New Phytologist

Research

The NADPH-oxidase *AtrbohB* plays a role in Arabidopsis seed after-ripening

Kerstin Müller[1], Anna Catharina Carstens[1], Ada Linkies[1], Miguel Angel Torres[2] and Gerhard Leubner-Metzger[1]

[1]Faculty of Biology, Institute for Biology II, Botany/Plant Physiology, University of Freiburg, Schänzlestr. 1, D–79104 Freiburg, Germany; [2]Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Universidad Politécnica de Madrid, ES–28223 Pozuelo de Alarcón, Madrid, Spain

Author for correspondence:
Gerhard Leubner-Metzger
Tel: +49 761 2032936
Email: gerhard.leubner@biologie.uni-freiburg.de

Received: *30 April 2009*
Accepted: *13 July 2009*

New Phytologist (2009) **184**: 885–897
doi: 10.1111/j.1469-8137.2009.03005.x

Key words: abscisic acid (ABA), after-ripening, alternative splicing, *Arabidopsis thaliana,* cress (*Lepidium sativum*), seed dormancy, seed germination, NADPH-oxidase (Rboh).

Summary

- Seeds can enter a state of dormancy, in which they do not germinate under optimal environmental conditions. Dormancy can be broken during seed after-ripening in the low-hydrated state.
- By screening enhancer trap lines of Arabidopsis, we identified a role for the NADPH-oxidase *AtrbohB* in after-ripening. Semiquantitative PCR was used to investigate *AtrbohB* transcripts in seeds. These methods were complemented with a pharmacological approach using the inhibitor diphenylene iodonium chloride (DPI) and biomechanical measurements in the Brassicaceae seed model system cress (*Lepidium sativum*) as well as protein carbonylation assays.
- *atrbohB* mutants fail to after-ripen and show reduced protein oxidation. *AtrbohB* pre-mRNA is alternatively spliced in seeds in a hormonally and developmentally regulated manner. AtrbohB is a major producer of superoxide in germinating Arabidopsis seeds, and inhibition of superoxide production by diphenylene iodonium (DPI) leads to a delay in Arabidopsis and cress seed germination and cress endosperm weakening.
- Reactive oxygen species produced by *AtrbohB* during after-ripening could act via abscisic acid (ABA) signalling or post-translational protein modifications. Alternative splicing could be a general mechanism in after-ripening: by altered processing of stored pre-mRNAs seeds could react quickly to environmental changes.

Introduction

Seeds from most species that grow in temperate environments are adapted to weather changes over the seasons by a mechanism that prevents germination at times when the conditions for seedling establishment and subsequent plant growth would be unfavourable: seeds can enter dormancy and even cycle in and out of varying depths of the dormant state in accordance with their environment (Bewley, 1997; Baskin & Baskin, 1998; Cadman *et al.*, 2006; Finch-Savage & Leubner-Metzger, 2006; Holdsworth *et al.*, 2008).

In the field, dormancy is broken by a species-specific set of conditions, including changes in ambient temperature, light penetration into the soil and soil hydration. In the laboratory, dormancy of Arabidopsis seeds is often broken by cold stratification of dark-imbibed seeds, which is known to raise levels of gibberellins (GA; Penfield *et al.*, 2005). Other means are light regimes whose action is mediated by phytochrome-induced GA biosynthesis, provision of the seeds with nutrients such as nitrate or the direct application of hormones counteracting dormancy, usually GA. Dormancy release also occurs during seed after-ripening (i.e. air-dry storage of seeds at room temperature for several months).

After-ripening involves changes in gene expression patterns, which is remarkable as the tissue is low-hydrated (Bove *et al.*, 2005; Leubner-Metzger, 2005; Leymarie *et al.*, 2007). It has been suggested that low-hydrated seeds contain pockets of higher hydration in which enzymatic catalysis and transcription might be possible (Leubner-Metzger, 2005; Holdsworth *et al.*, 2007).

In addition to breaking dormancy and widening the window of environmental conditions in which seeds will complete germination, characteristics of after-ripening are a loss of sensitivity to the germination-inhibiting hormone abscisic acid (ABA) and an increase in sensitivity to germination-

promoting hormones such as GA or ethylene (Finch-Savage & Leubner-Metzger, 2006). Fresh and after-ripened seeds differ not only in hormone signal transduction, but also in hormone contents. Changes in hormone contents are regulated both on the biosynthesis and degradation levels (Kucera *et al.*, 2005; Cadman *et al.*, 2006).

After-ripening leads to more synchronized and faster seed germination. In several species and conditions, dormant seeds do not germinate at all, while after-ripened seed populations quickly complete germination to 100%. Examples are the grains of wild oat (Poaceae) described by Adkins & Ross (1981) and barley (Poaceae), which do not germinate in the light in the dormant state in contrast to the after-ripened state (Gubler *et al.*, 2008). In all these cases, after-ripening takes place at room temperature in seeds stored in the air-dry state. It has been suggested that after-ripening and loss of dormancy are two distinct processes, as ABA-deficient, nondormant Arabidopsis seeds still show transcriptome changes characteristic for after-ripening after several months of storage at room-temperature (Carrera *et al.*, 2008; Holdsworth *et al.*, 2008).

Arabidopsis ecotypes vary widely in the depth of their dormancy, as measured in their capacity for after-ripening. Deeply dormant ecotypes such as Cape Verde Islands (Cvi) require many months of after-ripening to lose dormancy, while the ecotype Columbia (Col), commonly used in laboratories around the world and in this study, has a very shallow dormancy. The ecotype Cvi was used by Bentsink *et al.* (2006) for a quantitative trait locus (QTL) analysis of seed dormancy. In this QTL analysis, *delay of germination 1* (*DOG 1*) was identified as a major player in seed dormancy control. It later emerged that in addition to expression differences between accessions differing in dormancy, a complex pattern of alternative splicing is needed for DOG1 function (Schwab, 2008).

Once dormancy is broken, germination can proceed if conditions are favourable. Arabidopsis and the closely related Brassicaceae species garden cress (cress, *Lepidium sativum*) germinate in two sequential steps: first the testa ruptures, revealing the underlying endosperm still covering the radicle. Only after a lag of several hours or even days is the endosperm ruptured and the radicle emerges (Müller *et al.*, 2006). By definition, endosperm rupture is the end of the germination process which begins with seed imbibition (Bewley, 1997). Endosperm rupture of Arabidopsis and cress can be specifically delayed by the addition of physiological concentrations of ABA to the germination medium, an effect that can be countered by GA and ethylene (Kucera *et al.*, 2005; Müller *et al.*, 2006). This delay is correlated with a delay in weakening of the endosperm layer which covers the radicle tip. Endosperm weakening is a prerequisite for radicle emergence (Müller *et al.*, 2006). Both radicle growth potential and endosperm properties determine germination behaviour.

Seed after-ripening and germination can alter gene expression in a tissue-specific manner. In the work described here, we used Arabidopsis enhancer trap lines to identify genes regulated specifically during germination in an after-ripening and/or ABA-dependent manner in the embryo or endosperm. Enhancer trap techniques use a reporter gene construct driven by a minimal promoter which is only activated if the insertion site is close to an enhancer element in an endogenous promoter of the transgenic plant. A large-scale approach using a CaMV35S–minimal promoter–Glucuronidase (GUS) construct in Arabidopsis was initiated almost 10 yr ago with the aim of characterizing gene expression patterns in inflorescences (Campisi *et al.*, 1999). Seeds from this library were used by Liu *et al.* (2005a) to screen for seeds with a GUS signal during germination. This helpful resource has already been used to identify a transcription factor which positively regulates seed germination (Liu *et al.*, 2005b).

By using seeds from this collection we found, in the present work, that the NADPH-oxidase *AtrbohB* plays a role in seed germination and after-ripening. NADPH-oxidases, also known as respiratory burst oxidase homologues (Rboh) are named after a homology with the gp91phox domain of the animal respiratory burst oxidase which is involved in the immune response (Keller *et al.*, 1998). Rbohs localize to the plasma membrane, where they can transfer electrons from cytosolic NADPH or NADH to apoplastic oxygen, leading to the production of apoplastic superoxide (Sagi & Fluhr, 2006). The gene family has 10 known members in Arabidopsis; *AtrbohA-J* (Torres & Dangl, 2005) and homologues have also been found in a number of other angiosperm species (Simon-Plas *et al.*, 2002; Yoshioka *et al.*, 2003; Sagi *et al.*, 2004; Kobayashi *et al.*, 2006; Sagi & Fluhr, 2006). The individual Rbohs differ in their expression pattern across plant tissues and organs and have been shown to be involved in a range of processes including pollen tube elongation (Potocký *et al.*, 2007), response to pathogen attack (Torres *et al.*, 2002) and wounding (Sagi *et al.*, 2004) and root elongation and root hair development (Foreman *et al.*, 2003). Superoxide and other reactive oxygen species (ROS) have been proposed to play roles in seed germination and dormancy (Bailly, 2004; Oracz *et al.*, 2007; Müller *et al.*, 2009). However, it is not known which roles Rbohs play in seeds.

Materials and Methods

Germination tests

Three lots of 50 seeds of Arabidopsis (Col-0; *atrbohA-I* (from Miguel Angel Torres and Jonathan Jones); enhancer trap lines (Liu *et al.*, 2005a) ordered from NASC) or *Nicotiana tabacum* L. (cv Xanthi, WT and *ntrbohD*; Simon-Plas *et al.*, 2002), were placed on 1/10 Murashige–Skoog (MS)

salts solidified with 1% (w : v) agar. Germination took place at 24°C in continuous light. *Lepidium sativum* L. seeds (cress, 'Gartenkresse einfache'; Juliwa, Heidelberg, Germany) were incubated in Petri dishes on two layers of filter paper soaked with 1/10 MS salts at 18°C in continuous light as described by Müller *et al.* (2006). Where indicated, 1 μM (Arabidopsis) or 10 μM (cress) *cis*-S(+)-abscisic acid (ABA; Duchefa, Haarlem, the Netherlands) was added to the medium or cold stratification was conducted for 24 h at 4°C in darkness. Germination was scored under a binocular microscope by counting the incidence of endosperm rupture in the seed populations. Seeds were considered fresh for 3 wk after harvest and after-ripened after at least 6 months of air-dry storage at room temperature. Diphenylene iodonium chloride (DPI; Sigma-Aldrich) was dissolved in dimethyl sulphoxide (DMSO) as a 10 mM stock and added to the germination media in the concentrations indicated. Control media for comparison had an equal volume of DMSO added.

GUS staining and photographic documentation

Seeds were placed in Petri dishes on two layers of filter paper and covered in 5 ml staining solution (100 mM phosphate buffer (pH 7.0), 0.1% (v : v) Triton X-100 and 2 mM X-Gluc). After 24 h of incubation (dark, 24°C) the testa was removed. The staining was examined and photographed another 24 h later. All photographs of seeds were taken with the software IM1000 (Leica Microsystems, Wetzlar, Germany) and a Leica DCF480 digital camera attached to a Leica12.5 binocular microscope.

Identification and verification of T-DNA insertion sites in enhancer trap lines

Genomic DNA was extracted using the DNeasy Plant Mini kit (Qiagen). Genome-walking PCR was performed using the GenomeWalker and Advantage 2 Polymerase Mix Kits (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). Genomic DNA of CS24365 was digested with *Stu*I. Two specific primers for the T-DNA insert were designed, the first (5′-AACTTAATCGCCTTGCAGCACA-3′) being used in the first round of PCR, the second (5′-ATGAGACCTCAATTGCGAGCT-3′) serving as a nested primer in the second round of PCR. The resulting fragments were cloned into pGEM-TEasy vectors and sequenced. Thermal asymmetric interlaced PCR and arbitrary degenerate primer design was carried out according to Liu & Huang (1995). The two primers mentioned above plus another nested primer (5′-ATCGCCTTGCAGCACATCCC-3′) were used as insert-specific primers. Fragments were purified from an agarose-gel using the DNA Gel Extraction Kit (Fermentas, St Leon-Rot, Germany) and directly sequenced.

Reverse transcription and semiquantitative PCR

RNA was extracted from seedlings of Arabidopsis wild type (WT) and mutants with the Plant RNeasy Kit (Qiagen) including the optional DNAse step and reverse transcribed (SuperscriptIII; Invitrogen) with a mix of oligodT and random hexamer primers (1 : 1). The resulting cDNA was used as a template with rRNA-primers RR-FOR (5′-CGAGCTGATGACTCGCGCTTA-3′) and RR-REV (5′-GAGTGGAGCCTGCGGCTTA-3′) and *AtrbohB*-primers B-FOR (5′-GGAATTCC CGTCAGAAGGTGAGACAAACA-3′) and B-REV (5′-CGGGATCCCG CCAGAATTCAAACAATTCAGTTTT-3′). To confirm splice variants of *AtrbohB-ß*, we used forward primers B-intr-1-for (5′-CACTGCAATACTCAACTTGTTT-3′) and B-intr-1-for-2 (5′-CTCTCTGTAATTTCACTGCAA-3′) with reverse primers B-ex2/ex3-rev (5′-GTCCAAATTTTTGTCCACCATA-3′) and B-ex3/ex4-rev (5′-GCAATTATCT CCTTAACCTCAT-3′). Primers used for testing alternative splicing in other Atrbohs were A-FOR (5′-ACGGATTATTACACCGATCTAGA-3′), A-REV (5′-ATAA TCTCTCTTACTTCGGCTT-3′), D-FOR (5′-CCTC TTACTCTCTGCCAAGT-3′), D-REV (5′-AATAATCTCAGCCACCTCTTC-3′), E-FOR (5′-GGATTGCGATT GCGATCAGA-3′), E-REV (5′-AAGTTCTTTGATTTCTTCTCTAG-3′), F-FOR (5′-TCGCTCCGATTTCGCTCAAT-3′), F-REV (5′-ATTATCTCTTTTACTTCCTCTTC-3′), I-FOR (5′-GGAATCGTTGATTGGAACGAT-3′) and I-REV (5′-TCTCTTTTACTTCATTCTC TGTG-3′). For all primer pairs, we used an annealing temperature of 60°C. A total of 35 PCR cycles were used for rboh-primers and 25 cycles for the rRNA. Both were confirmed to be in the linear amplification phase.

In-situ RNA hybridization

In-situ RNA hybridization was performed as described previously (Mayer *et al.*, 1998). The *AtrbohB*–probe cDNA was amplified from reverse-transcribed RNA of seedlings using primers B-FOR and B-REV (see earlier). After digestion with *Eco*RI and *Bam*HI, the cDNA was ligated into pBluescript II KS$^+$. For the antisense probe, the plasmid was linearized with *Eco*RI and transcribed with T7 RNA polymerase (Promega) using a digoxigenin-labelling kit (Roche Diagnostics); for the sense probe, the plasmid was linearized with *Bam*HI and transcribed with T3 RNA polymerase (Promega).

Puncture force measurements

Puncture force was measured as described previously (Müller *et al.*, 2006). In short, cress seeds were cut in half, the radicle removed without damaging the endosperm cap and a metal probe slowly lowered into the empty endosperm

cap. The force it took to rupture the endosperm was recorded with a custom-made machine.

Genotyping of *atrbohB*

The *atrbohB* insertion mutant was identified by performing a PCR reaction on genomic DNA extracted from pools of Col-0 plants containing *dSpm* transposon insertions (Tissier *et al.*, 1999). Primers used include dSpm11 and dSpm1 (Tissier *et al.*, 1999) and specific primers for *AtrbohB* 154B (5′-GAATAATGTAATTGTAGTGAATGCG-3′) and 75 B (5′-ACAAATTCGCTAGATTCAACCAT-3′). The PCR products, 154B/dSpm1 and 75B/dSpm1, were 150 bp and 500 bp respectively. Insertions in the gene were confirmed by sequencing PCR products spanning the insertion. The line identified was homozygous and derived from a BASTA-resistant heterozygous parent.

Superoxide localization by nitroblue tetrazolium (NBT) staining

Arabidopsis embryos were dissected from imbibed seeds under a binocular microscope, equilibrated for 10 min in 20 mM phosphate buffer, pH 6.5, and transferred to a staining solution containing 5 mM NBT in 20 mM phosphate buffer, pH 6.5. Seeds were left in the staining solution until staining was visible.

Extraction of total soluble proteins and Oxyblots

Two biological replicates of 100 μl Arabidopsis seeds were ground with mortar and pestle in liquid nitrogen and thawed in thiourea–urea buffer (Harder *et al.*, 1999) with the protease inhibitor cocktail 'Complete Mini' (Roche), 60 U ml^{-1} DNAse and 6 U ml^{-1} RNAseA. After shaking for 30 min at 4°C, samples were centrifuged at 14800 g for 15 min at 4°C until the supernatant was clear. The final supernatant corresponds to the soluble protein extract. Protein concentration was determined with a Bio-Rad assay kit.

Bovine gamma globulin was used as a standard protein samples (10 μg) proteins were separated in one-dimensional electrophoresis on 11% polyacrylamide gels and stained with Coomassie brilliant blue. 2,4-Dinitrophenylhydrazone (DNP) labelling and detection of carbonylated proteins was performed as described in Job *et al.* (2005). Sodium dodecyl sulphate (SDS) was added to the extracts with a final concentration of 0.8% (w : v) and dialysis against water performed over night at 4°C. Four volumes of 10 mM dinitrophenylhydrazine (DNPH) in 2 mM HCl were added after dialysis and samples mixed for 30 min at room temperature. The labelled proteins were then precipitated with 20/80 TCA/acetone containing 1 mM dithiothreitol (DTT) and resolubilized in thiourea–urea buffer (Harder *et al.*, 1999) with 2% (v : v) Triton X and 20 mM DTT. After separation on one-dimensional gels, proteins (80 μg) were transferred by semi-dry blot onto nitrocellulose membranes. The DNP-labelled carbonylated proteins were detected with a rabbit anti-DNP antibody (Serologicals, Norcross, GA, USA) followed by a secondary antibody coupled to horseradish peroxidase (Sigma-Aldrich). Detection was performed with the ECL Plus kit (GE Healthcare, Little Chalfont, UK).

Sequence analyses

Sequences were aligned and analysed with the software GENEIOUS 4.0.2 (Biomatters Ltd, Auckland, New Zealand). The implemented MUSCLE algorithm (Edgar, 2004) was used for alignments.

Results

After-ripening promotes seed germination in Brassicaceae and other families

We compared the germination process of fresh and after-ripened cress seeds, fresh and after-ripened cress seeds by time-course analyses of their endosperm rupture. Mature cress seeds displayed only a shallow dormancy. Even immediately after harvest, *c.* 80% of the seeds germinated readily within 4 d (Fig. 1). After the seeds were after-ripened for several months, the final germination percentage rose to almost 100%, the kinetics of endosperm rupture was more uniform and the overall germination speed increased: all seeds completed germination within 1 d. The effect of after-ripening on germination can be demonstrated in a range of different species. We compared the $t_{50\%}$ values of fresh and after-ripened seeds (i.e. the mean times required for the populations to reach 50% endosperm rupture) of four endospermic species. After-ripening reduced the $t_{50\%}$ values about twofold for the Brassicaceae seeds of cress (Fig. 1) and Arabidopsis (Fig. 2). After-ripening also promoted the germination of Solanaceae seeds such as tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*), where the after-ripened : fresh $t_{50\%}$ ratios were 0.81 and 0.23, respectively (Groot & Karssen, 1992; Leubner-Metzger, 2003).

The extent of the after-ripening mediated changes is not only species specific, but also ecotype specific and to a certain extent even batch specific. Seed after-ripening is thus an important developmental process that occurs in low-hydrated seeds and determines the seeds' responses to environmental cues. We aimed to identify genes that play a role in this process by screening a selection of enhancer trap lines with reporter-gene expression in seeds for their germination phenotypes in the fresh and after-ripened state.

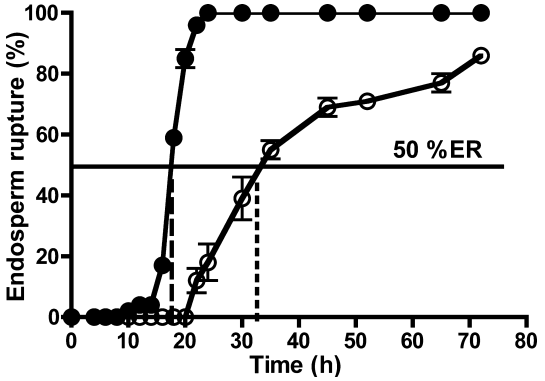

Fig. 1 Seed after-ripening releases dormancy and promotes germination. Time-course of cress (*Lepidium sativum*) endosperm rupture at 18°C in continuous light. Seeds just after harvest (fresh seeds, open circles) and after 6 months of air-dry storage (after-ripened (ar), closed circles) at room temperature. The black line indicates 50% endosperm rupture of the seed populations, the dotted line connects it to corresponding times $t_{50\%}$ (fresh) - 32 h, the dashed line to $t_{50\%}$ (after-ripened) - 17 h; i.e. the after-ripened : fresh $t_{50\%}$ ratio is 0.53). Means of three lots of 50 seeds ± SE are given.

Seeds of an enhancer trap line with an insertion in *AtrbohB* have an after-ripening phenotype

We obtained several lines from the seed–GUS-expression library donated by the group of Hiro Nonogaki to the Arabidopsis Biological Resource Center. These seeds had been prescreened for GUS staining during germination (Liu *et al.*, 2005a,b). We propagated and rescreened these lines for their GUS expression patterns in order to determine the spatial activity of the trapped enhancer in after-ripened germinating seeds. Seeds were dissected to clearly localize staining and grouped as showing staining in the embryo and/or the endosperm (Table S1). All lines were also screened for a germination phenotype, and only those that differed in the kinetics of endosperm rupture when compared with the wild type (WT, Col-0) were analysed further. As after-ripening includes changes in ABA-sensitivity, we conducted germination kinetics of fresh as well as after-ripened seeds on medium with and without ABA, respectively. Table S1 lists lines, localization of staining and the germination phenotypes.

Line CS24365 showed a particularly interesting phenotype with regard to after-ripening (Fig. 2): this is evident from detailed time-course analyses of the endosperm rupture of fresh and after-ripened seeds of CS24365 in medium without (CON; Table S1) and with ABA (Fig. 2). We did not observe differences in CON, but fresh seeds completed germination earlier than WT seeds on medium with ABA (Fig. 2a). Moreover, while WT seeds became less sensitive to ABA during after-ripening, the germination kinetics of CS24365 did not change: for after-ripened seeds, the insertion line completed germination later than the WT (Fig. 2b). A comparison of the associated $t_{50\%}$ values

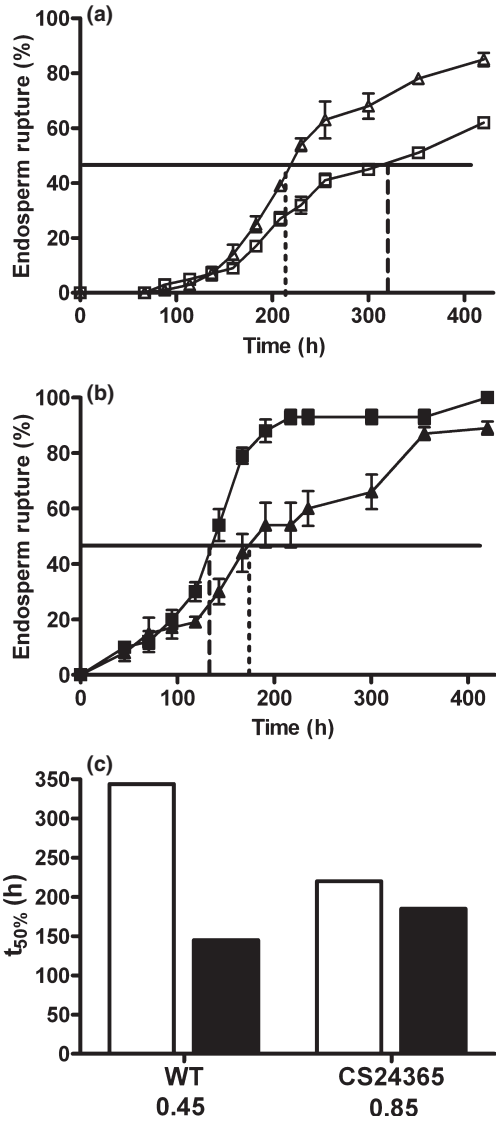

Fig. 2 The Arabidopsis enhancer trap line CS24365 (insertion in *AtrbohB*) exhibits an after-ripening phenotype: the ABA sensitivity of endosperm rupture does not change during after-ripening. (a,b) Germination kinetics of fresh (a) and after-ripened (ar) (b) seeds of wild type (WT; squares) and CS24365 (triangles) in the presence of 1 μM ABA. The black line indicates 50% endosperm rupture, the dotted line connects it to the corresponding times $t_{50\%}$ (CS24365), the dashed line to $t_{50\%}$ (WT); $t_{50\%}$ represents the time needed to reach 50% endosperm rupture. Mean ± SE of three lots of 50 seeds germinated at 24°C in continuous light are shown. (c) Comparison of $t_{50\%}$ values of fresh (open bars) and after-ripened (closed bars) seeds for WT and CS24365 (*AtrbohB* insertion mutant). Ratios of $t_{50\%}$ (after-ripened) : $t_{50\%}$ (fresh) are given below the *x*-axis.

showed that while the $t_{50\%}$ decreased over twofold for the WT, hardly any change could be observed for the mutant (Fig. 2c). The after-ripening mediated decrease in the ABA sensitivity of endosperm rupture thus seems to be blocked in the CS24365 line.

Genome walking led to the identification of the T-DNA insertion sites in enhancer trap line CS24365 (Fig. 3a),

which was confirmed by thermal asymmetric interlaced (TAIL)-PCR. The GUS-construct localized to chromosome 1, basepair 2932468, which is in the promoter region 300 bp upstream of the NADPH-oxidase *AtrbohB* (At1g09090). CS24365 showed a clear GUS staining in the whole embryo (Fig. 3b) but not the endosperm layer. Semi-quantitative PCR confirmed that the expression of *AtrbohB* is lower in imbibed after-ripened CS24365 seeds than in the corresponding WT (Fig. 3c). The transcript of *BIP3* (At1g09080), the other gene close to the insertion site, was not affected (data not shown). In order to confirm that the phenotype was actually caused by the insertion in the *AtrbohB* gene promoter, we compared the germination phenotype of CS24365 with that of the insertion mutant *atrbohB*, which carries a *dSpm* insertion in the coding region itself (Torres *et al.*, 2002). Both lines behaved in the same way (see *atrbohB*-mutant in Table 1). Thus, the reduced expression of *AtrbohB* led to an after-ripening phenotype, where the ABA sensitivity does not change during after-ripening.

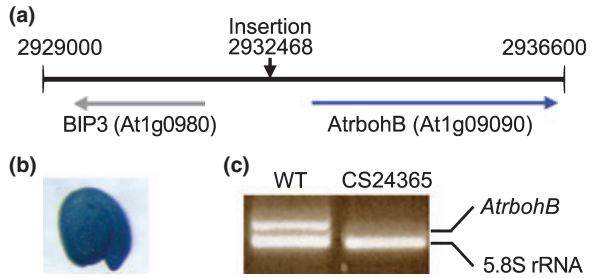

Fig. 3 The enhancer trap in line CS24365 disrupts the expression of *AtrbohB* (At1g09090). (a) Localization of the insertion in enhancer trap line CS24365 insertion to chromosome 1, bp 2932468, in the promoter region of *AtrbohB* (At1g09090). (b) Glucuronidase (GUS) staining of imbibed seeds leads to a strong blue colouring of the whole embryo. An isolated embryo is shown. (c) Semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) comparing expression of *AtrbohB* transcripts in 24 h imbibed seeds of wild type (WT) and the enhancer trap line CS24365. The *AtrbohB* transcript is only detected in the WT but not in the mutant; 5.8S rRNA served as internal standard.

AtrbohB is differentially expressed and the pre-mRNA alternatively spliced in germinating Arabidopsis seeds depending on after-ripening status and ABA

We investigated the expression pattern of the *AtrbohB* transcript by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) on WT seed RNA extracted from fresh and after-ripened seeds imbibed for 24 h in the absence and presence of 1 μM ABA. At this point, neither fresh nor after-ripened seeds exhibited endosperm rupture. Interestingly, we observed two bands of *AtrbohB*-transcripts (Fig. 4a) which seem to be the products of alternative mRNA splicing (Fig. 4b): sequencing of the two fragments showed that in the transcript leading to the larger band (635 bp – *AtrbohB-β*), intron 1 is retained, while intron 1 is spliced out in the smaller fragment (485 bp – *AtrbohB-α*). In order to confirm the identity of the larger band as a product of alternative splicing and exclude the possibility of genomic DNA contamination, we did a PCR with a forward primer in intron 1 and reverse primers spanning the exon border of exons 2/3 and exons 3/4, respectively. Both sets of primers yielded PCR bands of the expected sizes, confirming that *AtrbohB-α* and *AtrbohB-β* are alternative splicing variants of the *AtrbohB* pre-mRNA. A sequence alignment of the *AtrbohB* genomic sequence with the *AtrbohB-α* and *AtrbohB-β* cDNAs showed that the retention of intron 1 leads to a premature termination codon (PTC; Figs 4b and S1). It is highly unlikely that *AtrbohB-β* is translated into a protein, as a PTC at such an early point

Table 1 Some *atrboh* mutants germinate differently from wild type (WT) seeds

Line	Germination fresh CON	Germination fresh ABA	Germination after-ripened CON	Germination after-ripened ABA	RNA localization in WT after-ripened CON
atrbohA	–	0	0	–	ND
atrbohB	0	+	0	–	EM
atrbohC	0	0	0	0	EM
atrbohD	0	0	–	–	EM, ES
atrbohE	+	+	+	0	EM, ES
atrbohF	–	–	–	–	ND
atrbohG	0	0	0	0	ND
atrbohH	+	0	+	0	EM
atrbohI	+	+	+	+	EM
atrbohD/F	0	0	–	–	

Fresh and after-ripened Arabidopsis mutant seeds were germinated at 24°C in continuous light without (CON) or with 1 μM ABA added and after 1 d of stratification at 4°C in darkness. Symbols: +, germinated faster than WT; 0, germination equals WT; –, germinated slower than WT. RNA localization of the corresponding gene by *in situ* hybridization: EM, embryo; ES, endosperm; ND, not determined.

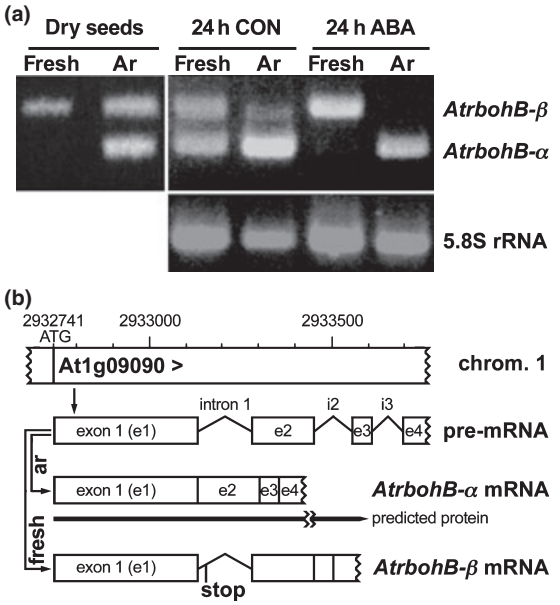

Fig. 4 Alternative *AtrbohB* mRNA splicing is hormonally and developmentally regulated in Arabidopsis wild type (WT) seeds. (a) Semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) of fresh or after-ripened (ar) seeds. RNA was extracted from dry seeds and from seeds imbibed for 24 h in medium without (CON) or with 1 μM ABA added (24°C, continuous light). Two *AtrbohB* RT-PCR fragments were obtained: *AtrbohB-α* (485 bp) and *AtrbohB-β* (635 bp). The relative abundance of the two transcript types changed in the presence of ABA depending on the developmental state of the seeds. 5.8S rRNA was used as a loading control. Note that the RT-PCR results of dry and imbibed seeds can only be compared qualitatively. As we cannot expect 5.8 S rRNA to be constant between these two states a semiquantitative comparison of dry and imbibed seeds is not possible. (b) Diagram illustrating the alternative splicing of *AtrbohB* mediated by seed after-ripening. The upper parts show the location of the *AtrbohB* gene (At1g09090) on chromosome 1 and the first four exons and three introns of the transcribed pre-mRNA. In ABA-treated after-ripened seeds splicing of this pre-mRNA leads to the *AtrbohB-α* mRNA for which the predicted protein is drawn below. In ABA-treated fresh seeds, intron 1 of the pre-mRNA is retained in the *AtrbohB-ß* mRNA. Introns 2 and 3 are spliced out. Retention of intron 1 leads to a premature termination codon.

will almost certainly lead to nonsense-mediated mRNA decay (NMD; Kertész *et al.*, 2006).

Figure 4a shows that the expression of the two mRNA splicing variants *AtrbohB-α* (without intron 1) and *AtrbohB-β* (including intron 1) is regulated differentially in fresh and after-ripened WT-seeds in the presence and absence of ABA (Fig. 4a): both transcript variants are expressed in fresh seeds imbibed in CON medium. The addition of 1 μM ABA to the medium caused the accumulation of *AtrbohB-ß* mRNA in fresh seeds and prevented *AtrbohB-α* mRNA expression. In after-ripened seeds, in which *AtrbohB-α* was found to be the dominant splicing form in both CON and ABA medium, ABA prevented *AtrbohB-β* mRNA expression. Thus, it appears that developmental factors (after-ripening) and hormonal regulation (ABA) both affect the relative expression of the two *AtrbohB* splicing variants. Alternative splicing of intron 1 was not evident for other Atrbohs investigated which demonstrates a specificity of the effect for AtrbohB.

We also checked which splicing variants are present in dry seeds. We observed that only splice variant *AtrbohB-ß* or pre-mRNA, which would yield the same band, is present in dry fresh seeds (Fig. 4a). Thus, 24 h imbibition of fresh seeds probably activated splicing of intron 1 in medium without, but not with ABA. In after-ripened dry seeds, we observed both bands in approximately equal strength. The transcript profiles of after-ripened imbibed seeds can thus to result from altered splicing efficiency. Nonsense-mediated mRNA decay may play an additional role in removing *AtrbohB-ß* transcripts.

A database search on NCBI for *AtrbohB* cDNAs provided two sequences, in which a second instance of alternative splicing was found: NM_100780.3 retains intron 9, which is not present in NM_202070.1. Retention of intron 9 also leads to a premature translation stop (Fig. S2), leaving out the NADPH binding sites. It is still unclear which of the splicing variants are translated into proteins.

AtrbohB is a major producer of superoxide in embryos of germinating Arabidopsis seeds

RNA *in-situ* hybridization in WT seeds showed that *AtrbohB* transcript expression localizes to the embryo (Fig. 5a). This finding is in agreement with the GUS-staining in the enhancer-trap line (Table S1).

While we cannot quantify the amount of ATRBOHB-protein as no specific antibodies are available and we cannot extract sufficient membranes from seeds, we can detect the product of their enzymatic activity: RBOHs are NADPH-oxidases that produce apoplastic superoxide. Superoxide reacts with NBT to a blue-ish precipitate. Histostains with NBT showed that superoxide is produced in the embryo of 24 h-imbibed Arabidopsis WT seeds (Fig. 5b). Superoxide production was strongly reduced in the insertion mutant *atrbohB* (Fig. 5b) and the enhancer trap line CS24365 (data not shown), which points to a major role of RBOHB in the superoxide production in the embryo during seed germination. In accordance with this, DPI, an inhibitor of NADPH-oxidases and other flavin-containing enzymes, inhibited superoxide production in WT seeds (Fig. 5c).

After-ripened *atrbohB* seeds show reduced protein carbonylation

Our findings indicate that ATRBOHB is largely responsible for superoxide production in Arabidopsis embryos. Oracz *et al.* (2007) showed that targeted protein oxidation occurs during after-ripening as well as artificial breakage of dormancy. A possible explanation for the after-ripening

phenotype of the *atrbohB* mutant is thus that its reduced superoxide production during the after-ripening period leads to a reduction of the required protein oxidation.

We therefore decided to study protein carbonylation, an identifier of protein oxidation (Möller *et al.*, 2007), in fresh and after-ripened WT and *atrbohB* seeds by immunoblots with antibodies against derivatives of carbonyls in soluble proteins (Fig. 6). We compared dry and 6-h imbibed seeds. Levels of protein carbonylation were slightly higher in fresh dry WT seeds than in atrbohB seeds. This difference became more pronounced during after-ripening: we observed a lower overall protein oxidation signal in dry *atrbohB* seeds compared with WT seeds that had after-ripened for the same amount of time. After 6 h imbibition, protein oxidation levels were reduced in WT seeds, presumably by proteolysis of the carbonylated proteins, while those of the mutants remained constant or even increased slightly. Thus, superoxide production by ATRBOHB could contribute to protein oxidation involved in seed after-ripening.

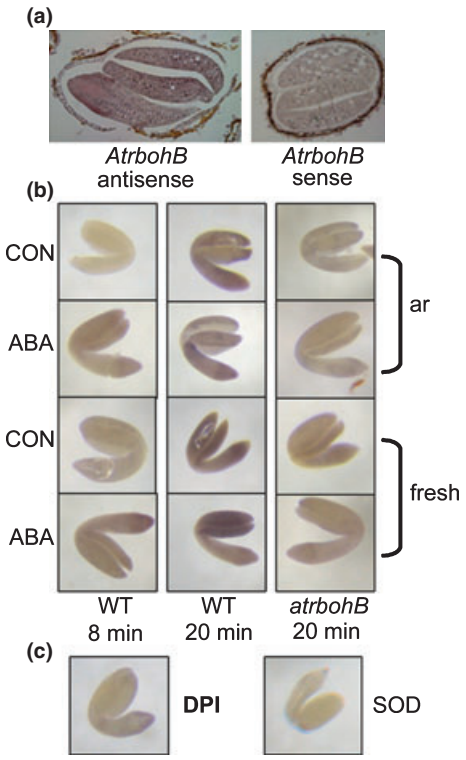

Fig 5 *AtrbohB*-transcripts and superoxide production localize to the embryo of germinating Arabidopsis seeds. (a) RNA *in-situ* hybridization shows the localization of the *AtrbohB* transcript to the embryo, but not the endosperm, of 24-h imbibed Arabidopsis seeds. No signal was visible with the corresponding sense probe. This probe was designed to bind to both the *AtrbohB-α* and *AtrbohB-ß* transcript splice variant. (b) Superoxide production in embryos of 24-h imbibed fresh and after-ripened (ar) Arabidopsis wild type (WT) and *atrbohB*-seeds was visualized with an nitroblue tetrazolium (NBT) tissue stain. Superoxide production is indicated by a blue-ish precipitate in the tissue. Staining time is given below the respective columns of pictures. ABA, 1 μM ABA in germination medium. (c) Even after 30 min, no NBT-staining was observed in WT embryos in the presence of 50 μM diphenylene iodonium chloride (DPI), an inhibitor of NADPH-oxidases and other flavin-containing enzymes. The addition of 10 U ml^{-1} superoxide dismutase (SOD), which leads to the disappearance of superoxide by its dismutation to hydrogen peroxide and oxygen, was used as a control for the specificity of the stain. No staining was visible after 30 min.

Insertion mutants in other *Atrboh* genes show a germination phenotype

In order to test if other *Atrboh* genes are involved in seed germination, we also tested germination of *dSpm* insertion mutants for nine other known Arabidopsis *rbohs* (Table 1). Indeed, while *atrbohB* was the only mutant in which germination in the presence of ABA was accelerated in fresh seeds, mutants carrying an insertion in *AtrbohE*, *AtrbohH* or *AtrbohI* germinated faster than WT in the fresh and after-ripened state on medium without ABA, and on medium with ABA in the after-ripened state. Interestingly, *atrbohH* showed a very strong sensitivity to ABA in the fresh state, when it germinated slower than any other mutant or the WT. *AtrbohF* was observed to germinate slower than the WT in all conditions tested, while *atrbohD* germinated more slowly only in the after-ripened state. No clear germination phenotype was detected for the remaining *atrbohs* in the conditions we used. We performed *in-situ* RNA hybridization to check for expression of the *rbohs* whose mutation

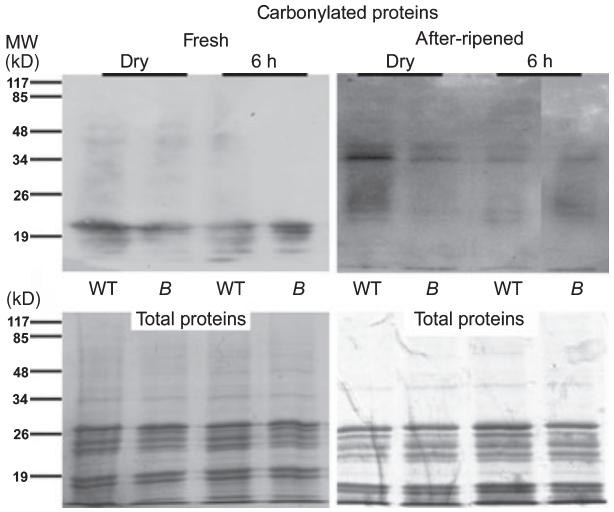

Fig. 6 Protein carbonylation is reduced in after-ripened seeds of the *atrbohB* mutant compared with Arabidopsis wild type (WT). Immunoblots with antibodies against derivatives of carbonylated (oxidized) proteins. Protein extracts from dry seeds and seeds after 6 h imbibition of WT and *atrbohB* (*B*). The seeds had been grown together and after-ripened in the same location for 2 yr. Below the blot, a Coomassie stain is shown as a loading control. The majority of the stained proteins most likely correspond to the very abundant globulin storage protein. MW, apparent molecular mass in kDa.

led to a germination phenotype in after-ripened WT seeds (Table 1). There did not appear to be a connection between RNA localization and phenotype. The fact that the germination phenotypes differ between these mutants points to a variety of roles the Atrbohs might play during germination.

We examined whether alternative splicing of intron 1 in fresh and after-ripened seeds is evident for the Atrbohs for which we found germination phenotypes of the mutants (*AtrbohA*, *D*, *E*, *F*, *I*; Table 1). In this independent RT-PCR experiment with forward primers in exon 1 and reverse primers over the exon3/exon4 border, *AtrbohB* was also included as a positive control and, as expected, there was an amplification of *AtrbohB-β* (intron 1 retained) and *AtrbohB-α* (intron 1 spliced) as shown in Fig. 4. By contrast, under the same experimental conditions no PCR bands for *AtrbohA*, *D*, *E*, *F* and *I* were amplified with the predicted size of a transcript retaining intron 1 (β-type). The PCR bands with the expected size for the α-type transcripts (intron 1 spliced) of *AtrbohA*, *D*, *E*, *F* and *I* were amplified in after-ripened seeds in the dry and 24-h imbibed states without and with ABA added. The α-type transcripts, but no β-type transcripts, were detected for *AtrbohD*, *E*, *F* and *I* in 24 h-imbibed fresh seeds. α-Type *AtrbohE* transcripts (but no β-type) were also detected in dry fresh seeds. *AtrbohE* appeared to be most abundant, whereas *AtrbohF* and *AtrbohI* were of low abundance, and *AtrbohA* was not detected in fresh seeds. This demonstrates that the after-ripening status and ABA did not cause a general effect on intron 1 splicing, but that this effect is specific for *AtrbohB*.

After-ripened seeds of the tobacco mutant *ntrbohD* (Simon-Plas *et al.*, 2002) germinated slower than the corresponding WT on media with and without ABA, just as the corresponding Arabidopsis mutant *atrbohD* does (data not shown). Thus, Rbohs might play a role in seed germination in other species besides Arabidopsis.

DPI delays Arabidopsis and cress endosperm rupture and cress endosperm weakening

In a pharmacological approach, we tested the effect of the NADPH-oxidase inhibitor DPI on seed germination. 100 μM DPI delayed the germination of after-ripened Arabidopsis WT seeds in the presence and absence of ABA, while *atrbohB*-mutant seeds showed a strongly reduced sensitivity to DPI (Table 2). We conclude that rbohB is a major target of DPI in Arabidopsis seed germination, although other *Atrbohs* may also contribute to the regulation of seed germination.

Some Atrbohs, such as *AtrbohD* (Penfield *et al.*, 2006), are known to be expressed in the Arabidopsis endosperm layer. We therefore examined whether endosperm weakening is affected in DPI-treated seeds. As Arabidopsis seeds are too small to allow for biomechanical approaches, we used the closely related species cress whose seeds are larger than those of Arabidopsis but have a very similar seed anatomy with one to two cell layers of endosperm and similar germination behaviour (Müller *et al.*, 2006). Treatment with DPI delayed the endosperm rupture of cress seeds just as that of Arabidopsis seeds, although a higher concentration (150 μM) had to be used. We measured a strong effect on endosperm weakening: at 18 h after imbibition, it took a force of 19.5 mN to puncture the endosperm cap covering the radicle in untreated seeds, but 29.8 mN in DPI-treated seeds (Table 2). We hypothesize that Atrbohs play roles both in the embryo and the endosperm of germinating Brassicaceae seeds.

Table 2 Diphenylene iodonium chloride (DPI) delays endosperm rupture of Arabidopsis and cress and endosperm weakening of cress

Time and treatment	Endosperm rupture (%)	Endosperm resistance (mN)
Arabidopsis thaliana, wild type after-ripened		
30 h control	95 ± 1	ND
30 h 100 μM DPI	51 ± 2	ND
374 h 1 μM ABA	76 ± 2	ND
374 h 1 μM ABA 100 μM DPI	22 ± 1	ND
Arabidopsis thaliana, *rbohB* after-ripened		
374 h 1 μM ABA	25 ± 1	ND
374 h 1 μM ABA 100 μM DPI	16 ± 0	ND
Lepidium sativum (cress), after-ripened		
18 h control	78 ± 3	19.5 ± 2.6
18 h 150 μM DPI	19 ± 5	29.8 ± 2.6
96 h 10 μM ABA	75 ± 4	21.0 ± 1.3
96 h 10 μM ABA 150 μM DPI	12 ± 4	Not measured

ND, not determinable (seeds are too small). Endosperm rupture (%): means of three lots of 50 seeds ± SE are given, germination at 24°C (Arabidopsis) : 18°C (cress) in continuous light. Endosperm resistance: means of at least three lots of 25 seeds ± SE are given.

Discussion

Seed after-ripening is a process of great relevance both in wild species and crops (Baskin & Baskin, 1998; Cadman *et al.*, 2006; Finch-Savage & Leubner-Metzger, 2006). While the first might rely on after-ripening to time their germination with changing environmental conditions, dealing with dormancy and after-ripening of the latter is an important aspect of seed management in agriculture and *ex-situ* seed banks. It is thus relevant to further our understanding of this important process and the genes and proteins involved.

By screening a set of Arabidopsis enhancer trap lines for after-ripening and germination phenotypes, we found that a decrease in At*rbohB*-transcript in embryos of mature seeds leads to changes in after-ripening: mutant seeds do not

after-ripen with respect to their sensitivity to ABA. There are two possible interpretations for this phenotype, and these are not mutually exclusive. (1) AtrbohB plays a role in ABA perception or signalling. The signal would in this case be either superoxide, produced by AtrbohB or the product of its dismutation, hydrogen peroxide. Hydrogen peroxide would be more likely to serve as messenger in a signalling cascade because of its comparatively long lifespan and ability to cross membranes (Hancock, 2001; Laloi *et al.*, 2004). The associated sensors could either react directly to the hydrogen peroxide molecule or sense changes in the redox state of the cell. Other Atrbohs have already been shown to be involved in hormone signalling: AtrbohD and AtrbohF have been shown to be involved in ABA-signal transduction pathways in guard cells (Kwak *et al.*, 2003), while AtrbohF is also involved in ethylene action on stomatal closure (Desikan *et al.*, 2006). (2) AtrbohB promotes after-ripening by producing superoxide, which leads to post-translational modifications by carbonylation of proteins. After-ripening and loss of dormancy have been associated with an increase in reactive oxygen species (ROS; superoxide and hydrogen peroxide) production in sunflower axes (Oracz *et al.*, 2007). This accumulation of ROS leads to targeted protein carbonylation during after-ripening as well as during artificial breaking of dormancy by the application of cyanide or methylviologen. Our Oxyblots showed that *atrbohB* mutant seeds indeed have a lower overall protein carbonylation after an equal period of after-ripening than the corresponding WT.

We found that the *AtrbohB* pre-mRNA is alternatively spliced in fresh and after-ripened seeds, with variant *AtrbohB-ß* retaining intron 1. This process is developmentally and hormonally regulated: it depends on the state of the seed (fresh/after-ripened) and is ABA-dependent. The only other published report of alternative splicing of a plant *Rboh* is, to our knowledge, *ZmrbohB* (highest sequence similarity in Arabidopsis with *AtrbohF*), which occurs in two splicing variants in different maize tissues (Lin *et al.*, 2009). Interestingly, the expression pattern of the splicing variants could be changed by subjecting the plants to stress, which is known to lead to ABA production. One of the variants retains intron 11, which leads to a PTC, and the authors suggest that this leads to NMD. Just as the splicing variant of *AtrbohB* with a retained intron 9 we found on NCBI, the variant of *ZmrbohB* retaining intron 11 would miss its NADPH-binding domain. As the domains relevant to electron transport are still present, this protein might still display activity, if the mRNA is translated and not degraded by NMD mechanisms. Interestingly, the animal NADH-Oxidase NOX1 also displays alternative splicing with one variant leading to a protein without NADH binding sites (Geiszt *et al.*, 2004). While intron retention is rare in animal systems (Kan *et al.*, 2002; Nagasaki *et al.*, 2005), it has been found to be the most common form of alternative splicing in Arabidopsis (Ner-Gaon *et al.*, 2004; Nagasaki *et al.*, 2005; Kertész *et al.*, 2006).

Our discovery that AtrbohB alternative mRNA splicing is differentially regulated in fresh and after-ripened seeds is the first instance of alternative splice forms being differentially expressed in seeds of different after-ripening status. *DOG1*, a gene cloned for a major QTL of Arabidopsis seed dormancy (Bentsink *et al.*, 2006), also occurs in various splice forms, but no relation to after-ripening is known. It seems that the ratios of *DOG1* transcript variants in combination with the actual amounts of the different forms contribute to the regulation of dormancy, and these ratios change during seed maturation when dormancy is induced (Schwab, 2008). The changes in transcript levels of *AtrbohB-α* and *AtrbohB-ß* we observed in our semiquantitative PCR involve changes both to the ratio and possibly also to the total amount of *AtrbohB* transcripts.

We hypothesize that alternative splicing is an attractive mechanism for dormancy and after-ripening regulation in seeds: seeds are known to store mRNAs. A global analysis of stored mRNAs in seeds by Nakabayashi *et al.* (2005) identified over 12 000 mRNAs in dry seeds, among which those with ABA responsive elements (ABREs) in the 1 kb promoter region were over-represented. A scan for promoter motifs of *AtrbohB* with the PLACE signal scan software (Higo *et al.*, 1999) showed two ABRE motives. If seeds stored pre-mRNAs, those could be alternatively spliced depending on the developmental status and environmental conditions, when the seed encounters conditions allowing rehydration and germination. This would be a flexible and fast way of reacting to changes in the environment.

Several cases in which ABA plays a role in the regulation of alternative splicing have been published. For example, PIMT2, an L-isoaspartyl methyltransferase involved in repairing proteins damaged in stress conditions has two splicing variants whose expression is regulated by ABA (Xu *et al.*, 2004). L-Isoaspartyl methyltransferases also play important roles in maintaining seed vigour and longevity during seed aging (Oge *et al.*, 2008). Thus, in accordance with our hypothesis, alternative splicing is used by the plants to react quickly to changes in their environment, especially to stress in which ABA signalling is known to play a major role. This fits with the fact that ABREs are over-represented in promoters of genes whose RNA is stored in seeds.

We show that DPI delays germination of after-ripened Arabidopsis and cress seeds and endosperm weakening of cress. A possible interpretation of these effects is that superoxide, together with its dismutation product hydrogen peroxide, can react at apoplastic peroxidases (Chen & Schopfer, 1999) and/or transition metal ions in the cell wall (Fry *et al.*, 2002) to form hydroxyl radicals in the Fenton

reaction. These highly reactive radicals can in turn cleave cell wall polymers, leading to cell wall loosening, which is a prerequisite of endosperm weakening as well as radicle growth (Müller *et al.*, 2009). As AtrbohB is expressed in the embryo and not in the endosperm, its superoxide production could act on radicle elongation, implying that a different *Rboh* is responsible for effects in the endosperm. The possibility has to be kept in mind that the delaying effect of DPI on germination has to be interpreted separately from the role of *Rbohs* in after-ripening, as DPI leads to a decrease in ROS production during the actual germination process as opposed to a decrease during after-ripening in the low-hydrated state caused by a mutated rboh.

The embryo of an imbibed seed reacts to ABA with a decrease in growth potential (Schopfer & Plachy, 1985). The endosperm, on the other hand, reacts to ABA with a delay in tissue weakening as was shown in cress (Müller *et al.*, 2006) and tomato (Toorop *et al.*, 2000). We could detect *AtrbohB* RNA only in the embryo, but not the endosperm both with the GUS-reporter in the enhancer trap lines and by *in-situ* RNA hybridization in the WT. One other Atrboh, namely *AtrbohD*, has been investigated, if briefly, in connection with seed germination. After-ripened seeds of the double mutant *atrbohD/F* have a lowered sensitivity to ABA in the germination medium (Kwak *et al.*, 2003). Penfield *et al.* (2006) pinpointed this phenotype to being caused by AtrbohD only. Their transcriptome data showed that the *AtrbohD* transcript is much more abundant in the endosperm than in the embryo of seeds treated with ABA and in seeds just after radicle protrusion. Based on these data, they suggested a role for AtrbohD in ABA signalling in the Arabidopsis endosperm. Under our conditions, *atrbohD* seeds germinated at the same speed as the WT in the fresh state and more slowly when they were after-ripened, thus also displaying an after-ripening phenotype. It would be an interesting option if different Rbohs mediated ABA signalling in different seed tissues and under different environmental conditions.

Acknowledgements

We are indebted to Jonathan Jones for his cooperation on behalf of the *atrboh*-mutants. We would like to thank Lydia Buller for expert technical assistance and the German Research Foundation (DFG) and the Wissenschaftliche Gesellschaft Freiburg for funding (DFG LE720/6). M.A.T. received the BIO 2007-66806 Grant from the Plant Nacional de I + D (Spain) and the IRG RTD REG/T.2 (2007)D/562971 from the European Community. We are grateful to Kai Gräber and Peter Schopfer for comments and discussions. Seeds of the tobacco mutant *ntrbohD* were kindly provided by Francoise Simon-Plas (INRA, Dijon, France).

References

Adkins SW, Ross JD. 1981. Studies in wild oat seed dormancy: I. The role of ethylene in dormancy breakage and germination of wild oat seeds (*Avena fatua* L.). *Plant Physiology* **67**: 358–362.

Bailly C. 2004. Active oxygen species in seed germination. *Seed Science Research* **14**: 93–107.

Baskin CC, Baskin JM. 1998. *Seeds – ecology, biogeography, and evolution of dormancy and germination*. San Diego, CA, USA: Academic Press.

Bentsink L, Jowett J, Hanhart CJ, Koornneef M. 2006. Cloning of *DOG1*, a quantitative trait locus controlling seed dormancy in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **103**: 17042–17047.

Bewley JD. 1997. Seed germination and dormancy. *Plant Cell* **9**: 1055–1066.

Bove J, Lucas P, Godin B, Ogé L, Jullien M, Grappin P. 2005. Gene expression analsis by cDNA-AFLP highlights a set of new signaling networks and translational control during the dormancy breakage in *Nicotiana plumbaginifolia*. *Plant Molecular Biology* **57**: 593–612.

Cadman C, Toorop P, Hilhorst H, Finch-Savage W. 2006. Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. *Plant Journal* **46**: 805–822.

Campisi L, Yang Y, Yi Y, Heilig E, Herman B, Cassista AJ, Allen DW, Xiang H, Jack T. 1999. Generation of enhancer trap lines in *Arabidopsis thaliana* and characterization of gene expression patterns in the inflorescence. *Plant Journal* **17**: 699–707.

Carrera E, Holman T, Medhurst A, Dietrich D, Footitt S, Theodoulou FL, Holdsworth MJ. 2008. Seed after-ripening is a discrete developmental pathway associated with specific gene networks in Arabidopsis. *Plant Journal* **53**: 214–224.

Chen S, Schopfer P. 1999. Hydroxyl-radical production in physiological reactions. A novel function of peroxidase. *European Journal of Biochemistry* **260**: 726–735.

Desikan R, Last K, Harrett-Williams R, Tagliavia C, Harter K, Hooley R, Hancock JT, Neill S. 2006. Ethylene-induced stomatal closure in Arabidopsis occurs via AtrbohF-mediated hydrogen peroxide synthesis. *Plant Journal* **47**: 907–916.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**: 1792–1797.

Finch-Savage WE, Leubner-Metzger G. 2006. Seed dormancy and the control of germination. *New Phytologist* **171**: 501–523.

Foreman J, Demidchick V, Bothwell JHF, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JDG *et al.* 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* **422**: 442–446.

Fry SC, Miller JG, Dumville JC. 2002. A proposed role for copper ions in cell wall loosening. *Plant and Soil* **247**: 57–67.

Geiszt M, Lekstrom K, Leto TL. 2004. Analysis of mRNA transcripts from the NAD(P)H oxidase 1 (Nox1) gene. *Journal of Biological Chemistry* **279**: 51661–51668.

Groot SPC, Karssen CM. 1992. Dormancy and germination of abscisic acid-deficient tomato seeds: studies with the sitiens mutant. *Plant Physiology* **99**: 952–958.

Gubler F, Hughes T, Waterhouse P, Jacobsen J. 2008. Regulation of dormancy in barley by blue light and after-ripening: effects on abscisic acid and gibberellin metabolism. *Plant Physiology* **147**: 886–896.

Hancock JT. 2001. Role of reactive oxygen species in cell signalling pathways. *Biochemical Society Transactions* **29**: 345–350.

Harder A, Wildgruber R, Nawrocki A, Fey SJ, Larsen PM, Görg A. 1999. Comparison of yeast cell protein solubilization procedures for two-dimensional electrophoresis. *Electrophoresis* **20**: 826–829.

Higo K, Ugawa Y, Iwamoto M, Korenaga T. 1999. Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Research* **27**: 297–300.

Holdsworth M, Finch-Savage W, Grappin P, Job D. 2007. Post-genomics dissection of seed dormancy and germination. *Trends in Plant Science* **13**: 7–13.

Holdsworth M, Bentsink L, Soppe W. 2008. Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. *New Phytologist* **179**: 33–54.

Job C, Rajjou L, Lovigny Y, Belghazi M, Job D. 2005. Pattern of protein oxidation in Arabidopsis seeds and during germination. *Plant Physiology* **138**: 790–802.

Kan Z, States D, Gish W. 2002. Selecting for functional alternative splices in ESTs. *Genome Research* **12**: 1837–1845.

Keller T, Damude H, Werner D, Doerner P, Dixon R, Lamb C. 1998. A plant homolog of the neutrophil NADPH oxidase gp91phox subunit gene encodes a plasma membrane protein with Ca^{2+} binding motifs. *Plant Cell* **10**: 255–266.

Kertész S, Kerényi Z, Mérai Z, Bartos I, Pálfy T, Barta E, Silhavy D. 2006. Both introns and 3′-UTRs operate as *cis*-acting elements to trigger nonsense-mediated mRNA decay in plants. *Nucleic Acids Research* **34**: 6147–6157.

Kobayashi M, Kawakita K, Maeshima M, Doke N, Yoshioka H. 2006. Subcellular localisation of Strboh proteins and NADPH-dependent O_2-generating activity in potato tuber tissue. *Journal of Experimental Botany* **57**: 1373–1379.

Kucera B, Cohn MA, Leubner-Metzger G. 2005. Plant hormone interactions during seed dormancy release and germination. *Seed Science Research* **15**: 281–307.

Kwak J, Mori C, Pei Z, Leonhardt N, Torres M, Dangl JL, Bloom R, Bodde S, Jones D, Schroeder JI. 2003. NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO Journal* **22**: 2623–2633.

Laloi C, Apel K, Danon A. 2004. Reactive oxygen signalling: the latest news. *Current Opinion in Plant Biology* **7**: 323–328.

Leubner-Metzger G. 2003. Functions and regulation of ß-1,3-glucanase during seed germination, dormancy release and after-ripening. *Seed Science Research* **13**: 17–34.

Leubner-Metzger G. 2005. ß-1,3-Glucanase gene expression in low-hydrated seed tissues as a novel mechanism for after-ripening. *Plant Journal* **41**: 133–145.

Leymarie J, Bruneaux E, Gibot-Leclerc S, Corbineau F. 2007. Identification of transcripts potentially involved in barley seed germination and dormancy using cDNA-AFLP. *Journal of Experimental Botany* **58**: 425–437.

Lin F, Zhang Y, Jiang M-Y. 2009. Alternative splicing and differential expression of two transcripts of nicotine adenine dinucleotide phosphate oxidase B gene from *Zea mays*. *Journal of Integrative Plant Biology* **51**: 287–298.

Liu Y-G, Huang N. 1995. Efficient amplification of insert end sequences from bacterial artificial chromosome clones by thermal asymmetric interlaced PCR. *Plant Molecular Biology Reporter* **16**: 175–181.

Liu P-P, Koizuka N, Homrichhausen T, Hewitt J, Martin R, Nonogaki H. 2005a. Large-scale screening of Arabidopsis enhancer-traps lines for seed germination-associated genes. *Plant Journal* **41**: 936–944.

Liu P-P, Koizuka N, Martin RC, Nonogaki H. 2005b. The BME3 (Blue Micropylar End 3) GATA zinc finger transcription factor is a positive regulator of Arabidopsis seed germination. *Plant Journal* **44**: 960–971.

Mayer KFX, Schoof H, Haecker A, Lenhard M, Jürgens G, Laux T. 1998. Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. *Cell* **95**: 805–815.

Möller IM, Jensen PE, Hansson A. 2007. Oxidative modifications to cellular components in plants. *Annual Review of Plant Biology* **58**: 459–481.

Müller K, Tintelnot S, Leubner-Metzger G. 2006. Endosperm-limited Brassicaceae seed germination: abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant and Cell Physiology* **47**: 864–877.

Müller K, Linkies A, Vreeburg RAM, Fry SC, Liszkay A, Leubner-Metzger G. 2009. *In vivo* **cell wall** loosening by hydroxyl radicals during cress (Lepidium sativum *L.*) seed germination and elongation growth. *Plant Physiology* **150**: 1855–1865.

Nagasaki H, Arita M, Nishizawa T, Suwa M, Gotoh O. 2005. Species-specific variation of alternative splicing and transcriptional initiation in six eukaryotes. *Gene* **364**: 53–62.

Nakabayashi K, Okamoto M, Koshiba T, Kmiya Y, Nambara E. 2005. Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: epigenetic and genetic regulation of transcription in seed. *Plant Journal* **41**: 697–709.

Ner-Gaon H, Halachmi R, Savaldi-Goldstein S, Rubin E, Ophir R, Fluhr R. 2004. Intron retention is a major phenomenon in alternative splicing of Arabidopsis. *Plant Journal* **39**: 877–885.

Oge L, Bourdais G, Bove J, Collet B, Godin B, Granier F, Boutin J-P, Job D, Jullien M, Grappin P. 2008. Protein repair L-isoaspartyl methyltransferase1 is involved in both seed longevity and germination vigor in Arabidopsis. *Plant Cell* **20**: 3022–3037.

Oracz K, Bouteau HE, Farrant JM, Cooper K, Belghazi M, Job C, Job D, Corbineau F, Bailly C. 2007. ROS production and protein oxidation as novel mechanisms for seed dormancy alleviation. *Plant Journal* **50**: 452–465.

Penfield S, Josse E, Kannangara R, Gilday A, Halliday K, Graham I. 2005. Cold and light control seed germination through the bHLH transcription factor SPATULA. *Current Biology* **15**: 1998–2006.

Penfield S, Li Y, Gilday A, Graham S, Graham I. 2006. Arabidopsis ABA INSENSITIVE4 regulates lipid mobilization in the embryo and reveals repression of seed germination in the endosperm. *Plant Cell* **18**: 1887–1899.

Potocký M, Jones AM, Bezvoda R, Smirnoff N, Zárský V. 2007. Reactive oxygen species produced by NADPH oxidase are involved in pollen tube growth. *New Phytologist* **174**: 742–751.

Sagi M, Fluhr R. 2006. Production of reactive oxygen species by plant NADPH oxidases. *Plant Physiology* **141**: 336–340.

Sagi M, Davydov O, Orazova S, Yesbergenova Z, Ophir R, Stratmann JW, Fluhr R. 2004. Plant respiratory burst oxidase homologs impinge on wound responsiveness and development in *Lycopersicon esculentum*. *Plant Cell* **16**: 616–628.

Schopfer P, Plachy C. 1985. Control of seed germination by abisic acid. III. Effect on embryo growth potential (minimum turgor pressure) and growth coefficient (cell wall extensibility) in *Brassica napus* L. *Plant Physiology* **77**: 676–686.

Schwab M. 2008. *Identification of novel seed dormancy mutants in Arabidopsis thaliana and molecular and biochemical characterization of the seed dormancy gene DOG1*. PhD thesis, University of Cologne, Cologne, Germany.

Simon-Plas F, Elmayan T, Blein JP. 2002. The plasma membrane oxidase *NtrbohD* is responsible for AOS production in elicited tobacco cells. *Plant Journal* **31**: 137–147.

Tissier AF, Marillonnet S, Klimyuk V, Patel K, Torres MA, Murphy G, Jones JDG. 1999. Multiple independent defective suppressor-mutator transposon insertions in Arabidopsis: a tool for functional genomics. *Plant Cell* **11**: 1841–1852.

Toorop PE, van Aelst AC, Hilhorst HWM. 2000. The second step of the biphasic endosperm cap weakening that mediates tomato (*Lycopersicon esculentum*) seed germination is under control of ABA. *Journal of Experimental Botany* **51**: 1371–1379.

Torres MA, Dangl JL. 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Current Opinion in Plant Biology* **8**: 397–403.

Torres MA, Dangl JL, Jones JDG. 2002. Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive

oxygene intermediates in the plant defense response. *Proceedings of the National Academy of Sciences, USA* **99**: 517–522.

Xu Q, Belcastro MP, Villa ST, Dinkins RD, Clarke SG, Downie AB. 2004. A second protein L-isoaspartyl methyltransferase gene in Arabidopsis produces two transcripts whose products are sequestered in the nucleus. *Plant Physiology* **136**: 2652–2664.

Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, Jones J, Doke N. 2003. *Nicotiana benthamiana* gp91 phox homologs NbrbohA and NbrbohB participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. *Plant Cell* **15**: 706–718.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Alignment of AtrbohB genomic DNA, AtrbohB-α and AtrbohB-ß.

Fig. S2 Alignment of AtrbohB genomic DNA and the two cDNAs we found on NCBI: NM_202070.1 and NM_100780.

Table S1 Localization of GUS staining and germination phenotypes of selected Arabidopsis enhancer trap lines

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.