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Cross-species approaches to seed dormancy and germination: conservation and biodiversity of ABA-regulated mechanisms and the Brassicaceae *DOG1* genes

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Abstract Seed dormancy is genetically determined with substantial environmental influence mediated, at least in part, by the plant hormone abscisic acid (ABA). The ABA-related transcription factor *ABI3/VP1* (*ABA INSENSITIVE3/VIVIPAROUS1*) is widespread among green plants. Alternative splicing of its transcripts appears to be involved in regulating seed dormancy, but the role of *ABI3/VP1* goes beyond seeds and dormancy. In contrast, *DOG1* (*DELAY OF GERMINATION 1*), a major quantitative trait gene more specifically involved in seed dormancy, was so far only known from *Arabidopsis thaliana* (*AtDOG1*) and whether it also has roles during the germination of non-dormant seeds was not known. Seed germination of *Lepidium sativum* ('garden cress') is controlled by ABA and its antagonists gibberellins and ethylene and involves the production of apoplastic hydroxyl radicals. We found orthologs of *AtDOG1* in the Brassicaceae relatives *L. sativum* (*LesaDOG1*) and *Brassica rapa* (*BrDOG1*) and compared their gene structure and the sequences of their transcripts expressed in seeds. Tissue-specific analysis of *LesaDOG1* transcript levels in *L. sativum* seeds showed that they are degraded upon imbibition in the radicle and the micropylar endosperm. ABA inhibits germination in that it delays radicle protrusion and endosperm weakening and it increased *LesaDOG1* transcript levels during early germination due to enhanced transcription and/or inhibited degradation. A reduced decrease in *LesaDOG1* transcript levels upon ABA treatment is evident in the late germination phase in both tissues. This temporal and ABA-related transcript expression pattern suggests a role for *LesaDOG1* in the control of germination timing of non-dormant *L. sativum* seeds. The possible involvement of the ABA-related transcription factors ABI3 and ABI5 in the regulation of *DOG1* transcript expression is discussed. Other species of the monophyletic genus *Lepidium* showed coat or embryo dormancy and are therefore highly suited for comparative seed biology.

Keywords ABI3/VP1 transcription factor · Coat and embryo dormancy · Comparative Brassicaceae seed biology · Delay of Germination 1 (DOG1) · Endosperm weakening · Reactive oxygen species · Seed biomechanics

Web: 'The Seed Biology Place': www.seedbiology.de

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Introduction

Dormancy has been defined by Lang et al. (1987) as a temporary suspension of visible growth of any plant structure containing a meristem. Dormancy may be associated with diverse plant structures that appeared at different times during evolution and may have evolved in adaptation to a variety of habitats and climates. The evolutionary ecology of dormancy associated with whole

plants (Shefferson 2009) or plant structures including vegetative buds (Horvath et al. 2003) and seeds (Baskin and Baskin 2004) has been reviewed. The bud was probably born more than 400 million years ago (MYA), in the Early Devonian, in the very early land plants. The origin of the seed habit goes back to the Late Devonian, about 385 MYA. Recent work with model species strongly suggests that similar molecular mechanisms are underlying dormancy induction and release of seeds and vegetative buds (Chao et al. 2007; Finch-Savage and Leubner-Metzger 2006; Holdsworth et al. 2008; Kucera et al. 2005; Rohde and Bhalerao 2007). Seed dormancy and endodormancy of vegetative buds are innate properties that define the environmental conditions in which the seed is able to germinate and the bud is able to sprout. Innate blocks have evolved differently across species through adaptation to the prevailing environment so that seed germination occurs when conditions for establishing a new plant generation are likely to be suitable and that bud sprouting occurs in the right season. The ecologist definition of dormancy proposed by Baskin and Baskin (2004) can be applied to seed and bud dormancy: a dormant seed or endodormant bud does not have the capacity to germinate or sprout in a specified period of time under any combination of normal physical environmental factors that are otherwise favorable for its germination or sprouting, i.e., after the seed or bud becomes non-dormant.

Germination of a non-dormant seed requires water, oxygen, an appropriate temperature, and in some cases light. It commences with the uptake of water by imbibition of the dry seed, followed by embryo expansion. The germination process is completed when the embryo (usually the radicle) emerges from the seed by protrusion of all covering layers. In Solanaceae like tobacco and in the Brassicaceae *Arabidopsis thaliana* (hereafter Arabidopsis) and *Lepidium sativum* ('garden cress') a 'two-step' germination process is evident with testa rupture and endosperm rupture as two subsequent visible events (Liu et al. 2005; Manz et al. 2005; Müller et al. 2006; Petruzzelli et al. 2003a). The embryo elongation leading to radicle protrusion (endosperm rupture) is due to cell elongation and in Arabidopsis a discrete cell elongation region that is immediately proximal to the radicle, i.e., the lower hypocotyl and hypocotyl-radicle transition zone, was identified (Sliwinska et al. 2009). Radicle protrusion is considered as visible sign for the completion of the germination process, and is followed by seedling growth, which constitutes a physiologically and molecularly distinct developmental program. The covering layers, testa and endosperm in many seeds, contribute to their dormancy and to germination timing after dormancy has been released (e.g., Bethke et al. 2007; Debeaujon and Koornneef 2000; Leubner-Metzger 2002, 2005; Piskurewicz et al. 2009). Seed structure is therefore, in addition to the ecophysiology of the whole seed, an important attribute of seed dormancy mechanism(s) (Finch-Savage and Leubner-Metzger 2006). Therefore, a distinction between *embryo dormancy* and *coat dormancy* is described later in the section about seed germination and dormancy of the genus *Lepidium* ('cress'). A major issue arising from the importance of seed structure is that the expression analysis of dormancy and germination genes should be based on tissue-specific level analyses. This tissue-specific aspect is very important for the regulation of down-stream gene expression by the 'ancient' plant hormone abscisic acid (ABA) (e.g., Leubner-Metzger 2002; Linkies et al. 2009; Millar et al. 2006). ABA is known to be involved in dormancy induction of seeds and buds, but also inhibits the germination of non-dormant seeds (Finch-Savage and Leubner-Metzger 2006). In species with a 'two-step' germination process like Arabidopsis and *L. sativum* (Linkies et al. 2009; Müller et al. 2006, 2009b) or tobacco (Manz et al. 2005) ABA did not affect the kinetics of testa rupture, but it specifically inhibited endosperm weakening and rupture of non-dormant seeds.

Seed and bud dormancy induction, maintenance, and release, as well as germination and sprouting share some interesting similarities in the molecular components that are involved (reviewed by Bailly 2004; Bentsink and Koornneef 2008; Chao et al. 2007; Finch-Savage and Leubner-Metzger 2006; Holdsworth et al. 2008; Matilla and Matilla-Vazquez 2008; Rohde and Bhalerao 2007). Regulation of bud dormancy by the environment involves the circadian clock and recent work by Penfield and Hall (2009) show that this is also the case for Arabidopsis seeds. In both developmental processes the environmental signals are mediated, at least in part, by hormone-related changes. The involvement of the plant hormones ABA, GA and ethylene in seed and bud biology is supported by recent transcriptome analyses of poplar bud (Ruttink et al. 2007) and Arabidopsis seed (e.g. Cadman et al. 2006; Carrera et al. 2008; Finch-Savage et al. 2007; Nakabayashi et al. 2005) dormancy cycling, as well as ABA-inhibited endosperm weakening (Linkies et al. 2009). Genes associated with these hormones, e.g., the *ABI3/VP1*-type transcription factors that mediate bud and seed ABA responses, are often widespread and mutations in these genes usually cause pleiotropic phenotypes. ABA constitutes an ancient signaling pathway and changes in transcriptional regulators and their gene promoter targets provide predominant mechanisms for the generation of novel phenotypes (Santner and Estelle 2009; Wasilewska et al. 2008). ABA is not restricted to plants since it also exists in metazoans ranging from the most primitive to the advanced on the evolution scale (sponges to humans). Wasilewska et al. (2008) state that cross-kingdom comparisons have shed light on the surprisingly ancient origin of ABA and its

attendant mechanisms of signal transduction. Intermediates in the signal transduction chain are also conserved between animals and plants including reactive oxygen species (ROS), but not the *ABI3/VP1* (*ABA INSENSITIVE3/VIVIPAROUS1*)-type transcription factors which are plant-specific and belong to the B3 DNA binding domain superfamily (Holdsworth et al. 2008; Rohde et al. 2002, 2007; Romanel et al. 2009).

Maize *viviparous1* (*vp1*) mutant seeds are non-dormant and germinate precociously due to the reduced ABA sensitivity in developing embryos (McCarty et al. 1989). The *VP1* genes of cereals, Arabidopsis *ABA Insensitive3* (*ABI3*) and their orthologs in gymnosperms and core eudicots encode transcription factors that are highly expressed in developing seeds. They are central components of the network that controls the expression of ABA responsive genes during seed maturation (Bentsink and Koornneef 2008; Holdsworth et al. 2008; Suzuki and McCarty 2008). This includes the ABA-promoted deposition of seed storage proteins, the induction and maintenance of seed dormancy, and the acquisition of desiccation tolerance. ABI3 is a major component of the ABA signaling pathway in seeds and a major determinant of embryo ABA sensitivity. Examples for ABI3/VP1 gene expression during seed maturation and dormancy induction include gymnosperms like yellow-cedar (*CnABI3, Callitropis nootkatensis* = *Chamaecyparis nootkatensis*, Lazarova et al. 2002), monocots (several cereals, e.g., Holdsworth et al. 1999; Hollung et al. 1997; McCarty et al. 1989), asterids (e.g., Chandler and Bartels 1997; Shiota et al. 1998) and rosids (e.g., Gagete et al. 2009; Nambara et al. 1995; Rohde et al. 2002; Stephen et al. 2004). A VP1-dependent positive feedback mechanism regulates ABA-induced VP1 expression during maize and wheat seed maturation (Cao et al. 2007; Utsugi et al. 2008). Mutants with defective *ABI3/VP1* genes are not only affected in seed development, but often also exhibit a de-repression of germination and post-germination processes. VP1 represses the GA-induction of α-amylase genes in the aleurone layer of germinated cereal caryopses (Hoecker et al. 1995, 1999; Leubner-Metzger 2007; Utsugi et al. 2008). In barley caryopses HvVP1 and HvABI5 are both involved in the ABA induction of stress genes in the aleurone layer (Casaretto and Ho 2003). A GA-derived signal passing from the endosperm to the embryo which acts to promote ABI3 expression in the tomato embryo was postulated by Bassel et al. (2006). In two recent publications Piskurewicz et al. (2008, 2009) showed that Arabidopsis endosperm rupture is inhibited by ABA due to the stimulation of ABI3 and ABI5 expression. ABI3/VP1 regulates the expression of MIP-type water channel proteins during seed germination of *Mesembryanthemum crystallinum* (common ice plant, Caryophyllids, Fukuhara and Bohnert 2000). In imbibed dormant yellow-cedar seeds *CnABI3* transcripts and proteins are expressed in the embryo and megagametophyte, but disappear upon dormancy release treatments (Ren and Kermode 1999; Zeng et al. 2003). Dormancy release of these gymnosperm seeds was associated with reduced seed ABA sensitivity, reduced ABA levels in embryo and megagametophyte, as well as ABA degradation and leaching. Stratification broke the dormancy of yellow-cedar (Ren and Kermode 1999) and white spruce (Downie and Bewley 1996) seeds. In both species this was associated with mechanical weakening of the megagametophyte and an increase in the growth potential of the embryo. Taken together, these findings are indicative for a role of ABA in the gymnosperm coat dormancy associated with the megagametophyte. ABA is therefore an important positive regulator of the coat-mediated non-deep physiological dormancy in the seeds of gymnosperms and angiosperms (Finch-Savage and Leubner-Metzger 2006; Holdsworth et al. 2008; Kucera et al. 2005). ABI3/VP1-type transcription factors are involved in regulating ABA-related molecular mechanisms of gymnosperm and angiosperm dormancy and germination.

Hexaploid bread wheat (*Triticum aestivum*) caryopses are characterized by relatively weak dormancy and are susceptible to preharvest sprouting (PHS), a phenomenon that is phenotypically similar to the viviparous germination observed in the maize *vp1* mutant (Gerjets et al. 2009; Holdsworth et al. 2006; Leubner-Metzger 2007). Hexaploid wheat possesses three *VP1* homeologs (*TaVP-A1, TaVP-B1, TaVP-D1*) and analysis of their transcripts in European cultivars and their ancestral species showed that the majority is alternatively spliced in embryos from mature seeds and does not have the capacity to encode full-length proteins with normal VP1 function (McKibbin et al. 2002; Wilkinson et al. 2005). McKibbin et al. (2002) showed that developing embryos from transgenic wheat grains expressing the *AfVP1* gene showed enhanced responsiveness to applied ABA compared to the control and less susceptibility to PHS. They concluded that alternative splicing of wheat *VP1* transcripts contributes to susceptibility to PHS in modern hexaploid wheat varieties. Utsugi et al. (2008) analysed *TaVP1* transcripts in a deeply dormant Japanese wheat cultivar that is also resistant to PHS. Although some alternatively spliced transcripts were found during seed development, the majority of *TaVP1* transcripts were correctly spliced. The regulation of alternative splicing differed between the three homeologs of *TaVP1* and the *TaVP-B1* homeolog may have an important role in the deep dormancy of this cultivar (Utsugi et al. 2008). *TaVP-B1* alleles of PHS-susceptible and -tolerant cultivars differed in insertions or deletions in the third intron which might affect their expression and ABA sensitivity of the different cultivars (Yang et al. 2007).

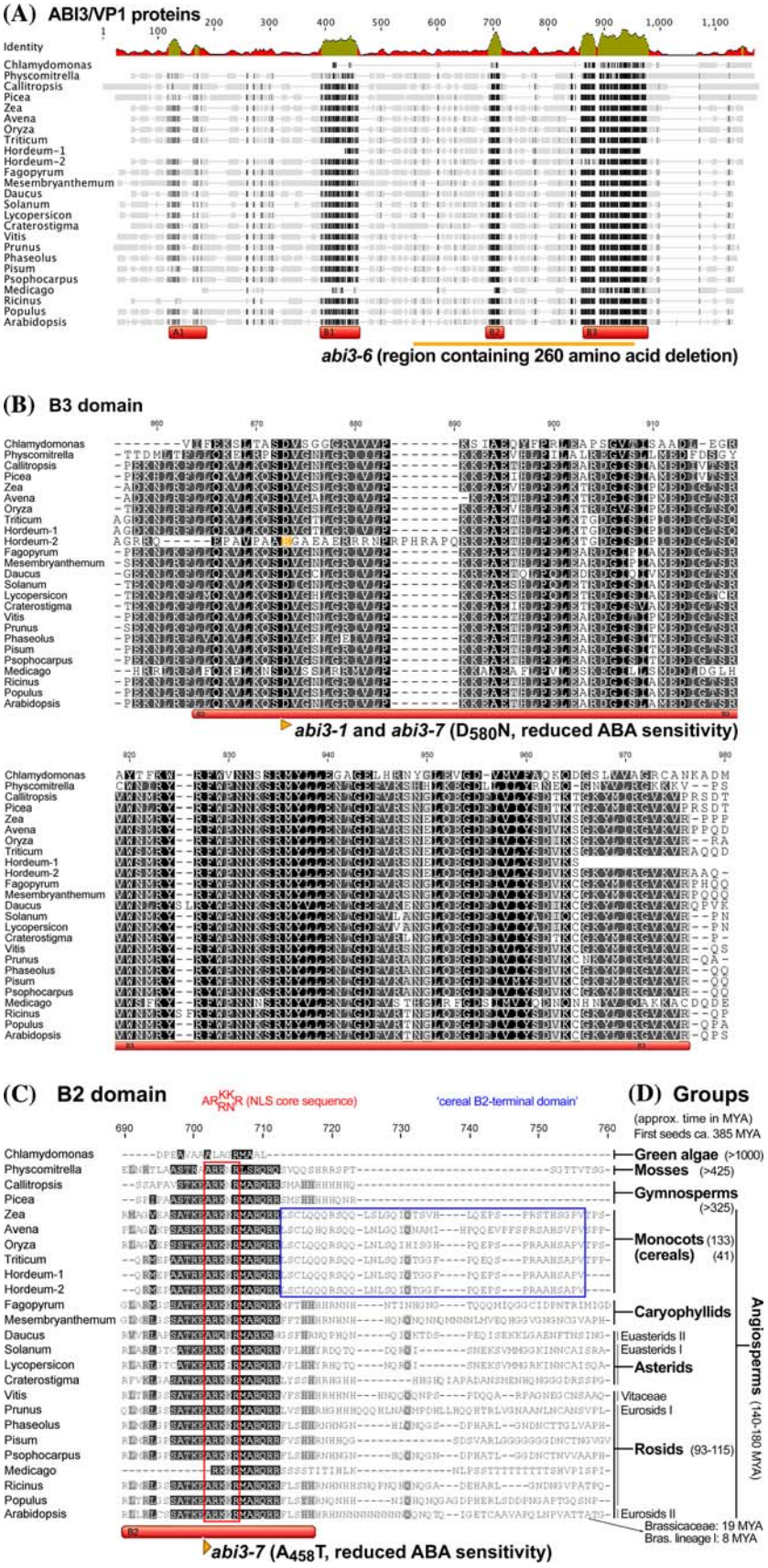
Although ABI3/VP1-type transcription factors are important in seed ABA signaling, they are not seed specific

and are involved in other developmental processes associated with meristems like dormancy of vegetative buds of Norway spruce (Footitt et al. 2003) and poplar (Rohde and Bhalerao 2007; Rohde et al. 2002). Poplar (*Populus trichocarpa*) ABI3 (PtABI3) is expressed in vegetative buds during natural bud set and this expression as well as growth cessation required for bud set are regulated by a critical day length. Overexpression or downregulation of PtABI3 results in altered bud development in short-day conditions. Constitutive overexpression of PtABI3 resulted in apical buds with large embryonic leaves and small stipules, whereas in antisense lines, bud scales were large and leaves were small. ABA levels peaked in buds concomitantly with PtABI3, suggesting that ABA and PtABI3 act simultaneously in bud set. PtABI3 is therefore an essential component of successful bud set that in turn is a precondition for the establishment of endodormancy. Formation of closed apical buds upon short-day induction is not only prevented by overexpression of ABI3, but also by expression of a dominant-negative version of the ethylene receptor ETR1 (Rohde et al. 2002, 2007; Ruonala et al. 2006). However, in both cases, at least some degree of dormancy is established, indicating that bud formation and dormancy *per se* are distinct, partially independent aspects of bud dormancy. ABI3/VP1-type transcription factors may have a general role in processes that require developmental arrest and quiescence including Arabidopsis apical dominance (paradormancy), but are also involved in plant stress tolerance and chloroplast/nuclear retrograde signaling (e.g., Footitt et al. 2003; Kotak et al. 2007; Rohde and Bhalerao 2007; Rohde et al. 2000).

ABI3/VP1-type transcription factors are not seed-plant specific, but are present in green plants from the green algae *Chlamydomonas reinhardtii* and the moss *Physcomitrella patens* to gymnosperms and angiosperms (see Fig. 1 for species and sequences). The evolution of the plant-specific B3 DNA binding superfamily, in which the ABI3/VP1-type transcription factors constitute one of the five families, showed that the ancestral B3 domain gave rise, after gene duplication events, to the different families (Romanel et al. 2009). The ABA-related transcription factor ABI3/VP1 genes are widespread among green plants and therefore, evolutionary old (origin $>$ 1,000 MYA). In Fig. 1 we have aligned the predicted protein sequences of ABI3/VP1-type transcription factors across the entire phylogenetic tree. Their different domains and roles in mediating ABA signaling in seeds is compared and discussed in the section 'Evolution and role of ABI3/VP1 transcription factors in seed and bud biology'. This provides a useful interpretation framework for the ABA-regulated molecular mechanisms of dormancy and germination presented in the subsequent sections.

Fig. 1 Alignment of deduced amino acid sequences of *ABI3/VP1* (*ABA INSENSITIVE3/VIVIPAROUS1*) proteins from plants. Alignments are comparisons of the **a** entire proteins showing the overview of the domain structure (with known domains A1, B1, B2, B3) and the regions of identity (visualized by the identity plot on top of *panel A* and by dark areas in the individual sequences), **b** B3 domain and the **c** B2 domain amino acid sequences. **d** The plant groups (*green* algae, mosses, gymnosperms and angiosperms) including the divergence time estimates of their crown subgroups (in 'million years ago', MYA) based on the current view of deep-level relationships (Franzke et al. 2009; Soltis et al. 2008; Wang et al. 2009). ABI3/VP1 protein sequences (NCBI accession number): *Chlamydomonas reinhardtii* CrABI3/VP1-like (XP_001693653.1), *Physcomitrella patens* PpABI3 (XP_001752973.1), *Callitropsis nootkatensis* CnABI3 (CAC19186.1), *Picea abies* PaVP1 (AAG22585.1), *Zea mays* ZmVP1 (NP_001105540.1), *Avena fatua* AfVP1 (CAA04553.1), *Oryza sativa* OsVP1 (EAY76922.1), *Triticum aestivum* TaVP1 (CAB91108.1), *Hordeum vulgare* HvVP1 ('Hordeum-1' AAO06117.1, 'Hordeum-2' CAD24413.1), *Fagopyrum esculentum* FeVP1 (BAC78904.1), *Mesembryanthemum crystallinum* McVP1 (BAA28779.1), *Daucus carota* DcABI3 (BAA82596.1), *Solanum tuberosum* StABI3-like (CAC84597.2), *Lycopersicon esculentum* LeABI3 (AAW84252.1), *Craterostigma plantagineum* CpVP1 (CAA04184.1), *Vitis vinifera* Vv(CAO63796.1), *Prunus avium* PaABI3 (AAQ03210.1), *Phaseolus vulgaris* PvABI3-like (BAB96578.1), *Pisum sativum* PsABI3-like (BAC10553.1), *Psophocarpus tetragonolobus* (BAD42433.1), *Medicago truncatula* MtABI3-like (ABD32571.1), *Ricinus communis* (EEF41288.1), *Populus trichocarpa* PtABI3 (CAA05921.1), *Arabidopsis thaliana* AtABI3 (CAA48241.1). Selected mutations of the Arabidopsis *ABI3* gene and conserved amino acids of a nuclear localization signal (NLS) core sequence are indicated

More general ABA-related molecular mechanisms include dormancy induction and maintenance which we have studied in different *Lepidium* species, as well as endosperm weakening and ROS production which we have studied in *L. sativum*. Expression of the *DOG1* (*DELAY OF GERMINATION1*) gene appears to be more or less seed-specific and the gene is probably evolutionary young since it has so far only been found in Arabidopsis (Bentsink et al. 2006) and other Brassicaceae (this work); the Brassicaceae evolved ca. 19 MYA (Franzke et al. 2009). Although the *DOG1* gene promoter contains *cis*-acting elements that strongly suggest regulation of *DOG1* expression by ABA (see the *DOG1* section of this work), direct evidence that ABA-inhibited endosperm weakening and rupture of non-dormant seeds is associated with altered *DOG1* gene expression was so far lacking. ABA is therefore a focus in our cross-species work on the molecular mechanisms of endosperm weakening and rupture and we present evidence that Brassicaceae *DOG1* gene expression during germination involves ABA signaling. Based on these results and the *DOG1* gene promoter motifs we propose that the ABA regulation of *DOG1* gene expression is mediated, at least in part, by the transcription factors ABI3 and ABI5. This extends the roles of *DOG1* from a dormancy gene to a Brassicaceae germination control gene.


(A) ABI3/VP1 proteins
Identity
Chlamydomonas
Physcomitrella
Callitropsis
Picea
Zea
Avena
Oryza
Triticum
Hordeum-1
Hordeum-2
Fagopyrum
Mesembryanthemum
Daucus
Solanum
Lycopersicon
Craterostigma
Vitis
Prunus
Phaseolus
Pisum
Psophocarpus
Medicago
Ricinus
Populus
Arabidopsis
A1
B1
B2
B3
abi3-6 (region containing 260 amino acid deletion)
(B) B3 domain
abi3-1 and abi3-7 (D580N, reduced ABA sensitivity)
(C) B2 domain
ARKKRN (NLS core sequence)
'cereal B2-terminal domain'
abi3-7 (A458T, reduced ABA sensitivity)
(D) Groups
(approx. time in MYA)
First seeds ca. 385 MYA
Green algae (>1000)
Mosses (>425)
Gymnosperms (>325)
Monocots (133)
(cereals) (41)
Caryophyllids
Euasterids II
Euasterids I
Asterids
Vitaceae
Eurosids I
Rosids (93-115)
Eurosids II
Angiosperms (140-180 MYA)
Brassicaceae: 19 MYA
Bras. lineage I: 8 MYA

Materials and methods

Plant material, germination and puncture-force measurements

Seeds were obtained from the following sources: *Lepidium sativum* L. FR1 ('Gartenkresse, einfache') and FR14 ('Keimsprossen') from Juliwa (Heidelberg, Germany); the *Lepidium* species *L. draba*, *L. campestre*, *L. didymus*, *L. ruderale*, *L. pedicellosum*, *L. pholidogynum* from B&T World Seeds (Aigues-Vives, France); *L. africanum*, *L. papillosum*, *L. bonariense*, *L. spinosum*, *L. rotundum*, and *L. phlebopetalum* were kindly provided by Prof. Dr. Klaus Mummenhoff (Fachbereich Biologie, Spezielle Botanik, University of Osnabrück, Germany); *L. divaricatum*, *L. hyssopifolium*, *L. pseudohyssopifolium*, *L. monoplocoides*, and *L. oxytrichum* were kindly provided by Dr. John L. Bowman (School of Biological Sciences, Monash University, Victoria, Australia).

For germination analysis, biomechanics and experiments that involved tissue separation (Figs. 2, 3d, 4, 6) after-ripened seeds of *L. sativum* FR1 and FR14 were incubated in petri dishes on two layers of filter paper with 6 ml 1/10 Murashige-Skoog salts in continuous white light (ca. 100 µmol s^{-1} m^{-2}) as described by Müller et al. (2006) at 24°C or as indicated. For germination and dormancy analysis of different *Lepidium* species (Fig. 3a–c; Table 1) seeds were incubated in continuous light on the same medium solidified with 1% (w/v) agar–agar at 24°C or as indicated. Where indicated, cis-S(+)-ABA (Duchefa, NL), gibberellin A_{4+7} (GA_{4+7}; Duchefa, NL) or 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma, Germany) were added in the concentrations indicated. Endosperm rupture and radicle emergence were scored using a binocular microscope. Puncture-force measurements were performed as described by Müller et al. (2006).

Tetrazolium assay testing embryo viability

After-ripened *Lepidium* spp. seeds were incubated as described for tissue separation for 4 h. Testa and endosperm were removed and embryos were stained according to the procedure described by Wharton (1955) in 1% (w/v) 2,3,5-triphenyltetrazolium chlorid (Sigma, Germany) in phosphate buffer (pH 7) at room temperature for the time indicated. As negative controls heat killed seeds (dry seeds were incubated at 100°C for 1 h) were used. Two biological replicates of 20 seeds each were analyzed per species.

Hydroxyl radical detection by EPR spectroscopy

Detection of apoplastic hydroxyl radicals was performed by electron paramagnetic resonance (EPR)-spectroscopy as described in Müller et al. (2009b). In brief, *L. sativum* FR1 seeds were dissected after incubation in the indicated media for 40 h. Isolated radicles or endosperm caps (100) were incubated for 3 h in spin-trapping solution [50 mM 4-POBN (α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone) containing 4% (v/v) ethanol] on a rotary shaker. EPR spectra were recorded for the incubation solution at room temperature in a flat cell with an ESR-300 X-band

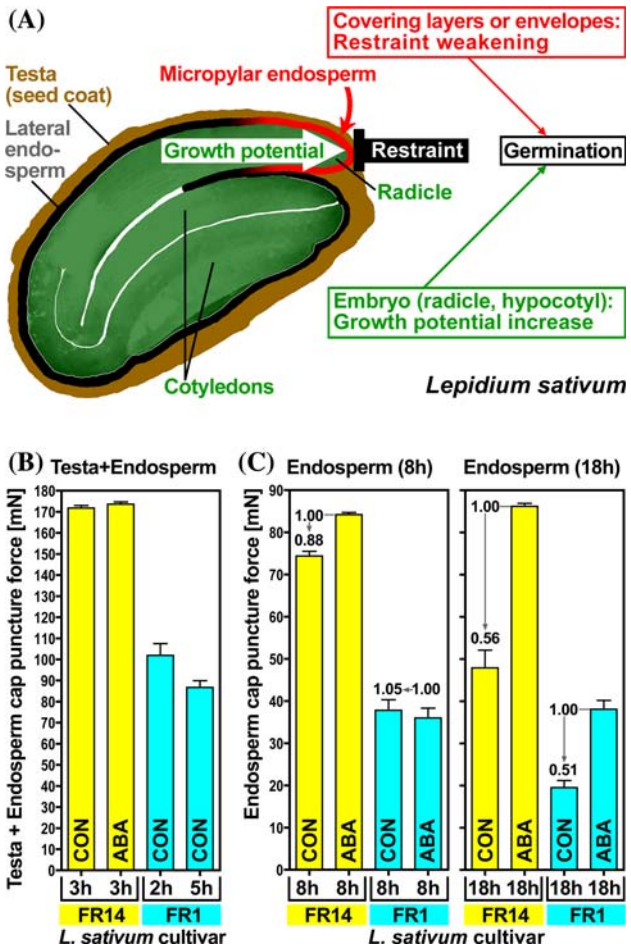


Fig. 2 *Lepidium sativum* ('garden cress') as a model species for seed biology research. **a** Mechanistic model for the control of germination and dormancy by opposing forces, the embryo growth potential and the restraint by the covering layers (micropylar endosperm and testa). **b**, **c** Comparative puncture-force analysis of restraint weakening and the effect of 10 µM ABA (abscisic acid) in the two *L. sativum* cultivars FR1 and FR14. **b** The combined puncture force of the testa plus the micropylar endosperm (cap) during early imbibition is ca. twofold higher in FR14 compared to FR1 and is not affected by ABA during the initial 3 h. Separate puncture-force analysis of the two tissues is technically not possible at these early time points. **c** Puncture-force analysis of endosperm cap weakening and the effect of ABA in FR1 and FR14. Endosperm cap resistance is ca. twofold higher in FR14 compared to FR1, but the relative changes (numbers above columns) and the inhibition of cap weakening by ABA are similar in both cultivars. Mean values ± SE of at least 50 puncture force measurements are presented

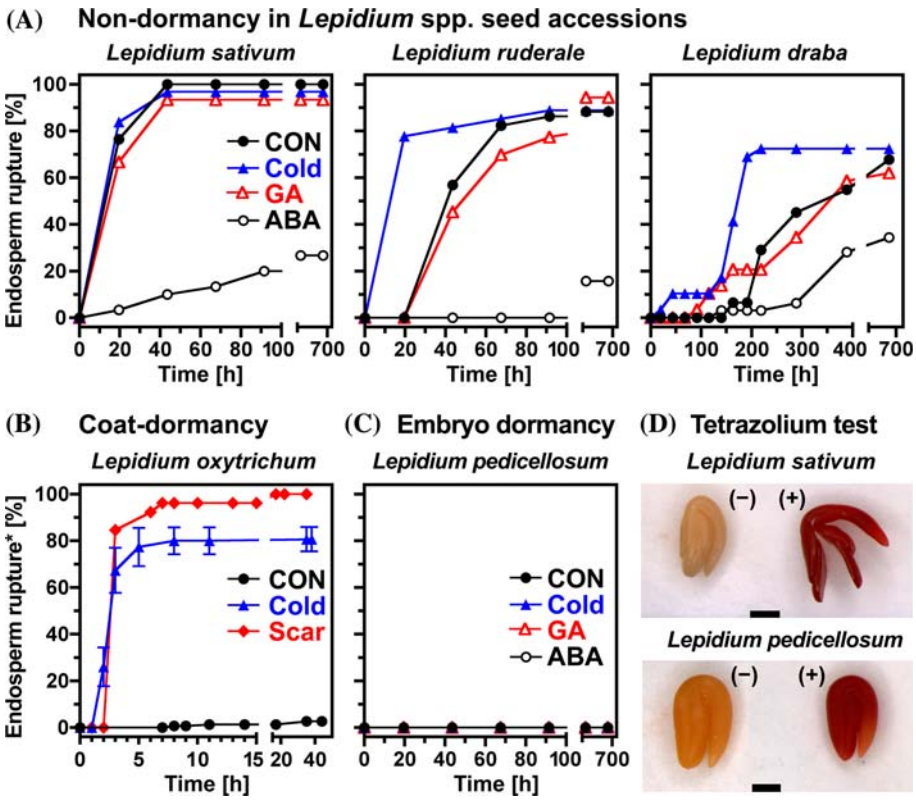


Fig. 3 Analysis of germination and dormancy in different *Lepidium* spp. ('cress') accessions. **a** The time course of endosperm rupture of non-dormant *Lepidium* spp. accessions and the effect of cold stratification (incubation of imbibed seeds in darkness at 4°C from −48 to 0 h), addition of gibberellin (GA = 10 µM GA_{4+7}), or abscisic acid (10 µM ABA) to the medium (CON = without hormone additions). **b** The effect of cold stratification and scarification (cutting of the covering layers) on the time course of endosperm rupture of *Lepidium oxytrichum*. Based on these results the *L. oxytrichum* accession has coat dormancy. * Initial radicle elongation was scored in the case of scarification. **c** The effect of cold stratification and hormone treatments on the germination of the *Lepidium pedicellosum* accession. Note that germination of this accession was also not induced by scarification. Based on these results and a positive tetrazolium test the *L. pedicellosum* accession has embryo dormancy. **d** Tetrazolium test of embryo viability for *L. sativum* FR14 and *L. pedicellosum*. *Left:* Embryos of heat-killed seeds used as negative controls did not stain (−). *Right:* Embryos of both accessions develop staining (+) after 15 min (*L. sativum* FR14) or 20 h (*L. pedicellosum*) and are therefore alive. Results are representatives of 2 biological replicates with 20 embryos each. *Scale bar* 1 mm. After-ripened seeds were used; for statistics (**a**–**c**) and results obtained with other *Lepidium* spp. accessions see Table 1

spectrometer from Bruker (Rheinstetten, Germany) at 9.7 GHz microwave frequency, 100 kHz modulation frequency, modulation amplitude 1 G and 63 mW microwave power as described (Renew et al. 2005). Signal size was calculated as signal to noise ratio.

RNA isolation from seed tissues

For each sample ca. 1,000 *L. sativum* FR1 micropylar endosperm caps (Cap) or ca. 100 radicals (Rad) were collected at the times indicated, frozen in liquid nitrogen and stored at −80°C. Total RNA extraction was carried out and followed by quantity and quality control analyses as described (Chang et al. 1993). Four biological replica RNA samples were used for downstream applications.

Quantitative (real-time) RT-PCR (qRT-PCR)

Transcript expression of selected genes was quantified by qRT-PCR, which was conducted according to the requirements described by Udvardi et al. (2008). Four biological replicates of *L. sativum* FR1 endosperm cap and/or radicle RNA were used for each time point and treatment. 3 µg RNA were reverse transcribed in a 20-µl reaction using random pentadecamers (3 µM) and digested with RNaseH according to the Superscript III kit instructions (Invitrogen, Carlsbad, CA, USA). 100 ng cDNA were then used for each quantitative PCR reaction. For quantification with the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Scoresby, Victoria, Australia) the ABsolute QPCR SYBR Green ROX Mix (ABgene, Epsom, Surrey, UK) was used according to the manufacturers' instructions.

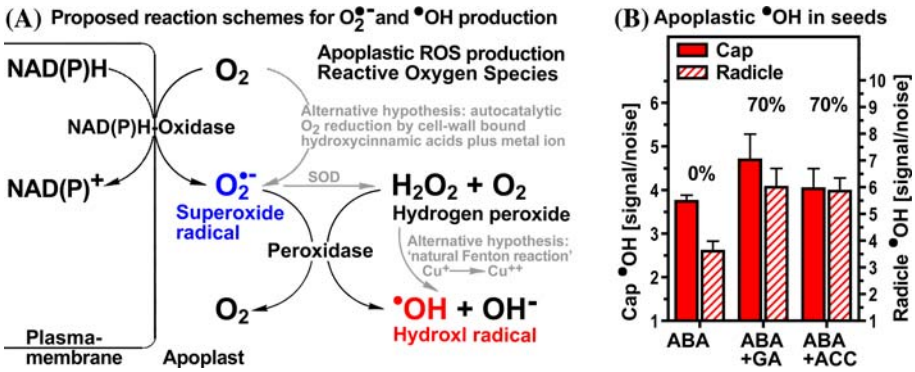


Fig. 4 Apoplastic hydroxyl radical ($^{\bullet}OH$) production in plant tissues. **a** Proposed hypotheses for the production of apoplastic $^{\bullet}OH$ as described in the main text. **b** In vivo detection of apoplastic $^{\bullet}OH$ production by EPR in *Lepidium sativum* FR1 endosperm caps and radicles incubated for 40 h in medium with 5 μM ABA alone (ABA) or combined with 10 μM GA_{4+7} (ABA + GA) or 1 mM ACC (ABA + ACC); ACC (1-aminocyclopropane-1-carboxylic acid) is the direct precursor of ethylene. Means of radicle samples (ABA vs. ABA + GA and ABA vs. ABA + ACC) differ significantly ($P < 0.05$) as calculated by one way ANOVA followed by Tukey's multiple comparison test (GraphPad Prism software). For comparison the endosperm rupture values are given as numbers above the columns. In addition to the ABA-GA antagonism (Müller et al. 2009b) an ABA-ACC antagonism is evident indicative for a role of ethylene in counteracting the ABA inhibition. Mean values ± SE of at least four replicates of 100 radicals or caps are shown

Table 1 Screening of *Lepidium* spp. ('cress') accessions for dormancy and germination characteristics

Species	Geographical origin	Dormancy of accession	Cold stratification	Seed batch size
Lepia s.l. (major clade)				
L. draba	EA	ND	+	L
L. campestre	EA	ND	+	L
Lepidium s.s. (major clade)				
L. africanum	AF	ND	n.d.	S
L. divaricatum	AF	ND	−	L
L. hyssopifolium	AU + NZ	ND	n.d.	S
L. pseudohyssopifolium	AU	ND	n.d.	S
L. monoplocoides	AU	ND	n.d.	S
L. oxytrichum	AU	CD*	+	L
L. papillosum	AU	ED	n.d.	S
L. didymus	SA	CD	+	L
L. bonariense	SA	CD	+	L
L. spinosum	EA	ND	n.d.	S
L. sativum FR14	EA	ND*	−	L
L. ruderale	EA	ND	+	L
Monoploca s.s. (major clade)				
L. rotundum	AU	ED	n.d.	S
L. pedicellosum	AU	ED*	−	L
L. phlebopetalum	AU	ED	n.d.	S
L. pholidogynum	AU	ED*	−	L

Seed populations were considered as non-dormant (ND) if they germinated under control conditions (CON = 24°C, continuous light, medium without hormone addition). Coat dormant (CD) seeds germinated after a scarification treatment (cutting or removal of the seed covering layers). Seed populations designated as embryo dormant (ED) did not germinate, also not after scarification; *Embryo viability was confirmed by a tetrazolium test (see Fig. 3c). '+' Means that cold stratification (incubation of imbibed seeds in darkness at 4°C for 48 h) caused dormancy release and/or promoted germination, '−' no effect, *n.d.* not determined. Geographic origin of the species: *EA* Europe + Asia, *AF* Africa, *AU* Australia, *NZ* New Zealand, *SA* South America. Geographical grouping of the species largely resembles phylogenetic relationship. Seed batch sizes: *L* Large seed batch used for screening, 1–3 plates with 50 seeds each were analysed (mean values ± SE in Fig. 3); *S* Small seed batch used for screening, usually one plate with 10–30 seeds was analysed

Single product amplification was validated by a melting curve analysis according to the manufacturers instructions. QPCR raw data was analysed and PCR efficiency (E) and cycle threshold (Ct) were calculated with the freely available software PCR Miner (http://www.miner.ewindup.info/; Zhao and Fernald 2005). E was calculated for each reaction individually and the average efficiency of each primer pair was used to calculate the efficiency-corrected expression level of each sample as E^{-Ct}. Expression data were normalized to the geometric mean (geomean) of the expression levels of three reference genes, *EF1-a* (*elongation factor 1a*), *Cyp1* (*Cyclophillin 1*) and 5.8S rRNA for which we have verified constitutive expression in both tissues during *L. sativum* seed germination (data not shown). The relative expression result for every sample was then calculated as $E^{-Ct\ sample}$/geomean $E^{-Ct\ references}$ (Pfaffl 2001). Mean values ± SE from the four biological replicates are shown (Fig. 6). Primer sequences (5′ to 3′) used for qPCR amplification: Dog1qPCR-F2 (CTTTGTGTGGCTCCGAAACT), Dog1qPCR-R4 (GCCGCGTCTTCTTGTAGG), cyp1qPCR-F2 (GGATCCTGTCGATGGCGAAC), cyp1qPCR-R2 (TCCACGACCTGCCCAAACAC), ef1aqPCR-F (TGAGCACGCTCTTCTTGCT), ef1aqPCR-R (GTGGCATCCATCTTGTTACA), LsrRNAqpcrFOR (CTTTGAAGCCAAGTTGCGC), LsrRNAqpcrREV (CGTCCCACACTCGTGAAAAT).

Cloning of genomic DNA and cDNA sequences

Genomic DNA (gDNA) from leaves of *L. sativum* FR14 was isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. Heterologous primers based on the *A. thaliana DOG1* sequence were used for PCR cloning and sequencing of short *L. sativum* gDNA fragments. Primers based on these *L. sativum* fragments were used in an inverse PCR approach to clone the unknown 5′ and 3′ regions of the *L. sativum DOG1* ortholog (*LesaDOG1*). To do this 1 µg of gDNA was digested with SacI (NEB, Frankfurt, Germany) in a 20 µl reaction at 37°C for 4 h and the enzyme inactivated by denaturing at 65°C for 20 min. The digested gDNA was religated using 100 U T4 Ligase (NEB, Frankfurt, Germany) in a 400 µl reaction at 4°C overnight according to the manufactures instruction. The enzyme was inactivated (as above) and the gDNA precipitated (EtOH/sodium-acetate method) and resuspended in water. Primers based on the short *L. sativum* sequences were used for PCR cloning of the unknown 5′ and 3′ regions of the religated genomic fragments. Sequence analysis of these clones allowed the localization of the ligation site and based on this sequence information primers were designed for PCR cloning of the *LesaDOG1* gene from intact, uncut gDNA. At least 5 overlapping clones from independent PCR reactions were sequenced to verify the *LesaDOG1* gene sequence.

For the cloning of *DOG1* cDNAs, RNA was extracted from whole dry seeds of *L. sativum* FR14 and *Brassica rapa* subsp. *pekinensis* 'Chiifu' (IPK, Gatersleben, Germany) as described above. First strand cDNA was synthesized in 20-µl reactions with 3 µM random pentadecamers and 5 µg total RNA as a template according to the instructions of the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). Primers for subsequent PCR cloning of *L. sativum* and *B. rapa* cDNAs were designed based on the cloned genomic *LesaDOG1* DNA sequence (see above) and the sequence of the *B. rapa* genomic clone KBrH003E13 (AC189537.2), respectively. 3′RACE was performed using the 3′RACE Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturers instructions. To verify the cDNA sequences, at least three independent cDNA clones were sequenced.

The *L. sativum* FR14 and *B. rapa* subsp. *pekinensis* 'Chiifu' DNAs isolated and described here have been deposited in GenBank/EMBL data libraries under the following accession numbers: *LesaDOG1* gDNA (GQ411193), *LesaDOG1* cDNA (GQ11192), *BrDOG1* cDNA (GQ411194).

Sequence alignments and molecular phylogenetic analysis

The bioinformatics software Geneious Pro 4.7.4 (Biomatters LTd., Auckland, New Zealand, www.geneious.com) was used as sequence analysis platform: the alignment of *ABI3/VP1* amino acid sequences (Fig. 1) from different species was build using the MUSCLE algorithm with its default settings. The alignment of *DOG1* genes of different Brassicaceae species (Fig. 5) was build using the ClustalW 2.0.11 algorithm (gap open cost 15; gap extension cost 6.66, IUB cost matrix). Percentage pairwise identity means the average percent identity over the alignment and is computed by looking at all pairs of residues at the same column and scoring a hit (one) when they are identical, divided by the total number of pairs.

Results and discussion

Evolution and role of the ABI3/VP1 transcription factors in seed and bud biology

ABA-related ABI3/VP1-type transcription factors are widespread in green plants. All known land plant ABI3/VP1 proteins, except one from *Medicago trunculata*, contain four conserved domains (Fig. 1): an acidic domain (A1) and three basic domains (B1, B2, B3). The N-terminal

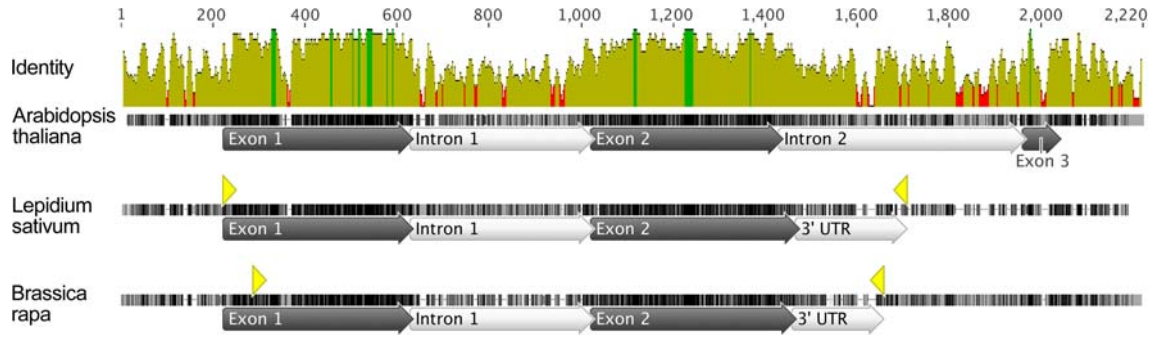


Fig. 5 Multiple sequence alignment of Brassicaceae *DOG1* (*DELAY OF GERMINATION1*) gene orthologs. The *DOG1* genomic sequences of *Arabidopsis thaliana* L*er* (*AtDOG1*), *Lepidium sativum* FR14 (*LesaDOG1*) and *Brassica rapa* subsp. *pekinensis* 'Chiifu' (*BrDOG1*) are compared. Their GenBank entries and references are: *AtDOG1* (EF028470.1, Bentsink et al. 2006), *LesaDOG1* (GQ411193, this work) and *BrDOG1* (AC189537.2, genomic clone KBrH003E13 from bp 8,949 to bp 10,992). The identity plot and the alignment of the individual genomic DNA sequences are presented. The exon/intron annotation is based on the Arabidopsis genomic DNA sequence for *AtDOG1*. For Arabidopsis splicing of the introns as they are drawn here generates the known *AtDOG1* α-type transcripts (Bentsink et al. 2006). To verify splice sites and to identify 3′ UTR regions we cloned *LesaDOG1* and *BrDOG1* cDNAs: the yellow triangles mark the verified cDNA sequence regions and their GenBank entry numbers are QG411192 (*LesaDOG1*) and QG411194 (*BrDOG1*). The identity plot displays the average identity across all genomic sequences for every position in a 10 bp window. High green bars indicate that the residues at the position of the window are the same across all sequences. Medium yellow bars represent less than complete identity and red low bars refer to very low identity for the given window position. Thick horizontal lines represent the individual genomic DNA sequences, thin horizontal lines represent gaps in the alignment. Different vertical shadings represent different levels of identity at this base position between the three sequences: *black* = all bases at this position are identical in all three sequences, *dark grey* = two of the three sequences have the same base at this position, *light grey* = this base at this position is only present in this sequence

A1 domain is responsible for ABA-dependent co-activation and repression activities and has the least sequence similarity between the diverse species (33.9, 54.6, 57.0, 78.4% pairwise identity for the A1, B1, B2, B3 domain, respectively, Fig. 1a). The B1 and B2 domains play important roles in nuclear location and interactions with other proteins and thereby can enhance the DNA binding activity of other transcription factors. The B2 domain is required for ABA-regulated gene expression and appears to facilitate the DNA binding capacity of a number of diverse DNA binding proteins. The core of the B2 domain of most angiosperm sequences contains ARKKR as a universal and prototypic nuclear localization signal (NLS, red box in Fig. 1c). The same sequence position is occupied by ARKNR in the gymnosperms and by ARRNR in the moss *Physcomitrella patens*. The B2 domain is required, but not sufficient, for nuclear localization of the angiosperm and Physcomitrella ABI3 orthologs (Gagete et al. 2009; Marella and Quatrano 2007; Marella et al. 2006). Another putative NLS sequence, RKRK, is located in the C-terminal part of the B1 domain of the gymnosperm but not the angiosperm sequences (Footitt et al. 2003; Zeng and Kermode 2004; Zeng et al. 2003). In the green algae *C. reinhardtii* none of these NLS sequences are present, the B1 and B2 domains differ from the land plant ABI3/VP1 proteins and the A1 domain is absent (Fig. 1). Nothing is known about a role of ABI3/VP1 in this green algae, but the absence of an NLS sequence suggests that it may act differently as in land plants. ABA may act as a signal molecule to induce antioxidant reactions for elimination of reactive oxygen species (ROS) produced during oxidative stress in *C. reinhardtii*, but not to induce specific response reactions to mitigate water stresses (Yoshida et al. 2004). All the cereal VP1 proteins share a highly similar region following the B2 domain which we named 'cereal B2-terminal domain' (blue box in Fig. 1c). This domain is absent in the other groups, but the gymnosperms and core eudicots share at the same position a region of similarity that is distinct from the cereal B2-terminal domain.

The B3 domains of ABI3/VP1-type transcription factors are highly similar (Fig. 1b). The B3 domain binds DNA and targets the highly conserved RY motif (CATGCA(TG)), found at −100 from ATG in seed-specific gene promoters. The B3 DNA binding domain of Arabidopsis ABI3 has been shown to be necessary and sufficient for the specific interaction with the RY element (Mönke et al. 2004). Storage protein gene promoters of various angiosperms, ferns and gymnosperms contain RY motifs and their induction in Arabidopsis leaf and seed tissues (embryo and endosperm) has been demonstrated (Schallau et al. 2008). Different angiosperm ABI3/VP1 genes activate a wheat seed storage gene promoter in the moss *P. patens* (Marella and Quatrano 2007; Marella et al. 2006). Angiosperm and moss ABI3/VP1 transcription factors were shown to physically interact via the B1 domain with ABI5-type bZIP transcription factors in homologous and heterologous assays (Gagete et al. 2009; Hobo et al. 1999; Holdsworth et al. 2008; Kurup et al.

2000; Lopez-Molina et al. 2002; Nakamura et al. 2001; Piskurewicz et al. 2009). The ABI5-type transcription factors bind to the ABA-responsive elements (ABRE) in ABA-responsive gene promoters. ABI5 binding to ABRE elements may tether ABI3 to target promoters and facilitate the interaction of ABI3 with the RY motif and transcription complexes. The *P. patens* and *Callitropsis nootkatensis* ABI3 transcription factors partially complement the severe Arabidopsis *abi3–6* mutation (Fig. 1a). The finding that this complementation is partial can be explained by a weaker physical interaction between the B1 domain of the moss/gymnosperm ABI3 with the distantly related Arabidopsis ABI5 transcription factor (Marella and Quatrano 2007; Zeng and Kermode 2004). The ABI3/VP1 interaction with ABI5-type transcription factors appears to be a widespread mechanism (e.g., Casaretto and Ho 2003; Leubner-Metzger 2007; Marella et al. 2006; Piskurewicz et al. 2009). Non-ABI5-type ABI3-interacting proteins (AIPs) have been isolated from Arabidopsis (Kurup et al. 2000; Zhang et al. 2005) and AfVP1-interacting proteins (AfVIPs) involved in after-ripening of *Avena fatua* (wild oat, freshly harvested caryopses have deep dormancy, Jones et al. 2000). It is known for barley grains that dormancy release and/or after-ripening are associated with changes that occur in the coleorhiza that covers the radicle tip (Barrero et al. 2009). Enhanced ABA degradation, reduced ABA sensitivity and the transcript up-regulation of a putative ABI3-interacting protein is associated with the coleorhiza and the radicle. These findings highlight the importance of dynamic interactions to provide specificity and graded responsiveness to ABI3/VP1-mediated ABA signaling.

A weak dormancy and PHS susceptibility of wheat is associated with alternative splicing of *TaVP1* transcripts (see introduction). Figure 1 shows that a correctly spliced *TaVP1* sequence yields a canonical ABI3/VP1-type protein. Alternative splicing of *VP1* transcripts due to short, direct repeats has been published for rice (Fan et al. 2007), but so far not for wild oat, maize and barley. Figure 1 shows that the partial *Hordeum vulgare VP1* transcript sequence 'Hordeum-1' published by Casaretto and Ho (2003) seems to be properly spliced and yields a canonical putative VP1 protein sequence. In contrast, we found another barley VP1 sequence in the database ('Hordeum-2' in Fig. 1) that exhibits alternative splicing just in the B3 domain, but yields a canonical putative *VP1* protein sequence in all other domains. Interestingly, the altered B3 domain sequence contains a glutamic acid (E, marked in orange in Fig. 1b) at a highly conserved aspartic acid (D) found in all other ABI3/VP1 transcription factors (Fig. 1b). Two Arabidopsis mutants, *abi3–1* and *abi3–7*, have point mutations at this highly conserved aspartic acid (D_{580} in AtABI3, Fig. 1b; *abi3–7* has in addition a mutation in the B2 domain, Fig. 1c) and are characterized by reduced ABA sensitivity and impaired seed storage protein gene regulation (Bies-Etheve et al. 1999). Our finding for barley demonstrates that alternative splicing also occurs in cultivars of barley and can generate putative HvVP1 proteins that are apparently functional in the A1, B1 and B2 domains (based on having canonical domain sequences), but possess a nonfunctional B3 domain. Alternative splicing of ABI3/VP1 transcription factors is not restricted to cereals: *PsABI3* transcripts present in dry pea (*Pisum sativum*) seeds are degraded upon imbibition (Gagete et al. 2009). At least seven isoforms of *PsABI3* originate from alternative splicing in pea seeds, and this splicing is developmentally regulated and tissue-specific. The interactions of specific splice forms were tested by Gagete et al. (2009) and in some cases long, but inactive protein forms were imported into the nucleus. Taken together, these findings demonstrate that developmental regulation of alternative splicing appears to be important for ABI3/VP1 function in ABA signaling in seeds.

Lepidium as a useful Brassicaceae genus for studying seed germination and dormancy

The genus *Lepidium* L. ('cress') is one of the largest genera of the Brassicaceae with its ca. 175 species being distributed worldwide (Mummenhoff et al. 2001, 2004). *Lepidium* spp. are found on all continents and islands (except Antarctica), mainly in temperate and subtropical regions, but also in the mountain areas of tropical regions. Adaptation to different climatic environments and rapid radiation, e.g., by intercontinental dispersal of mucilaginous seeds adhering to birds seem to be a characteristic of this genus. This occurred ca. 2–4 MYA in the Pliocene and had the Mediterranean and the Irano-Turanian territory as origin which is an ecologically, altitudinally and geologically extremely diverse region (Mummenhoff et al. 2001, 2004). Molecular phylogenetic studies provided strong evidence that *Lepidium sensu lato* (*s.l.*) forms a natural monophyletic assemblage with three major clades: *Lepidium sensu stricto* (*s.s.*), *Monoploca s.s.* and *Lepia s.l.* Based on molecular studies the monophyletic clade *Lepidium s.s.* can be divided in several phylogenetically and continentally defined groups (Table 1). The monophyletic nature of *Lepidium* spp. is based on comparisons of several independent DNA sequences and this information has been successfully used to study the evolutionary changes in floral structures (Bowman et al. 1999; Lee et al. 2002; Mummenhoff et al. 2001, 2004). *Lepidium* species were successfully used as a model system for studying the evolution of fruit development in Brassicaceae (Mummenhoff et al. 2009). Similarly, in cross-species approaches focusing on endosperm weakening and seed after-ripening we utilized the combined

advantages of *Lepidium sativum* and Arabidopsis to study the effects of these processes on seed germination (Müller et al. 2006, 2009a, b). The Brassicaceae (rosid clade) evolved ca. 19 MYA and the lineage I Brassicaceae (Arabidopsis, *Lepidium* spp.) ca. 8 MYA (Franzke et al. 2009). The genus *Lepidium* is therefore closely related to the genus *Arabidopsis*, the phylogenetic relation *Arabidopsis-Lepidium* is much closer as the phylogenetic relation *Arabidopsis-Brassica* as the genus *Brassica* is within the lineage II (the split between the two lineages occurred ca. 11 MYA, Franzke et al. 2009). While their seed size differs considerably (Müller et al. 2006), the seed structure of *L. sativum* (big seeds) and Arabidopsis (tiny seeds) is similar (thin layer of endosperm); mature seeds of *Brassica* spp. are endospermless. The genus *Lepidium* is therefore highly suited for moving beyond Arabidopsis in comparative experiments regarding seed dormancy genes and the control of germination by plant hormones and environmental factors especially in tissue-specific approaches.

Figure 2a provides a general mechanistic model for seed germination and dormancy in which the net sum of the embryo growth potential and the weakening of the opposing restraint of the covering layers determines if a seed completes germination or not (Bentsink and Koornneef 2008; Bewley 1997b; Finch-Savage and Leubner-Metzger 2006; Nonogaki 2006). According to this model an increase in the growth potential of the embryo (radicle, hypocotyl) is an important requirement for radicle protrusion. The embryo growth potential is promoted by GA and inhibited by ABA. Cell elongation in a discrete region of the Arabidopsis embryo immediately proximal to the radicle (lower hypocotyl and hypocotyl-radicle transition zone) is required for the completion of germination (Sliwinska et al. 2009). The second requirement of the model is that the restraint conferred by the covering layers (testa, endosperm) is weakened (Bewley 1997a; Finch-Savage and Leubner-Metzger 2006; Nonogaki 2006). In general, this restraint weakening is also promoted by GA and inhibited by ABA. This restraint by the envelopes can be mechanical, prevention of inhibitor (ABA) leakage, or interference with water uptake or gas (O_2) exchange. A decrease in the mechanical restraint of the thin endosperm layer present in the mature seeds of Arabidopsis and *L. sativum* is a requirement for typical endosperm rupture (Bethke et al. 2007; Müller et al. 2006, 2009b). In all species that have been investigated, endosperm weakening preceded radicle protrusion and was promoted by GA (Bewley 1997a; Finch-Savage and Leubner-Metzger 2006). In contrast, the ABA response may differ between angiosperm clades. Biomechanical measurements of the ABA response in tomato (Toorop et al. 2000) and coffee (da Silva et al. 2004) suggest that endosperm weakening is biphasic in these seeds of the asterid clade: the first phase is ABA insensitive, the second phase is inhibited by ABA. In contrast a 'one-phase' ABA-inhibited endosperm weakening is observed in *L. sativum* seeds (rosid clade, Müller et al. 2006, 2009b). Based on our comparative seed biology approach with *L. sativum* and Arabidopsis, we speculate that the endospermic Brassicaceae seeds have evolved to retain the ABA-inhibitable mechanism(s) found in both clades, but not the ABA-insensitive phase of endosperm weakening. Figure 2 shows that there is natural variation for the endosperm cap resistance between the two *L. sativum* accessions FR1 and FR14. The endosperm cap puncture force of FR14 is ca. twofold higher when compared with FR1 (Fig. 2c). In both cases, a ca. 50% decrease was evident when seeds imbibed for 8 h were compared with seeds imbibed for 18 h, and ABA inhibited this decrease. FR14 and FR1 therefore differ in the absolute values, but not in the relative regulation of the endosperm cap weakening by ABA and time. Endosperm cap weakening of *L. sativum* seeds is a very localized event at the place of radicle protrusion. In the absence of the radicle (detached endosperm cap) a hole forms in the same position close to the very tip of the cap (Müller et al. 2009b). *L. sativum* endosperm cap weakening, endosperm rupture, and endosperm cap hole formation are thus constituents of a developmental process associated with the control of germination and are inhibited by ABA.

Physiological dormancy (PD) is the most abundant dormancy class and is found in seeds of all major gymnosperm and angiosperm clades (Baskin and Baskin 2004; Finch-Savage and Leubner-Metzger 2006). The great majority of seeds, including Arabidopsis, have non-deep PD; with 'non-deep' as one of three levels (non-deep, intermediate, deep) of the PD-class within the hierarchical classification system proposed by Baskin and Baskin (2004). Embryos excised from these non-deep dormant seeds produce normal seedlings and treatment with gibberellins (GA) can break this dormancy. Also, depending on species, this dormancy can be broken by natural or artificial scarification (abrasion or cutting of the covering layers), after-ripening during air-dry seed storage, and by cold or warm stratification (e.g., prechilling of imbibed Arabidopsis seeds in darkness). This hierarchical dormancy classification system accurately reflects the 'whole-seed' ecologists view of the control of germination by dormancy. A collection of after-ripened seed populations of different *Lepidium* species (Table 1) representing distinct phylogenetical positions of this genus (Mummenhoff et al. 2001; 2004) as well as different geographic origins were screened for their germination behavior (Fig. 3). Several of these accessions were non-dormant and ABA delayed radicle protrusion. GA did not affect their endosperm rupture, but they differed in their responses to cold stratification with respect to the timing of endosperm rupture (Fig. 3a).

However, we also found several accessions that were dormant (Fig. 3b, c; Table 1) and analysed their dormancy.

Studies in which seeds with non-deep PD were analysed regarding the intrinsic molecular mechanism(s) that are underlying this 'whole-seed' dormancy showed that these mechanism(s) can have an 'embryo' and/or a 'coat' component (e.g., Bentsink and Koornneef 2008; Bewley 1997a; Finch-Savage and Leubner-Metzger 2006; Holdsworth et al. 2008; Koornneef et al. 2002; Nonogaki 2006). The terms *embryo* and *coat dormancy* will therefore be used here to distinguish between these two mechanisms. Embryo dormancy and coat dormancy are components of PD; their sum and interaction determines the degree of 'whole-seed' PD. *Embryo dormancy* (ED) is characterized by an intrinsic block that inhibits extension growth. Excised dormant embryos therefore do not grow. *Coat dormancy* (CD) is characterized by an intrinsic block that is conferred by the covering layers. Non-dormant embryos excised from coat-dormant seeds will therefore extend and grow. 'Coat' is used here in a loose sense and can be any embryo covering structure, e.g., testa, megagametophyte (gymnosperm seeds), and endosperm (angiosperm seeds). For example, based on this distinction, the non-deep PD of Arabidopsis seeds is due to coat dormancy: testa (Debeaujon and Koornneef 2000) and endosperm (Bethke et al. 2007) prevent embryo growth in the dormant state. The work of Bethke et al. (2007) demonstrates the importance of the endosperm for Arabidopsis CD: when the testas of dormant seeds were removed, the endosperm prevented germination upon imbibition. Ultrastructural cellular changes in the micropylar endosperm including vacuolation and changes that suggest thinning and weakening of cell walls or even cell separation occur only when dormancy has been released. These processes are confined to the micropylar endosperm region and the vacuolation is inhibited by ABA (Bethke et al. 2007). Treatments known to release Arabidopsis seed dormancy, induced endosperm rupture and radicle emergence of these 'testa-less' seeds.

Embryos excised from deeply dormant Cvi and C24 or GA-mutant seeds grow or exhibit at least the initial extension growth required for the completion of germination (Bethke et al. 2007; Iuchi et al. 2007). This underlines the importance of the covering layers, but it does not mean that in the case of coat dormancy the excised embryos from the dormant and non-dormant seeds are undistinguishable. The excised embryos from dormant Arabidopsis seeds grow, but may exhibit a reduced growth potential and have a higher sensitivity for the germination-inhibiting hormone ABA as well as for osmotic stress (Bethke et al. 2007). The combination of an embryo having low growth potential with 'coat' constraints leads in its sum and interaction to a defined degree of 'whole-seed' PD which prevents germination if it is above a certain threshold level at given environmental conditions.

In contrast to the non-dormant accessions of *Lepidium* spp. (Fig. 3a, ND in Table 1), several other accessions were found to have CD: Fig. 3b shows that after-ripened seeds of *L. oxytrichum* do not germinate, but dormancy was released and germination induced by cold stratification or scarification. Since several other *Lepidium* species showed CD (Table 1) this might indicate that mechanisms and/or genes may be shared between the known *Lepidium* species with CD (this work) and Arabidopsis accessions (Bethke et al. 2007; Debeaujon and Koornneef 2000; Debeaujon et al. 2000). In contrast to this, embryo dormancy (ED) is not known in Arabidopsis. Figure 3c shows that *L. pedicellosum* has ED. Neither GA nor cold treatment nor scarification released their dormancy and induced germination. A tetrazolium assay (Fig. 3d) demonstrated that the embryos isolated from these seeds are not dead. It is interesting that several of the *Lepidium* species showing ED belong to the *Monoploca s.s.* clade endemic to Australia (Table 1). The common arid origin of these species led to the evolvement of similar xeromorphic characters and they all show shrubby growth forms and strongly developed mucosity of the testa (Hewson 1981). The thick translucent testa (present e.g., in *L. phlebopetalum*) might play a significant role in water absorption and retention thereby regulating germination timing as an adaptation to the dry environment. It is likely that the evolvement of ED in these species is also an environmental adaptation of common origin.

Thellung (1906) noticed that some *Lepidium* species have been dispersed as weeds in human-influenced habitats throughout the world whereas others have not. For example *L. ruderale*, *L. africanum*, *L. bonariense* and *L. draba* show a high colonization potential enabling those species to leave their typical habitats and invade environments dominated by human activities. The weediness of these species led to a dispersion on nearly all continents.

It is striking that these weedy *Lepidium* species seem to show a trend to not being dormant (Table 1; Fig 3a) whereas non-weedy *Lepidium* species like *L. oxytrichum* and *L. pedicellosum* seem to possess some level of dormancy (Table 1; Fig. 3b, c). This suggests that the ability of weedy *Lepidium* species to colonize human-influenced habitats might be correlated with their dormancy level and therefore with their germination strategy and that the apparent overrepresentation of ND in these species could support their weediness.

Taken together, the genus *Lepidium* offers interspecific variation in seed dormancy and germination characteristics. Some of these phenotypic traits are also found in Arabidopsis, while others appear to be novel for endospermic Brassicaceae seeds. It would therefore be interesting to

compare presence and expression of dormancy genes between the different *Lepidium* species and to Arabidopsis in cross-species experiments. This could provide insight into evolutionary conserved and species-specific mechanisms and genes.

ABA- and reactive oxygen species-associated mechanisms in seeds

As phylogenetically widespread and evolutionary conserved components like ABI3/VP1 are used in the ABA signaling pathways, it is not surprising that another 'ancient key player', namely 'Reactive Oxygen Species' (ROS), are involved in ABA signaling as one of their indirect modes of action (Bailly 2004; Cho et al. 2009; Wasilewska et al. 2008). ROS comprise very short-lived molecules like superoxide ($O_2^{\bullet -}$) and hydroxyl radicals ($^{\bullet}OH$) 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. ABA/ROS signaling in stomatal closure was investigated in detail. Increased ABA levels trigger H_2O_2 production, which in turn mediates the activation of Ca^{2+} channels at the plasma membrane, resulting in stomatal closure (Cho et al. 2009; Kwak et al. 2006). The ABA-INSENSITIVE1 (ABI1) and ABI2 protein phosphatase 2C enzymes and the NAD(P)H oxidases D and F ('*Arabidopsis thaliana* respiratory burst oxidases', AtrbohD and AtrbohF) are key components of the ABA/ROS signaling in Arabidopsis stomatal closure. It was recently found that ROS also contributed to ethylene signaling in Arabidopsis stomatal closure (Desikan et al. 2005, 2006).

Ethylene is known to promote the germination of non-dormant seeds by an unknown mechanism and an ABA-ethylene antagonism is evident for the germination of diverse species including Arabidopsis (Beaudoin et al. 2000; Ghassemian et al. 2000), *Sisymbrium officinale* (Brassicaceae, Iglesias-Fernandez and Matilla 2009), tobacco (Leubner-Metzger et al. 1998), pea (Petruzzelli et al. 2003b), sugarbeet (Hermann et al. 2007) and others (Kucera et al. 2005; Matilla and Matilla-Vazquez 2008). Ethylene-insensitive *etr1* mutant seeds of Arabidopsis germinate poorly, their dormancy is enhanced and their germination is ABA hypersensitive (Chiwocha et al. 2005). The downstream responses of ethylene are mediated by transcription factors of the 'Ethylene Response Factor (ERF)' family, whose expression in seeds is regulated by ABA, ROS and ethylene (Leubner-Metzger et al. 1998; Oracz et al. 2009).

The recent work of Oracz et al. (2007, 2008, 2009) with sunflower (*Helianthus annuus*, Asteraceae) as a model for embryo dormancy, shows that dormancy release is associated with ethylene signaling (ethylene receptors and the HaERF1 transcription factor), ROS production by Rbohs and ROS-mediated protein oxidation. Protein oxidation by carbonylation inhibits or alters protein activities and increases their susceptibility to proteolytic attack. Protein carbonylation is proposed to have specific functions during life cycle transitions in Arabidopsis plants (Johansson et al. 2004) and distinct protein carbonylation pattern in seeds are associated with dormancy release and/or after-ripening (Müller et al. 2009a; Oracz et al. 2007). The recent work of Müller et al. (2009a) shows that Arabidopsis seed after-ripening is impaired in the *atrbohB* mutant which also shows reduced protein oxidation. Interestingly, the *AtrbohB* pre-mRNA is alternatively spliced in seeds in a manner that depends on ABA and the seed after-ripening status.

In addition to their indirect mode of action in cellular signaling, a direct mode of action for hydroxyl radicals ($^{\bullet}OH$) by scission of cell wall polymers has been proposed (Fry et al. 2001; Schopfer et al. 2001). A recent publication by Müller et al. (2009b) reports direct in vivo evidence for a 'positive' developmental role of apoplastic $^{\bullet}OH$ during seed germination and seedling growth. These extremely reactive molecules can, if produced directly in the apoplast, attack cell wall polysaccharides and lead to breakage of load-bearing structures. Three hypotheses for $^{\bullet}OH$ production in the apoplast are known (Fig. 4a). Schopfer et al. (2001) proposed that $^{\bullet}OH$ is produced by apoplastic peroxidases from H_2O_2 and superoxide ($O_2^{\bullet -}$) as shown in Fig. 4a. Fry et al. (2001) and Kukavica et al. (2009) proposed alternative hypotheses for the apoplastic $^{\bullet}OH$ production which are also included in Fig. 4a. Electron paramagnetic resonance (EPR) spectroscopy has been used to show that $^{\bullet}OH$ is generated in vivo in the cell walls of maize and Arabidopsis seedlings during extension growth (Liszkay et al. 2003; Renew et al. 2005), and in *L. sativum* seed tissues (micropylar endosperm cap, radicle) during germination (Müller et al. 2009b). By using ^{3}H-fingerprinting in vivo cell wall polysaccharide attack by apoplastic $^{\bullet}OH$ was demonstrated for maize seedlings during extension growth and elongating *L. sativum* embryos during germination (Müller et al. 2009b), and for tissue weakening processes of pear fruits (Fry et al. 2001) and the *L. sativum* seed endosperm cap during germination (Müller et al. 2009b). An important conclusion from these results is that the in vivo $^{\bullet}OH$ production in the apoplast is developmentally and hormonally regulated and that the direct action of $^{\bullet}OH$ on cell wall polysaccharides has tissue-specific target sites.

Apoplastic $^{\bullet}OH$ production increases in the radicle and the endosperm cap of imbibed *L. sativum* seeds prior to endosperm cap weakening and rupture (Müller et al. 2006, 2009b). ABA inhibits this increase and delays endosperm cap weakening and rupture. Figure 4b shows that treatment of ABA-imbibed *L. sativum* seeds with GA or 1-amino-cyclopropane-1-carboxylic acid (ACC, the direct precursor

of ethylene) caused a substantial reversion of the ABA-inhibition of endosperm rupture. This reversion was associated with an increased apoplastic $^{\bullet}OH$ production in the radicle in the ABA + GA and ABA + ACC series (Fig. 4b). Ethylene-induced apoplastic $^{\bullet}OH$ production and direct action could therefore be a novel mechanism for counteracting the ABA inhibition of seed germination (Fig. 4b, Müller et al. 2009b). Ethylene is required for the initiation and progression of pear fruit softening (Hiwasa et al. 2003), suggesting that this hormone promotes the progressive $^{\bullet}OH$ attack on polysaccharides during the softening process (Fry et al. 2001). Based on the results shown in Fig. 4b both, GA and ethylene are antagonists of the ABA inhibition of seed $^{\bullet}OH$ production. Apoplastic ROS has a 'positive' developmental role and provides a novel interpretation frame for ROS production during seedling growth, seed germination and tissue weakening processes.

A cross-species comparison of the Brassicaceae *DOG1* genes and the analysis of the ABA-regulation of *DOG1* transcript expression in *L. sativum* seed tissues during germination

Intraspecific natural variation has been defined as the within-species phenotypic variation caused by spontaneously arising mutations that have been maintained in nature by any evolutionary process including artificial and natural selection (Alonso-Blanco et al. 2003, 2009; Tonsor et al. 2005). This is the basis of the enormous biodiversity present among wild plant species as well as most of the genetic variation that is found in domesticated plants. Some of the phenotypic differences existing in wild or cultivated plants are due to single-gene (monogenic) allelic variants. However, most of the natural variation is quantitative and determined by molecular polymorphisms at multiple loci and genes (multigenic), which are referred to as quantitative trait loci (QTL) and quantitative trait genes (QTG). Most wild annual plant species, including Arabidopsis, show substantial intraspecific natural variation for seed dormancy, after-ripening, longevity and germination properties, which is presumably involved in adaptation to different environments (Alonso-Blanco et al. 2009). Due to extensive influence of environmental effects on the expression of these seed characteristics and the involvement of many genes they are typical quantitative traits. Such traits are becoming more amenable to genetic analysis because the position of individual QTLs and the relative contribution of these loci can now be determined. QTL analysis of seed traits requires permanent mapping populations, such as recombinant inbred lines (RILs), because these allow the testing of a large number of genetically identical seeds in different environmental conditions. An Arabidopsis L*er*/Cvi RIL population was used for mapping of seed dormancy loci based on the natural genetic variation that exists between the ecotype L*er* (low dormancy) and the deeply dormant ecotype Cvi (Alonso-Blanco et al. 2003; Bentsink and Koornneef 2008; Koornneef et al. 2004). The individual QTLs can be further characterized and fine mapped by developing near-isogenic lines (NILs) with monogenic differences. Seven dormancy QTLs named *'DELAY OF GERMINATION'*, *DOG1* to *DOG7,* have been identified by Alonso-Blanco et al. (2003). Cvi alleles at six loci (*DOG1*, *DOG3-DOG7*) increased dormancy, while the Cvi allele at *DOG2* decreased dormancy, compared to L*er* alleles. Despite the strong QTL-environment interactions, several seed trait QTLs colocate in different mapping populations, and this is in particular true for the locus *DOG1*, which has been detected in three experimental populations (Alonso-Blanco et al. 2003; Clerkx et al. 2004; Laserna et al. 2008). Arabidopsis *DOG1*, for which the Cvi allele increases the level of seed dormancy, is responsible for 12% of the variance observed in seed dormancy. The *dog1* loss-of-function mutants lack dormancy and exhibit reduced longevity, but they do not show any obvious pleiotropic effects. The positional cloning of this major seed dormancy QTL has been reported by Bentsink et al. (2006). With the isolation of *DOG1* the first seed dormancy QTG accounting for genetic variation in natural populations has been identified at the molecular level. The Arabidopsis *DOG1* gene, *AtDOG1*, encodes a novel plant-specific protein of unknown mode of action, that is required for the seed dormancy of Arabidopsis. The *AtDOG1* alleles have been cloned from eight accessions that differ in dormancy (Cvi, L*er*, Col, An-1, Kond, Sha, Kas-2, Fei-0, Bentsink et al. 2006). The importance of the *AtDOG1* gene for the seed dormancy of Arabidopsis suggests that this gene and the dormancy mechanism that it mediates may be widespread among seed plants. However, Bentsink et al. (2006) only found sequences with partial and low similarities to *AtDOG1* in the available databases of other species. The situation for the *AtDOG1* gene is therefore completely different to the phylogenetically widespread ABI3/VP-type transcription factors (Fig. 1). The phylogenetic distribution and possible origin of the *DOG1* gene therefore remains unknown.

The *AtDOG1* alleles of Arabidopsis accessions with different degree of dormancy, including L*er* and Cvi, were compared by Bentsink et al. (2006): a few amino acid exchanges were found, but with no obvious pattern that allows an association of particular regions of the protein with the different dormancy levels. Several-fold differences in the transcript expression levels were found in seeds of different Arabidopsis accessions, with the tendency that deeper dormancy is associated with *AtDOG1*

transcript accumulation. Alternative mRNA splicing generates four *AtDOG1* transcripts that differ in relative abundance and may lead to the expression of three putative proteins that differ in their C-termini. Bentsink et al. (2006) showed that *AtDOG1* transcripts accumulate during seed development with a peak in the last phase of seed maturation. Since *AtDOG1* function requires ABA it was hypothesized that *AtDOG1* is associated with the induction of primary dormancy during seed development. *AtDOG1* transcripts are present in fresh (dormant) and after-ripened (non-dormant) dry seeds and disappear upon imbibition of fresh and after-ripened seeds. A recent transcriptome analysis with Arabidopsis Cvi seeds demonstrated that *AtDOG1* transcript expression is regulated in a complex manner during dormancy induction and release (Finch-Savage et al. 2007). In contrast to the results of Bentsink et al. (2006), the *AtDOG1* transcript content of imbibed primary dormant (fresh) seeds was higher compared to imbibed after-ripened seeds (Finch-Savage et al. 2007). Since light is required for Arabidopsis seed germination, incubation in darkness was used by this group to induce secondary dormancy in after-ripened (non-dormant) Cvi seeds. They found that increased *AtDOG1* transcript expression is associated with the dark-induced secondary dormancy. However, neither did cold stratification (used to release primary dormancy) cause *AtDOG1* transcript degradation during imbibition, nor was there a close quantitative association between the deepness of dormancy and the relative *AtDOG1* transcript levels. In agreement with the light-requirement of Arabidopsis seed germination, Laserna et al. (2008) showed that *AtDOG1* acts as a negative regulator of the VLFR (very-low fluence response) induction of seed germination. They also showed that the Cvi allele of *AtDOG1* caused increased germination percentages in darkness compared to the L*e*r allele. While these findings support a role of *AtDOG1* in Arabidopsis primary and secondary seed dormancy, and possibly in seed after-ripening, they also show that even small differences in the ambient experimental conditions may lead to different results. An important conclusion is that the *AtDOG1*-mediated seed dormancy of Arabidopsis is regulated, at least in part, at the level of the *AtDOG1* transcripts (Bentsink et al. 2006). As the presence of the *DOG1* gene has so far only been published for Arabidopsis, no conclusions can be drawn if these findings are of general importance for wild and crop plant seed dormancy or if they provide Arabidopsis-specific adaptations.

To address this problem, we investigated if *DOG1* orthologs are present in other Brassicaceae species. *Lepidium sativum* ('garden cress') and *Brassica rapa* ('chinese cabbage') are Brassicaceae crop species. *L. sativum* (Müller et al. 2006, 2009b) and Arabidopsis are close relatives, both belong to the Brassicaceae lineage I, whereas *B. rapa* belongs to the Brassicaceae lineage II (Franzke et al. 2009). We have used a PCR strategy and cloned a *DOG1* genomic sequence from *L. sativum* FR14 which we named *LesaDOG1* (Fig. 5). We also found a genomic sequence, *BrDOG1*, in the database, which is a *DOG1* gene from *B. rapa* subsp. *pekinensis* 'Chiifu'. The three genomic DNA sequences of *AtDOG1*, *LesaDOG1*, and *BrDOG1* were compared based on the exon/intron annotation of the Arabidopsis Genbank entry and the predicted putative ORFs for *LesaDOG1* and *BrDOG1*. A pairwise identity of 82.5% and 79.8% was evident for exon 1 and exon 2, respectively (Fig. 5). On the basis of this high sequence identity we propose that *LesaDOG1* and *BrDOG1* are the orthologs of *AtDOG1*. In contrast to these high identities of the exon regions, the 200-bp 5′-upstream region from the ATG, the intron 1 sequence, and the ca. 800-bp 3′-downstream region following exon 2 had lower pairwise identities of only 52.8, 47.0, and 51.0%, respectively (Fig. 5). Four alternative splice variants (α, β, γ, δ) of *AtDOG1* transcripts are published for Arabidopsis (Bentsink et al. 2006). They all have intron 1 spliced out and contain the entire exon 1 region, most of exon 2 (variability at the 3′ end of exon 2), and variable parts of exon 3. These transcripts differ at the 3′ region (domains A, B, C) due to the alternative splicing. Since domain A contains a 'stop codon' this leads to three ORFs and three putative proteins α, $\beta = \gamma$, and δ which differ in the C-terminal region (Bentsink et al. 2006). In contrast to this, the *LesaDOG1* and *BrDOG1* genes have only two predicted exons and one intron (Fig. 5). Comparing the putative amino acid (aa) sequences of these ORFs to the *AtDOG1* α-variant (292 aa) shows that the putative LesaDOG1 (279 aa) and BrDOG1 (280 aa) proteins have a similar size and a high aa sequence similarity of 88% and 85%, respectively. The aa similarity between the putative LesaDOG1 and BrDOG1 proteins is 88%. To verify the transcript sequences predicted from the genomic sequences we cloned *LesaDOG1* and *BrDOG1* cDNAs from seed RNA (regions between the yellow triangles in Fig. 5). Several independent cDNA clones were obtained and correspond to one transcript variant for each species. These transcripts contain exon 1 and exon 2 plus a ca. 250-bp 3′-UTR. In contrast to Arabidopsis, the *LesaDOG1* and *BrDOG1* genes did not contain a third exon and we did not find evidence for alternative splicing of the *LesaDOG1* and *BrDOG1* transcripts. The intron 1 pre-mRNA splice sites of all three species are conserved in position (Fig. 5) and highly similar in their splice site sequences. High similarity to typical plant consensus sequences for 5′ (**AG/GT**AAGT, exon/intron) and 3′ (TGC**AG/G**, intron/exon) splice sites (Lorkovic et al. 2000) were evident for the 5′ splice sites (CTTC in all three, followed by **AG/GT**AAGG (*AtDOG1*), **AG/GT**TAGA (*LesaDOG1*), **AG/GT**AACT (*BrDOG1*)) and 3′

splice sites (CAC**AG/G** (*AtDOG1*), TAT**AG/G** (*LesaDOG1*), CAC**AG/G** (*BrDOG1*)) of intron 1. A typical branch point sequence (CTRAY; R = A or G, Y = C or T; 19–50 nt upstream of the 3′ splice site) is evident for *AtDOG1* (CTAAT 45 nt upstream of the 3′ splice site of intron 1), but not for *LesaDOG1* and *BrDOG1*. Taken together, these results demonstrate that *DOG1* orthologous genes are present and their mRNAs are expressed in seeds of Brassicaceae lineages I and II species.

Bentsink et al. (2006) showed that the *AtDOG1* transcripts present in dry seeds disappear rapidly upon imbibition. Although *AtDOG1* has been hypothesized to be involved in the ABA-mediated sugar signaling in seedlings (Teng et al. 2008), the relation to ABA in seeds is not clear. According to Bentsink et al. (2006), *AtDOG1* is not specifically involved in ABA signal transduction and the *dog1* mutant has a normal sensitivity to applied ABA. *AtDOG1* function is, however, clearly related to ABA based on experiments with different *DOG1* alleles in the *aba1-1* mutant background. Differences in the *AtDOG1* gene promoter regions of the different ecotypes were detected. A RY repeat (CATGCA; −500 from the ATG) required for seed-specific and ABI3-mediated expression was identified in the promoter of the Cvi ecotype (Bentsink et al. 2006). This RY repeat is also present at approximately the same position in the Arabidopsis ecotypes An-1, Fei-0, Col, Kond, Sha, Kas-2 as verified by PLACE search. ABRE motifs (TACGTGTC; −1,671 and −1,650 from the ATG), known to be required for ABI5-mediated ABA responsiveness, were identified in the *DOG1* gene promoter of the Cvi ecotype (Bentsink et al. 2006) and at approximately the same positions in the other ecotypes mentioned above. We found a RY repeat in the 5′ region the *BrDOG1* gene at position −2,684 from the ATG, but no ABRE motifs were evident in the ca. 3 kb upstream region. Three ABRE-like motifs (ACGTG) were, however, found ca. 4 kb from the ATG of the *BrDOG1* gene (at −4,000, −4,019, −4,265). In Arabidopsis (Col) the intragenic region between *DOG1* (At5g45830) and its 5′ upstream gene At5g45840 is ca. 2.7 kb. While this synteny is conserved in *B. rapa*, the intragenic region is ca. 7.1 kb and contains two large duplications of the proximal 1-kb region of the *BrDOG1* gene promoter (dotplot analysis). This duplication of the proximal *BrDOG1* gene promoter during speciation may be the explanation why in *B. rapa* the RY and ABRE elements are only found in the distal upstream region 3–4 kb from the ATG. The sequence of the *LesaDOG1* gene promoter is not known, but since ABA inhibits *L. sativum* and Arabidopsis endosperm rupture and *L. sativum* endosperm cap weakening (Müller et al. 2006, 2009b) we investigated the possible effect of ABA on the *LesaDOG1* gene expression.

Figure 6a shows that *LesaDOG1* transcripts are present in dry micropylar seed ends (radicle plus endosperm cap region) of *L. sativum* FR1. As in Arabidopsis seeds, the transcripts are rapidly degraded upon imbibition (CON) and at 8 h a ca. 20-fold lower *LesaDOG1* transcript level was evident compared to 0 h. Interestingly, addition of 10 µM ABA to the medium caused a ca. twofold higher transcript level at 8 h compared to 0 h (Fig. 6a). This shows that transcription of the *LesaDOG1* gene is ABA-inducible upon *L. sativum* seed imbibition. Figure 6b shows that *LesaDOG1* transcripts are present in radicals and micropylar endosperms at 8 h and are rapidly degraded in medium without ABA (CON). Just prior to endosperm rupture (18 h CON) only very low *LesaDOG1* transcript levels were detected. ABA-induced *LesaDOG1* transcript accumulation was evident in radicals and caps at 8 h (ABA), but thereafter the *LesaDOG1* transcript levels decreased in both tissues and were low the ABA-96 h time point which is just prior to endosperm rupture. The 96 h time point of the ABA series corresponds physiologically to the 18 h time point of the CON series (Müller et al. 2006). Our results presented in Fig. 6 therefore show that there is a strict association of *LesaDOG1* transcript abundance with radicle elongation and weakening of the micropylar endosperm. ABA is known to inhibit germination by stimulating ABI3 and ABI5 activity (Piskurewicz et al. 2008, 2009). One possibility how ABA could act is therefore to enhance *LesaDOG1* transcript accumulation via the ABI3- and ABI5-type transcription factors. As ABA also inhibits radicle elongation and endosperm cap weakening, we propose that *LesaDOG1* has an inhibitory role in these processes during seed germination.

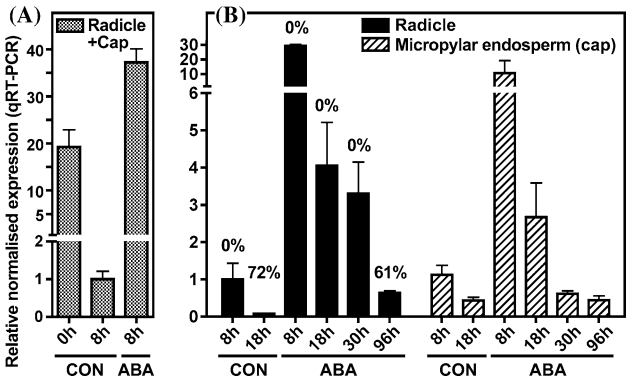


Fig. 6 *LesaDOG1* transcript expression pattern determined by qRT-PCR in specific *Lepidium sativum* FR1 seed tissues during incubation on medium without (CON) or with ABA added. Relative expression values based on the comparison with validated constitutive transcripts are presented. **a** Micropylar seed ends (radicle plus endosperm cap) of dry (0 h) and imbibed (8 h ± ABA) seeds are compared; 8 h-CON was set to 1. Note that tissue separation of dry seeds is not possible. **b** Radicles and micropylar endosperm caps dissected from seeds at the times indicated were analysed; radicle 8 h-CON was set to 1. Numbers above the columns are the percentage of endosperm rupture of the seed populations. Mean values +SE of 4 biological replicas; incubation of seeds at 24°C in continuous light, ± 10 µM ABA

Final conclusions

Lepidium sativum is an established model plant in seed germination research benefiting from its big seed size (facilitating tissue-specific analysis) and its molecular relatedness to Arabidopsis (enabling the utilization of the genome information). We showed that orthologs of the Arabidopsis dormancy gene *DOG1* are present in *L. sativum* and *Brassica rapa*. *DOG1* is expressed in seeds of these two species although they have only a shallow dormancy. *LesaDOG1* transcript expression in *L. sativum* seed tissues (radicle and micropylar endosperm) is regulated by imbibition and is ABA-inducible. *LesaDOG1* transcript abundance is associated with radicle extension growth and endosperm cap weakening. This strongly suggests novel role(s) for *DOG1* in germination timing. The monophyletic genus *Lepidium* with its high number of species offers future research possibilities for unexplored forms of coat and embryo dormancy. Moving beyond Arabidopsis to its relatives will advance our knowledge and will establish the Brassicaceae as a model family. Interdisciplinary and cross-species research approaches combining molecular genetics, physiology, biochemistry, ecology, biomechanics, engineering and technology with post-genomic, bioinformatic and modelling techniques will provide an integrated understanding of seed dormancy, after-ripening and germination. Comparative seed biology with model, horticultural and crop species representing important phylogenetic clades and important seed types will lead the way to the identification of evolutionary conserved and biodiverse mechanisms that control germination and dormancy.

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