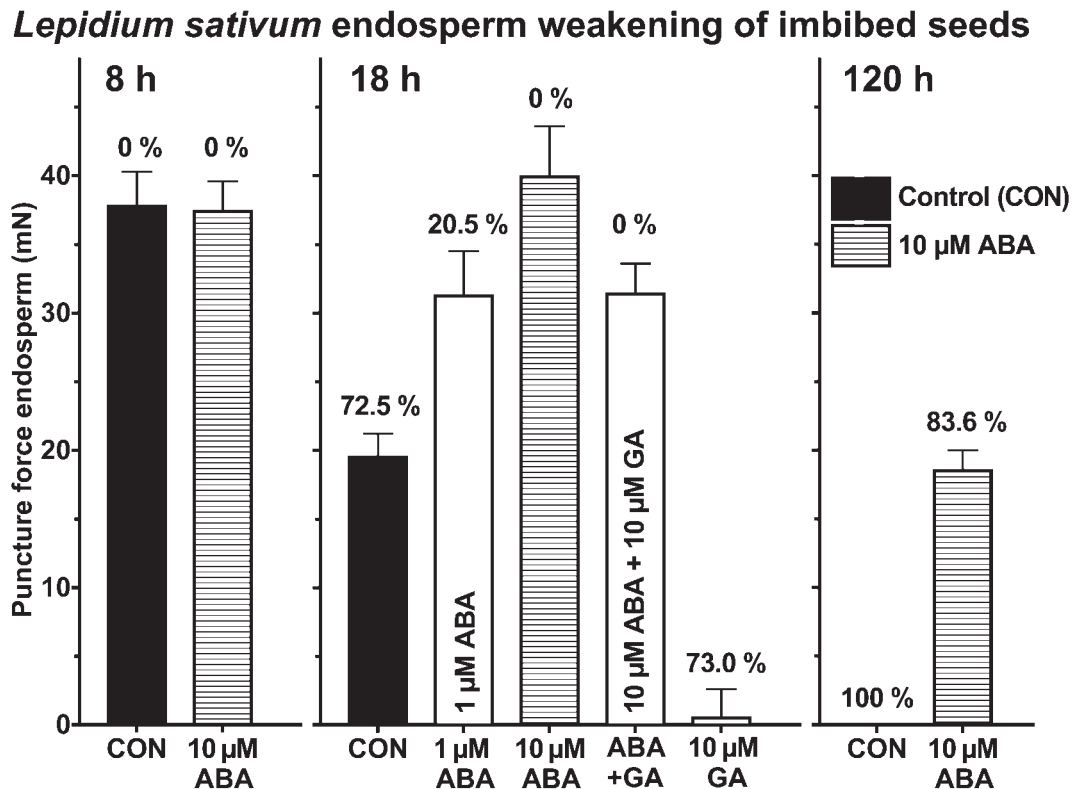


Fig. 6 Direct measurement of *Lepidium sativum* endosperm weakening of imbibed seeds by the puncture force method. Micropylar seed halves were dissected from whole seeds imbibed (continuous light, 18°C) for 8, 18 or 120 h without (control; CON) or with hormones (ABA and/or GA₄₊₇ in the concentrations indicated) added to the medium. Mean values \pm SE of at least 40 endosperm caps are presented. Note that all these seeds had completed testa rupture, but had intact endosperm. The numbers above the columns represent the percentage of seeds with ruptured endosperm in the corresponding seed populations.

showed that gibberellin is an antagonist of the ABA inhibition of endosperm weakening: a decrease in puncture force was already evident at 18 h (Fig. 6), and at 65 h a further decrease to 22.7 ± 2.5 mN was associated with approximately 70% endosperm rupture in the seed population treated with gibberellin plus ABA (only 5–10% for ABA-treated seeds). In agreement with a role for endosperm weakening in the control of endosperm rupture, gibberellin partially reversed the ABA inhibition of *Lepidium* endosperm rupture. The same pattern was obtained for *Arabidopsis*: at 84 h, endosperm rupture was >30% in the $10 \mu\text{M}$ GA_{4+7} plus $1 \mu\text{M}$ ABA-treated seed populations, whereas it was only 5–10% for the ABA-treated seeds. Thus, gibberellin is an antagonist of the ABA inhibition of *Lepidium* endosperm weakening and of *Lepidium* and *Arabidopsis* endosperm rupture.

Endosperm weakening is an organ-autonomous process, but requires induction by an early embryo signal, which can be replaced by gibberellin

It has been proposed that endosperm weakening requires induction by an embryo signal and that gibberellins are at least part of this signal (Bewley 1997a). In order to find out whether and if so in what time frame an embryo signal induces *Lepidium* endosperm weakening, we removed the embryo at different time points during the germination process. The empty seed halves with the micropylar endosperms were incubated separately in fresh medium. Puncture force was measured after 18 h, when endosperm weakening is evident in whole seeds (Fig. 7). Embryo removal at 8 h, subsequent incubation of the isolated seed halves for 10 h and puncture force measurement at the 18 h time point (after the start of seed

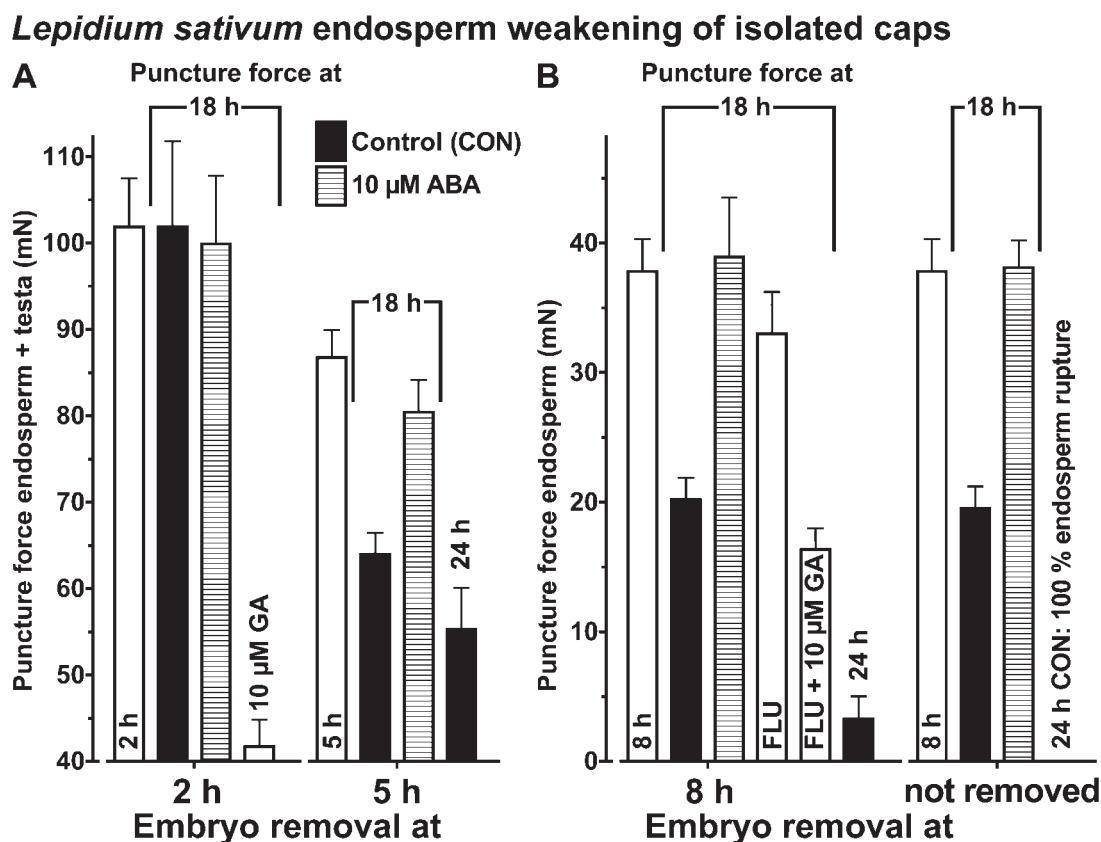


Fig. 7 Direct measurement of *Lepidium sativum* endosperm weakening of isolated micropylar caps by the puncture force method. (A) Embryos were removed from whole seeds imbibed for 2 and 5 h, respectively. The dissected micropylar seed halves were incubated until 18 h (i.e. for 16 or 13 h starting from the 2 or 5 h time point, respectively) in fresh medium without (control; CON) or with $10 \mu\text{M}$ ABA or $10 \mu\text{M}$ GA_{4+7} added. Note that the puncture force of endosperm plus testa was measured when 2 or 5 h caps were used. (B) Embryos were removed from whole seeds imbibed for 8 h, and the dissected micropylar seed halves were incubated until 18 or 24 h (i.e. for 10 or 16 h, respectively, starting from the 8 h time point) in fresh medium without (control; CON) or with $10 \mu\text{M}$ ABA, $10 \mu\text{M}$ GA_{4+7} or $100 \mu\text{M}$ flurprimidol (FLU; gibberellin biosynthesis inhibitor) added. Note that the puncture force of the endosperm was measured since 8 h caps from seeds after testa rupture were used. For direct comparison, puncture force values from seeds without embryo removed are given. Imbibition and incubation were in continuous light at 18°C . Mean values \pm SE of at least 40 endosperm caps are presented.

imbibition) resulted in the same degree of endosperm weakening as found in whole seeds at 18 h (Fig. 7B). The change in puncture force of isolated endosperms, i.e. a decrease of approximately 20 mN, corresponded to that found in whole seeds. The remaining puncture force at 18 h of approximately 20 mN decreased further when the isolated endosperms were incubated for a longer time; at 24 h, approximately 3 mN was measured (Fig. 7B).

No endosperm weakening took place if the embryo was removed after 2 h, but weakening of about 20 mN was measured at the 18 h time point if the embryo was removed after 5 h of seed incubation (Fig. 7A). A further decrease of approximately 10 mN was measured at the 24 h time point. An embryo–endosperm interaction in the time frame between 2 and 5 h is thus sufficient to induce weakening. After this period, the endosperm weakening does not require the presence of the embryo. The addition of 10 μ M GA₄₊₇ replaced the embryo signal and caused complete weakening of micropylar endosperms isolated at 2 h. Thus, an embryo signal is evident in *Lepidium* seeds between 2 and 5 h after the start of imbibition. It induces micropylar endosperm weakening and can be replaced by gibberellin.

Addition of ABA or the gibberellin biosynthesis inhibitor FLU to the incubation medium of endosperm caps isolated at the 8 h time point inhibited endosperm weakening, while simultaneous treatment with FLU plus gibberellin fully restored it (Fig. 7B). Endosperm weakening therefore appears to require de novo gibberellin biosynthesis in the micropylar endosperm cap. Thus, after the induction by an embryo signal, endosperm weakening is an organ-autonomous process evident in isolated micropylar endosperm caps and is controlled by the ABA/gibberellin ratio.

Discussion

The endosperm is known to act as a barrier for radicle protrusion and thereby the completion of germination in seeds from several angiosperm clades. Examples for this include *Trollius* (basal eudicots; Hepher and Roberts 1985), *Chenopodium* (core eudicots, caryophyllids; Karssen 1976), and several well studied species of the Solanaceae (e.g. tomato and tobacco) and Asteraceae (lettuce) (core eudicots, asterids; Hilhorst 1995, Bewley 1997a, Leubner-Metzger 2003). In contrast, only very little is known about species of the rosoid clade of the core eudicots. The rosoid clade includes the Brassicaceae with the model plant *Arabidopsis*, but it is not known whether the thin endosperm present in mature *Arabidopsis* seeds acts as a barrier for radicle protrusion. If it does, one would expect the endosperm to weaken before the radicle can protrude. At this point, *Arabidopsis* has its limits as a model system,

because of its small seed size, which makes it impossible to quantify endosperm weakening directly by measuring puncture force. We demonstrated by direct quantification of the puncture force that endosperm weakening occurs in the much bigger seeds of *L. sativum* (garden cress). *Lepidium* is a very close relative of *Arabidopsis* from the same subfamily (Brassicoideae, Brassicaceae; Hall et al. 2002, Koch et al. 2003). We showed that the seeds of *Lepidium* are highly similar in structure to those of *Arabidopsis*: a thin layer of endosperm cells is retained in the mature seeds and completely encloses the fully developed embryo. The micropylar endosperm surrounding the radicle tip is composed of one cell layer in *A. thaliana* (e.g. Brown et al. 1999, Debeaujon et al. 2000, Windsor et al. 2000, Liu et al. 2005), 1–2 cell layers in *L. sativum* (this work, Vaughan et al. 1971, Corner 1976) and a few cell layers in *Lepidium virginicum* (O'Brien and McCully 1969, Nguyen et al. 2000). Earlier work with *Arabidopsis* (reviewed in Koornneef et al. 2002, Yamaguchi and Kamiya 2002, Kucera et al. 2005) and *Lepidium* (e.g. Asami et al. 1998) together with our parallel experiments with both species (this work) strongly support the view that the seed germination physiology of *Arabidopsis* and *Lepidium* is highly similar.

Arabidopsis (Liu et al. 2005) and *Lepidium* (this work) exhibit a two-step germination, in which testa rupture and endosperm rupture are sequential events. Such two-step germination is widespread over the entire phylogenetic tree and has been described for many species, e.g. for *Trollius* (Ranunculaceae; Hepher and Roberts 1985), *Chenopodium* (Amaranthaceae; Karssen 1968, Karssen 1976), *Nicotiana* and *Petunia* (Cestroideae subfamily of the Solanaceae, Leubner-Metzger et al. 1995, Krock et al. 2002, Petruzzelli et al. 2003). In our present work, we utilized this distinction to demonstrate that endosperm rupture, but not testa rupture, of *Arabidopsis* and *Lepidium* is inhibited by the plant hormone ABA. This inhibitory effect of ABA is counteracted by gibberellin supporting the view that endosperm rupture is under the control of an ABA–gibberellin antagonism (Koornneef et al. 2002, Yamaguchi and Kamiya 2002, Leubner-Metzger 2003, Kucera et al. 2005). Experiments in lettuce with radioactive ABA showed that this substance is readily taken up from the medium within the initial 2–4 h of seed imbibition (McWha and Hillman 1973). We therefore can assume for our experiments with *Arabidopsis* that the 24 h stratification pretreatment ensures significant ABA uptake early during imbibition. We also utilized the permeation technique of dry seeds with dichloromethane (Meyer and Mayer 1971). Permeation of dry *Lepidium* seeds with ABA delayed endosperm rupture, but did not affect testa rupture; and, permeation of gibberellin had no effect (data not shown). The finding that endosperm rupture, but not testa rupture,

is the target site of the inhibition by ABA treatment is further supported by the fact that ABA does not inhibit testa rupture of endospermless *Brassica* seeds (Schopfer and Plachy 1984).

Further support for this conclusion is evident from our experiments with *Arabidopsis* mutants with altered endogenous ABA contents. Mutants such as *aba1-5* are ABA deficient and non-dormant (Debeaujon et al. 2000). Mutants that are impaired in ABA degradation such as *cyp707a1* and *cyp707a2* are characterized by seeds with enhanced dormancy, increased ABA contents and a longer period required for after-ripening (Kushiro et al. 2004, Millar et al. 2006). Although the endogenous ABA contents of dry *cyp707a1* and *cyp707a2* mutant seeds are increased 5- to 10-fold (Kushiro et al. 2004, Okamoto et al. 2006), testa rupture of after-ripened seeds of these mutants was not affected. ABA biosynthesis and ABA degradation in *Arabidopsis* seeds is localized in the embryo as well as in the endosperm (Lefebvre et al. 2006, Okamoto et al. 2006). The high abundance of *CYP707A2* mRNA in the dry seeds, and its transient expression pattern during early imbibition (6 h), suggest that ABA degradation in seeds is mainly achieved by the CYP707A2 isoform (Kushiro et al. 2004, Millar et al. 2006, Okamoto et al. 2006). This *CYP707A2* mRNA expression during early imbibition is localized in the radicle tip and the micropylar endosperm, suggesting that the ABA degradation during early imbibition is mediated by the CYP707A2 enzyme expressed in these tissues. These results are in agreement with our finding that ABA caused a stronger delay in *cyp707a2* endosperm rupture compared with WT and *cyp707a1*. We speculate that endosperm weakening is delayed in the *Arabidopsis cyp707a2* mutant due to impaired ABA degradation and that this is, at least in part, the reason for the higher ABA sensitivity of the *cyp707a2* endosperm rupture.

Sequential testa rupture and endosperm rupture is a major experimental advantage making it easier to assign enzymes, transcription factors and plant hormones to their target sites during germination. This is an important experimental feature of our system compared with tomato or pepper seeds (Solanaceae subfamily of the Solanaceae), where no visible distinction between the two landmarks is possible (Watkins and Cantliffe 1983, Groot and Karssen 1987, Petruzzelli et al. 2003). The micropylar caps of tomato and pepper consist of endosperm plus testa tissue covering the radicle tip. ABA treatment does not inhibit germination scored as initial radicle extension growth of detipped (surgical removal of the micropylar cap) tomato seeds (Liptay and Schopfer 1983, Groot and Karssen 1987, Groot and Karssen 1992). Detipping can also replace the requirement for treatment with gibberellin of gibberellin-deficient mutant seeds of tomato. These results support the hypothesis that the primary control of timing radicle emergence

resides in the micropylar endosperm cap, and that endosperm weakening is promoted by gibberellin and inhibited by ABA (Groot and Karssen 1987, Groot and Karssen 1992, Ni and Bradford 1993). Direct measurement of the weakening using the puncture force method demonstrated that it occurs prior to radicle protrusion and is promoted by gibberellin in tomato (Groot and Karssen 1987, Groot and Karssen 1992, Wu et al. 2000), pepper (Watkins and Cantliffe 1983), coffee (da Silva et al. 2005), *Fraxinus excelsior* (Finch-Savage and Clay 1997) and *Syringa* spp. (Junttila 1973). All of these species belong to the asterid clade and none exhibits a visible two-step germination. In *Lepidium*, endosperm weakening can be measured without interference of the testa. We found that endosperm weakening occurs prior to endosperm rupture of *Lepidium* seeds and that it is promoted by gibberellin. Thus, gibberellin-promoted endosperm weakening is also evident in endospermic Brassicaceae seeds, i.e. in seeds of the rosid clade.

Tomato and coffee endosperm weakening seems to be biphasic (Groot and Karssen 1992, Toorop et al. 2000, Wu et al. 2000, da Silva et al. 2004): the first phase of endosperm weakening is ABA insensitive; the second phase is inhibited by ABA. This suggests that in these seeds weakening is achieved by at least two distinct sets of molecular mechanisms. In contrast to these rosid species, we found only one phase of endosperm weakening in *Lepidium*, which can be inhibited by ABA in a dose-dependent manner. Interestingly, the overall change in puncture force of the *Lepidium* endosperm caps was equal at about 20 mN with and without ABA. The association of the percentage of endosperm rupture with equal puncture force values in control and ABA-treated seeds supports our working hypothesis that endosperm weakening is required for radicle protrusion and contributes to its timing.

The endosperm layers of mature seeds of *Lepidium*, *Arabidopsis*, *Chenopodium* and lettuce are thin compared with those of tomato, pepper and coffee. *Chenopodium* seeds are too small for puncture force measurements, but experiments with ABA and scarification are in agreement with the hypothesis that the endosperm weakens prior to endosperm rupture (Karssen 1968, Karssen 1976, Bewley 1997a). Numerous publications (e.g. Ikuma and Thimann 1963, Pavlista and Haber 1970, Jones 1974, Halmer et al. 1975, Pavlista and Valdovinos 1978, Tao and Khan 1979, Psaras and Paragamian 1984, Abeles 1986, Brooks and Mitchell 1988, Nijse et al. 1998, Toorop et al. 1999) are indirect support for the hypothesis that weakening of the thin lettuce endosperm occurs and is a prerequisite for endosperm rupture. Structural modifications in the endosperm of lettuce opposite the radicle tip were observed in whole seeds prior to radicle emergence and are in agreement

with the occurrence of endosperm weakening caused by cell wall-modifying proteins (Jones 1974). These structural modifications may occur as very local weakening in the micropylar endosperm and may include cell wall loosening, loss of cell-to-cell adhesion and cell autolysis (e.g. Pavlista and Haber 1970, Jones 1974, Pavlista and Valdovinos 1978, Psaras and Paragamian 1984, Nijse et al. 1998). ABA delays endosperm rupture of lettuce (Toorop et al. 1999), but the effect of ABA on lettuce endosperm weakening has not been measured directly.

Our results demonstrate that early during imbibition (between 2 and 5 h) an embryo signal is necessary and sufficient to induce *Lepidium* endosperm weakening, but that the subsequent molecular mechanisms are organ autonomous and under gibberellin-ABA control. Gibberellin treatment of early isolated endosperm caps can replace the embryo signal and causes endosperm weakening. This appears to be different from isolated lettuce endosperm, where gibberellin only modifies the endosperm when it is isolated from seeds during late imbibition (Psaras and Paragamian 1984). In agreement with our results for gibberellin, gibberellin induced and ABA inhibited the weakening of isolated micropylar caps of tomato that were dissected early during imbibition, i.e. at 3 h, and measured at 24 h (Groot and Karssen 1992). The hypothesis that gibberellin is an embryo signal for the induction of *Lepidium* endosperm weakening is consistent with published work on gibberellin biosynthesis and response during *Arabidopsis* seed germination (Yamaguchi et al. 2001, Yamaguchi and Kamiya 2002, Ogawa et al. 2003, Yamauchi et al. 2004). Gibberellin biosynthesis in the embryo is induced at specific sites during imbibition, and bioactive gibberellins accumulate just prior to radicle protrusion. This gibberellin also induces gibberellin-responsive genes at other locations including the *Arabidopsis* endosperm. These findings strongly suggest (i) that gibberellin moves as an embryo signal to the endosperm; (ii) that gibberellin induces gene expression in the endosperm; and (iii) that these genes might facilitate gibberellin-controlled endosperm weakening. Furthermore, low temperature pre-incubation, used to promote and synchronize *Arabidopsis* seed germination, further enhanced the expression of some gibberellin-responsive genes and caused additional expression of gibberellin biosynthesis genes in the endosperm (Ogawa et al. 2003, Yamauchi et al. 2004). In agreement with this, our experiments with isolated endosperm caps of *Lepidium* strongly suggest that de novo gibberellin biosynthesis is evident in the micropylar endosperm and is required for endosperm weakening. Taken together, these results show that gibberellin is not only an embryo signal, but also a locally generated signal for endosperm weakening of *Arabidopsis* and *Lepidium*.

In conclusion, our results clearly demonstrate that *Lepidium* and *Arabidopsis* endosperm rupture is promoted by gibberellin and inhibited by ABA, and that *Lepidium* endosperm weakening is promoted by gibberellin and inhibited by ABA. These results support the hypothesis that the gibberellin-ABA control of endosperm rupture is mediated, at least in part, by the antagonistic effects on endosperm weakening. The molecular mechanisms that control endosperm weakening might differ among seeds from distinct angiosperm clades. A 'one-phase' ABA-inhibited endosperm weakening is evident in *Lepidium* seeds. We speculate that during evolution the endospermic Brassicaceae seeds have retained ABA-inhibitable molecular mechanisms also found in asterid seeds (second phase of endosperm weakening), whereas the ABA-insensitive phase of endosperm weakening was lost. *Lepidium*, a close relative of *Arabidopsis*, is a new rosoid seed model system for endosperm weakening. The complementary advantages of both systems will be exploited in future experiments to investigate the molecular mechanisms of endosperm weakening.

Materials and Methods

Light microscopy

Seeds were imbibed for 2–3 h and fixed in a buffer containing 4% (w/v) para-formaldehyde, 0.25% (v/v) glutaraldehyde, 10 mM sodium phosphate and 100 mM NaCl overnight at 4°C. Fixation was followed by an ethanol dilution series and subsequent stepwise exchange of ethanol with xylol. Seeds were embedded in paraffin and cut into 10 µm sections. To remove the xylol, sections were incubated in a decreasing ethanol series. Staining was carried out in 0.05% (w/v) toluidine blue solution followed by an exchange of ethanol with xylol. An Axioplan 2 microscope (Zeiss, Oberkochen, Germany) was used for bright field microscopy. Images were taken with an EOS D30 digital camera (Canon, Krefeld, Germany).

Seeds and germination assays

Lepidium sativum L. 'Gartenkresse, einfache' seeds (Juliwa, Heidelberg, Germany) were incubated in Petri dishes containing 6 ml of 1/10 Murashige-Skoog medium without hormones or vitamins (Duchefa, Haarlem, The Netherlands), adjusted to pH 7.0, and two layers of filter paper. The Petri dishes were sealed with parafilm, placed in a Sanyo Versatile Environmental Test Chamber (MLR-350) and incubated at 18°C in continuous white light ($8.4 \mu\text{mol s}^{-1} \text{m}^{-2}$). Germination experiments were performed with fully after-ripened seeds of *L. sativum*. *Arabidopsis thaliana* (L.) Heyhn. seeds were incubated on the same medium solidified with 1% (w/v) agar-agar for 24 h at 4°C and subsequently in an MLR-350 chamber at 24°C in continuous white light ($8.4 \mu\text{mol s}^{-1} \text{m}^{-2}$). *Arabidopsis thaliana* ecotype Columbia (WT) and mutant seeds in the Columbia background were used. Homozygous seeds of the *aba1-5* (CS155, The Nottingham Arabidopsis Stock Centre), *cyp707a1* and *cyp707a2* (Dr. Eiji Nambara, RIKEN Institute, Japan) mutants were compared with WT from the same harvest. Germination experiments were performed with conditionally dormant (Table 1), not fully after-ripened (2–3 months of dry storage; Fig. 3) and fully

after-ripened (>9 months of dry storage; all other experiments) *A. thaliana* seed batches. Testa rupture and endosperm rupture were scored using a binocular microscope. If indicated, ABA (Sigma, Taufkirchen, Germany), GA₄₊₇ (Duchefa) or the gibberellin biosynthesis inhibitor FLU (Duchefa) was added to the medium. Seeds were photographed using a Leica DCF480 digital camera (Bensheim, Germany) attached to a stereomicroscope (Leica Mz 12.5). The software used was IM 1000 (Leica) and Adobe Photoshop.

Puncture force measurements

Puncture force was measured using a custom-made machine. Lepidium seeds were cut in half after the indicated incubation period, the embryo carefully removed and the empty, intact endosperm cap placed in a seed-shaped mould. A metal probe (diameter 0.3 mm) was driven into the endosperm cap (2 mm min⁻¹), and the force it took to rupture the tissue registered as a peak on an attached recorder. The corresponding force was calculated based on a calibration curve using defined masses of water. For the experiments on the embryo signal (Fig. 7), seeds were incubated under control conditions (MLR-350 chamber as described above) for the indicated periods of time and dissected. The empty seed halves with the micropylar endosperm were then transferred to fresh medium and further incubated under the indicated conditions. Puncture force was measured on the seed halves at the times indicated.

Acknowledgments

Our research is supported by the Deutsche Forschungsgemeinschaft (DFG grant LE 720/6-1). We thank Eiji Nambara (RIKEN Institute, Japan) for kindly providing the *cyp707a* mutant seeds, Ada Linkies for critical reading of the manuscript, and Kai Gräber for expert technical assistance.

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