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DARWIN REVIEW

First off the mark: early seed germination

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Abstract

Most plant seeds are dispersed in a dry, mature state. If these seeds are non-dormant and the environmental conditions are favourable, they will pass through the complex process of germination. In this review, recent progress made with state-of-the-art techniques including genome-wide gene expression analyses that provided deeper insight into the early phase of seed germination, which includes imbibition and the subsequent plateau phase of water uptake in which metabolism is reactivated, is summarized. The physiological state of a seed is determined, at least in part, by the stored mRNAs that are translated upon imbibition. Very early upon imbibition massive transcriptome changes occur, which are regulated by ambient temperature, light conditions, and plant hormones. The hormones abscisic acid and gibberellins play a major role in regulating early seed germination. The early germination phase of *Arabidopsis thaliana* culminates in testa rupture, which is followed by the late germination phase and endosperm rupture. An integrated view on the early phase of seed germination is provided and it is shown that it is characterized by dynamic biomechanical changes together with very early alterations in transcript, protein, and hormone levels that set the stage for the later events. Early seed germination thereby contributes to seed and seedling performance important for plant establishment in the natural and agricultural ecosystem.

Key words: Abscisic acid, cold stratification, energy metabolism, gibberellins, imbibition, novel techniques, testa rupture, transcriptome.

Introduction

> '… I have had one experiment some little time in progress which will, I think, be interesting, namely, seeds in salt water, immersed in water of 32°–33° […] I have in small bottles out of doors, exposed to variation of temperature, cress, radish, cabbages, lettuces, carrots, and celery, and onion seed—four great families. These, after immersion for exactly one week, have all germinated, which I did not in the least expect (and thought how you would sneer at me); for the water of nearly all, and of the cress especially, smelt very badly, and the cress seed emitted a wonderful quantity of mucus (the 'Vestiges' would have expected them to turn into tadpoles), so as to adhere in a mass; but these seeds germinated and grew splendidly. The germination of all (especially cress and lettuces) has been accelerated, except the cabbages, which have come up very irregularly, and a good many, I think, dead. One would have thought, from their native habitat, that the cabbage would have stood well. The Umbelliferae and onions seem to stand the salt well.' (April 13th, 1855, cited from: Darwin, 1887).

Charles Darwin's interest in seed germination was a focus within his wider interest in plant development. He published several papers about the above findings in the *Gardeners' Chronicle* and *Agricultural Gazette* (including Darwin, 1855*a*, *b*, *c*, *d*). His interest in seed germination was indeed well founded: seed germination is a crucial process in the seed plant life cycle. It determines when plants enter natural or agricultural ecosystems and is the basis for crop production. This review deals with the early events during this important life cycle transition. Early seed germination is defined here as imbibition plus the early plateau phase of water uptake. It is thus positioned between the dry state of the seed and the late phase of germination. Germination is

completed by visible radicle protrusion through the seed covering layers, and followed by seedling establishment (Fig. 1). Late germination has been the focus of seed research for many decades (summarized in recent reviews, e.g. Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.*, 2008; Nonogaki *et al.*, 2007; North *et al.*, 2010). It is believed that unravelling the mechanisms underlying germination requires the integration of all of its facets including early events.

Most mature angiosperm seeds consist of an embryo surrounded by covering layers such as the maternal testa (seed coat) and the triploid endosperm. Seeds exhibit species-specific differences in their structure and the composition of their storage compounds (Obroucheva and Antipova, 1997; Linkies *et al.*, 2010). Interestingly, Charles Darwin already worked with some species that would later become model species in seed biology, namely lettuce, on which the red/far red light-induced reversibility of phytochrome effects was discovered (Borthwick *et al.*, 1952), and cress, radish, and cabbages, which are members of the Brassicaceae family for which the first plant genome was sequenced (*Arabidopsis thaliana*; Koornneef and Meinke, 2010). This review will focus on a range of orthodox eudicot model systems of seed germination. Pea (*Pisum sativum*, Fabaceae, Fig. 2A) seeds store mainly proteins and starch in the embryo's storage cotyledons; mature pea seeds have no endosperm (Obroucheva and Antipova, 1997; Melkus *et al.*, 2009). The Brassicaceae oil-seeds of *Arabidopsis* (Fig. 1A) and garden cress (*Lepidium sativum*, 'cress') contain a thin endosperm layer (Haughn and Chaudhury, 2005; Müller *et al.*, 2006), while the oil-seeds of tobacco (*Nicotiana tabacum*, Solanaceae) contain a thicker endosperm layer (Leubner-Metzger, 2003; Manz *et al.*, 2005). This review will follow the physiological timeline of events during early seed germination, from the dry seed to fully reactivated metabolism. This is supported by corresponding figures and in addition by Supplementary material available at *JXB* online.

The dry seed stage: moisture content, after-ripening, and the stored transcriptome

Seed maturation and desiccation were recently reviewed (Holdsworth *et al.*, 2008; Angelovici *et al.*, 2010). This discussion will start at the end-point of these reviews: with the biochemical properties of the desiccated mature orthodox seed, which constitutes a desiccation-tolerant state of

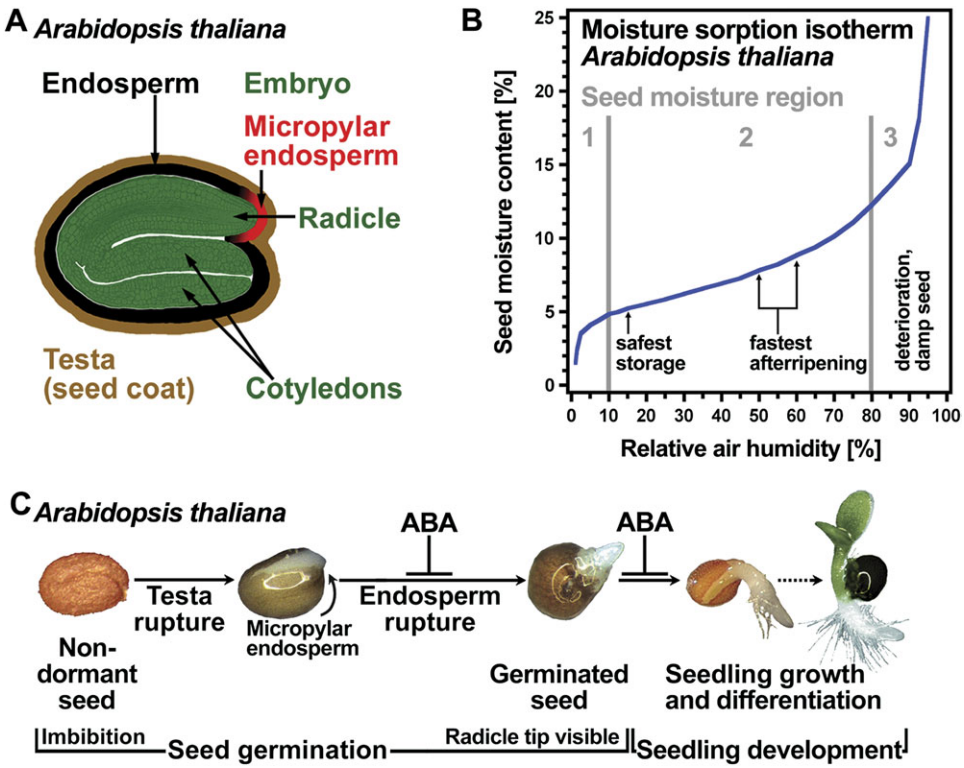

Fig. 1. Comparison of morphological and physiological key processes during the germination of typical endospermic (e.g. *Arabidopsis thaliana*, *Lepidium sativum*, and tobacco) eudicot seeds. (A) Morphology of a mature seed of *A. thaliana* with a single layer of endosperm between the testa (seed coat) and the embryo. (B) Typical moisture sorption isotherm of an oil-seed at room temperature. Region 1 represents strongly bound water (monolayer) which is unavailable for water-dependent biochemical reactions. Region 2 represents weakly bound, multilayered water, which leads to a limited availability for water-dependent biochemical reactions. Only water represented in region 3 is freely available and may allow molecular biochemical events that occur during seed imbibition. (C) Visible events during two-step germination: testa and endosperm rupture. Abscisic acid (ABA) inhibits endosperm rupture, but not testa rupture, of after-ripened seeds. The seed image is from Müller *et al.* (2006) with permission of the publisher. The moisture sorption isotherm diagram is based on quantitative data of Hay *et al.* (2003) and Manz *et al.* (2005).

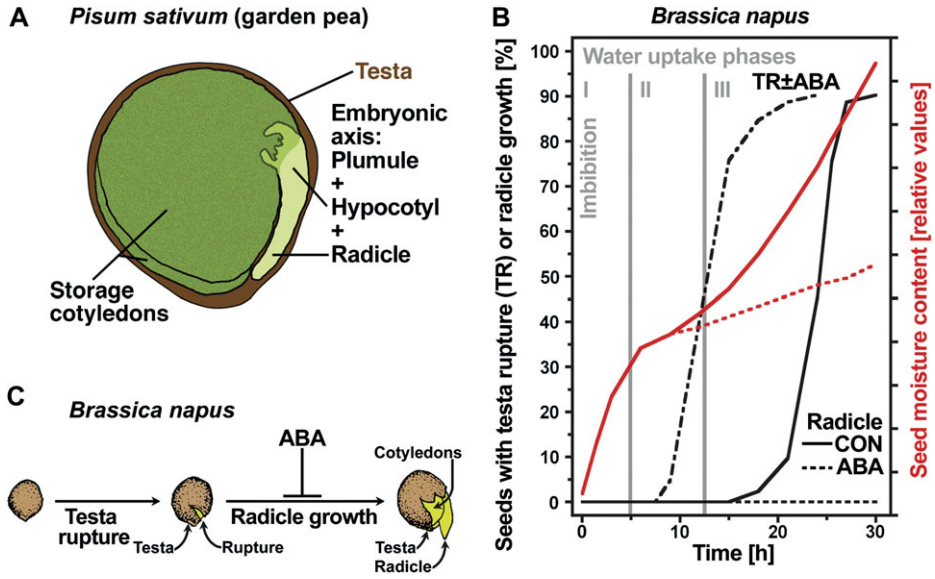

Fig. 2. Comparison of morphological and physiological key processes during the germination of typical endospermless (e.g. *Brassica napus*, pea, and many other legumes) eudicot seeds. (A) Morphology of a mature pea seed which is endospermless. (B) Time courses of *B. napus* seed water uptake, testa rupture, radicle growth >2 mm, and the effect of abscisic acid (ABA); control without added hormone (CON). (C) Visible events during one-step germination typical for endospermless species. Seed images (A, C) are from Finch-Savage and Leubner-Metzger (2006) with permission of the publishe. Diagram (B) is based on quantitative data by Schopfer and Plachy (1984).

the sporophyte with typical average water contents of ~10%. This 'dry' seed state is therefore in fact a 'low-hydrated' state, and dry seeds are not completely metabolically inert. The physiological state of dry seeds changes during after-ripening (i.e. a prolonged period of dry storage at room temperature of freshly harvested, mature seeds). After-ripening storage is associated with a loss of dormancy, although dormancy release and after-ripening may be separate pathways (Carrera *et al.*, 2008; Holdsworth *et al.*, 2008). After-ripening depends on temperature and seed moisture content. The optimal moisture content for after-ripening is lower for oil-storing compared with starchy seeds, but in general after-ripening takes place at seed moisture contents between 8% and 15% (Probert, 2000; Bazin *et al.*, 2011). Moisture sorption isotherms of seeds (Fig. 1B) show that water molecules are weakly bound at this water content, which means its availability for biochemical reactions is limited. The shape of the moisture sorption isotherm curves is similar for oil-seeds (e.g. tobacco and *Arabidopsis*; Hay *et al.*, 2003; Manz *et al.*, 2005) and starchy seeds (e.g. pea; Chen, 2003); even though absolute values differ slightly. However, water distribution is inhomogeneous within seeds, and seed tissues differ in their moisture sorption isotherms (see references in Hay *et al.*, 2003; Manz *et al.*, 2005; Wojtyla *et al.*, 2006). ^{1}H-nuclear magnetic resonance (NMR) spectroscopic imaging of tobacco seeds suggests that there are local pockets of higher hydration, in which water may be freely available for biochemical reactions. This may lead to differing biochemical capabilities between seed organs and tissues. Although evidence is still fragmentary, there are several reports that indicate that low-level transcription, post-transcriptional processing, and translation may be possible during seed after-ripening of tobacco (Leubner-Metzger, 2002, 2005), *Arabidopsis* (Müller *et al.*, 2009), barley (Leymarie *et al.*, 2007), and other species (Holdsworth *et al.*, 2008). Holdsworth *et al.* (2008) also provide a critical review of this issue which is recommended for further reading. Future experiments to elucidate these highly debated findings are required and could include the use of novel imaging techniques as well as tissue-specific transcriptome and proteome analyses during after-ripening storage.

There are indications that after-ripening includes protein oxidation by reactive oxygen species (ROS). ROS are formed in the dry state as can be shown by the redox state of dry seeds shifting towards a more oxidized cellular environment (Kranner *et al.*, 2006, 2010*b*). Antioxidant enzyme activity will be limited or impossible in most parts of the seeds due to the lack of available water. The seeds therefore rely on small antioxidant molecules for their protection from oxidative damage, with the glutathione (GSH) system probably playing a major role. ROS oxidize GSH to its dimer GSSG, which accumulates during seed storage (Kranner and Grill, 1993). In addition, the lipophilic antioxidant tocopherol, which protects membranes from lipid peroxidation, is essential for seed longevity and germination characteristics, as was shown in a mutant approach in *Arabidopsis* (Sattler *et al.*, 2004; Mene-Saffrane *et al.*, 2010). A third major antioxidant, ascorbate, is only present in small amounts in dry seeds and therefore probably plays a minor role in regulating the redox situation in the dry state (Wojtyla *et al.*, 2006; Dowdle *et al.*, 2007).

Oracz *et al.* (2007, 2009) proposed a causal role for ROS in sunflower embryo dormancy release, and Müller *et al.* (2009) showed that the *Arabidopsis* mutant *atrbohB* which

does not after-ripen has an altered pattern of protein oxidation in the dry seeds. In this context, the concept of an oxidative window has been suggested which assumes that oxidative processes in seeds first lead to after-ripening and loss of dormancy, but later tip the scale towards oxidative damage, deterioration, and loss of viability (Bailly, 2004).

The processes that take place in dry seeds and lead to after-ripening or deterioration are an important aspect of seed biology and the topic of active research. As even small changes in overall seed moisture content influence storability and longevity of seeds (Buitink *et al.*, 2000; Finch-Savage and Leubner-Metzger, 2006), understanding these processes is an issue of economic importance and a major concern of seed banks (Nagel and Börner, 2010). New non-invasive imaging approaches (see last section) will be very helpful in elucidating this stage of the plant life cycle.

Dry seeds contain mRNAs stored during maturation, also called long-lived transcripts to indicate that they survived desiccation (Rajjou *et al.*, 2004). Over 10 000 different stored mRNAs have been identified in transcriptome analyses of *Arabidopsis* (Nakabayashi *et al.*, 2005; Kimura and Nambara, 2010; Okamoto *et al.*, 2010). Similar numbers were found in barley and rice (Howell *et al.*, 2009; Sreenivasulu *et al.*, 2010). In *Arabidopsis*, abscisic acid (ABA)-responsive elements are over-represented in the promoters of genes whose transcripts are stored (Nakabayashi *et al.*, 2005), in accordance with the major role of ABA during seed maturation (Nambara *et al.*, 2010; Radchuk *et al.*, 2010). So far the published transcriptomes are from whole dry seeds, but it is known that the different seed compartments, for example the endosperm and embryo, accumulate different transcripts during seed development (Le *et al.*, 2010). Kimura and Nambara (2010) showed that major portions of the dry seed transcriptomes of the non-dormant *Arabidopsis* ecotype Columbia (Col) and the dormant ecotype Cape Verde Island (Cvi) are very similar. The majority of stored mRNAs are of the LEA ('late embryogenesis abundant') group or transcripts of storage proteins, supporting the view that the dry seed transcriptome mirrors the process of seed maturation as well as prepares the seed for the following germination. The transcriptomes of the two ecotypes differ in an over-representation of heat shock proteins and ROS-related transcripts in Col and of phosphate and lipid metabolism as well as cytoskeleton-associated transcripts in Cvi. Larger transcriptome differences between these dormant and non-dormant seeds only develop after imbibition. A comparison between the dry seed transcriptomes of near isogenic lines (NILs) representing 'Delay of Germination' (DOG) quantitative trait loci (QTLs) of *Arabidopsis* that differ in after-ripening and/or dormancy suggests that natural variations for these traits are mainly controlled by additive genetic and molecular pathways, rather than epistatic interactions (Bentsink *et al.*, 2010). It will be interesting to see if, as is the case for the comparison of Col and Cvi (Kimura and Nambara, 2010), the differences between the transcriptomes of the NILs become larger during imbibition and if these distinct DOG pathways remain independent. This review will come back to the dry seed transcriptome and its changes upon imbibition when metabolism is explored in a later section.

Physical, morphological, and physiological aspects of imbibition and testa rupture

Seed germination begins when the dry seeds come into contact with water under favourable conditions. It comprises three phases of water uptake. Dry seeds have very low water potentials (Woodstock, 1988; Obroucheva and Antipova, 1997) which cause rapid water influx during phase I (imbibition, Figs 2B, 3). As this process is driven by the matrix potential, it also occurs in dead seeds (Krishnan *et al.*, 2004). During imbibition the seed rapidly swells and changes in size and shape (e.g. Robert *et al.*, 2008; Preston *et al.*, 2009). ^{1}H-NMR image analyses of imbibition with pea, tobacco, and other species demonstrate that there are major entry points for water uptake such as the micropyle, and that the progress of imbibition differs between seed tissues (e.g. Manz *et al.*, 2005; Wojtyla *et al.*, 2006). In *Arabidopsis* the shape of the imbibed wild-type seed approximates a prolate spheroid during this period. Robert *et al.* (2008) showed that ethylene mutants differ in seed shape and imbibition behaviour from wild-type seeds. These differences in changes in size and shape of imbibing seeds could in the future be used for large-scale mutant screens, as computational approaches facilitate the high-throughput analysis of image time series (e.g. Joosen *et al.*, 2010).

The *Arabidopsis* testa contains volcano-shaped cell wall structures on the seed surface known as columellae (Fig. 4A). Upon first contact with water of *Arabidopsis* and other mucilaginous seeds, mucilage is released quickly from the columellae—the 'wonderful quantity of mucus' that Darwin observed on his cress seeds is a further example. *Arabidopsis* mucilage is composed mainly of rhamnogalacturonan pectins and cellulose arranged in an outer water-soluble layer and an inner layer covalently bound to the testa by cellulose microfibrils (Windsor *et al.*, 2000; Macquet *et al.*, 2007). Possible functions of the mucilage are the adherence to surfaces and animals for seed dispersal (Mummenhoff *et al.*, 2004) and aiding germination in osmotically and saline-stressful environments (Yang *et al.*, 2010) as the mucilage is very hydrophilic and delays water loss.

Initial imbibition is often accompanied by a massive leakage of cellular solutes. Similar phenomena can be observed in resurrection plants and pollen that rapidly return from a dry quiescent state to a fully hydrated state (Hoekstra *et al.*, 1999). While leakage can accelerate germination by lowering inhibitor concentrations within seeds (Matilla *et al.*, 2005), it is also a sign of damage to membranes and cellular compartments caused by fast and/or inhomogeneous rehydration (Powell and Matthews, 1978). In order to deal with the damage imposed during dehydration, storage, and, most significantly, rehydration, seeds activate a number of repair mechanisms during imbibition (Fig. 3). This includes the repair of membranes

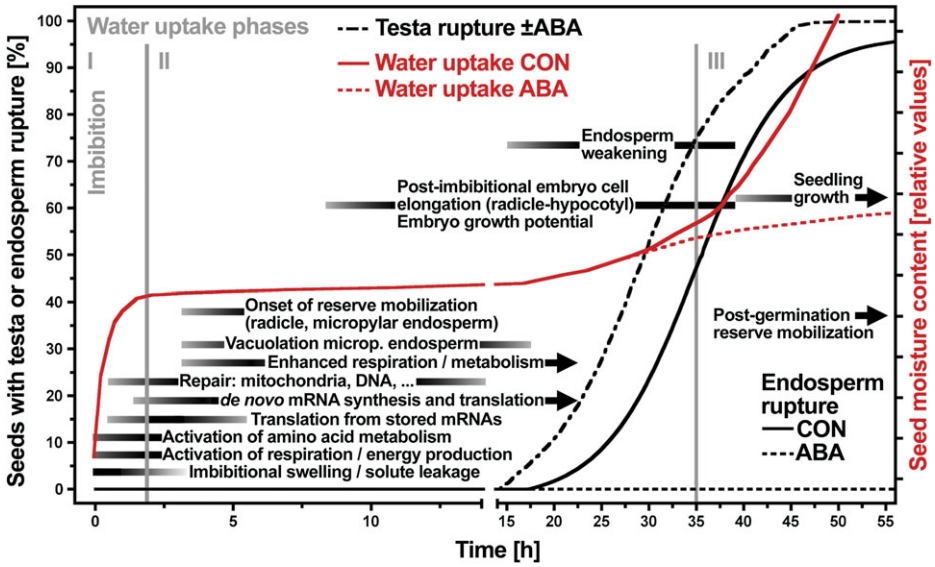

Fig. 3. Key processes during the germination of typical endospermic eudicot seeds with separate testa and endosperm rupture (two-step germination). Time courses of *Arabidopsis thaliana* seed water uptake, testa and endosperm rupture, and the effect of abscisic acid (ABA) on these processes; control without added hormone (CON). Important biophysical, biochemical, and cellular events during seed germination are triggered, at least in part, by water uptake and are depicted in the diagram. The diagram is based on quantitative data by Preston *et al.* (2009), Vander Willigen *et al.* (2006), and Manz *et al.* (2005). Events were added based on Bewley, (1997), Nonogaki *et al.* (2007), and Obroucheva and Antipova (1997).

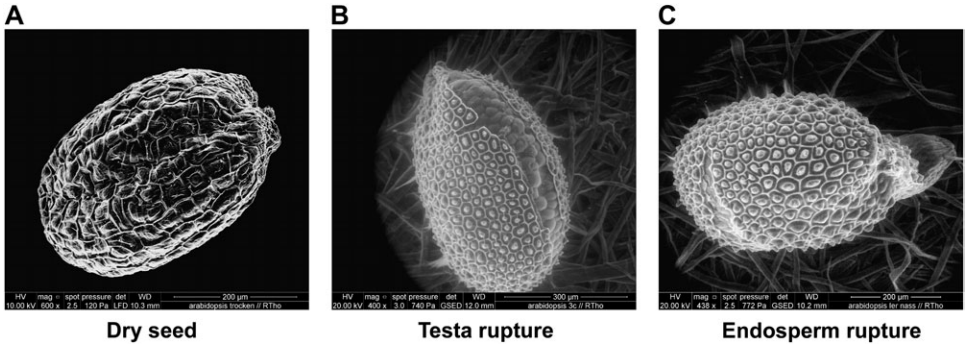

Fig. 4. Scanning electron microscopy (SEM) and environmental scanning electron microscopy (eSEM) images of *Arabidopsis thaliana* seed germination. (A) Air-dry seed of *Arabidopsis* (SEM) showing the hexagonal testa cells on the surface with the mucilage packed into the middle elevation resulting in the columella. (B) Imbibed *Arabidopsis* seed (eSEM) in testa rupture state; the micropylar endosperm covering the radicle is visible. (C) *Arabidopsis* seed (eSEM) in endosperm rupture state; the emerged radicle is visible and designates the completion of germination. eSEM works without freezing, coating, fixing, or embedding and in a relative humidity of ⩾90 %. It can thus be used to image a living organism with high magnification (Windsor *et al.*, 2000 Muscariello *et al.*, 2005). Images taken by Dr Ralf Thomann, Freiburg Materials Research Center (FMF).

as well as of proteins in which aspartyl residues were damaged by conversion to isoaspartyl. The latter can be reversed by isoaspartyl methyltransferase (Oge *et al.*, 2008).

Damage to genomic DNA includes progressive loss of telomeric sequences during prolonged dry storage (Boubriak *et al.*, 2007) as well as strand breaks and other types of DNA damage that result from cumulative effects of temperature, moisture, oxygen, and ROS levels (reviewed by Bray and West, 2005). The accumulation of chromosomal damage and/or an inability to repair such damage during the imbibition period appear to be significant factors contributing to loss of seed viability during storage. Seed dehydration during maturation and rehydration during imbibition lead to the appearance of a large number of DNA single-strand breaks in maize seeds, most of which can be attributed to imbibitional damage. This includes the appearance and repair of apurinic/apyrimidinic sites in DNA during early germination. DNA damage, which would obviously be a major obstacle during germination, can be repaired by DNA ligases. DNA ligase expression is activated quickly upon imbibition of *Arabidopsis* seeds, and high levels of *de novo* DNA synthesis have been observed in the absence of nuclear DNA replication or cell division, indicating a role in DNA repair. In aged seeds, which have suffered more severe damage during storage, enhanced early DNA synthesis has been observed (Bray and West, 2005). Insertional knock-out

mutants of two DNA ligases, AtLIG4 and the plant-specific AtLIG6, consequently showed a delay in germination under optimal conditions which was aggravated under cold stress conditions and in the presence of ROS (Waterworth *et al.*, 2010). Bray and West (2005) state that the seed provides an ideal 'model' system for investigating the effects of a variety of endogenous DNA-damaging agents and environmental stresses on genome integrity.

The permeability of the testa, being the part of the seed that comes into contact with the ambient water in most seeds, plays a central role in the rate of water uptake (Chachalis and Smith, 2000; Wojtyla *et al.*, 2006; Koizumi *et al.*, 2008). *Brassica* seeds with different testa morphology show altered germination characteristics (Zeng *et al.*, 2004; Matilla *et al.*, 2005). *Arabidopsis* testa mutant seeds with reduced pigmentation are more permeable to tetrazolium salts than the wild type, and the seeds show a lower dormancy and differ in hormone sensitivities during germination (Debeaujon and Koornneef, 2000; North *et al.*, 2010).

Once the rate of water uptake and changes in seed size and shape start to stagnate, germinating seeds move into water uptake phase II, during which the water content remains stable and which can vary widely in duration. In species with a 'two-step germination' process such as *Arabidopsis*, cress, and tobacco (Liu *et al.*, 2005; Manz *et al.*, 2005; Müller *et al.*, 2006), phase II encompasses testa rupture (Figs 1C, 3, 4B). This is followed by phase III water uptake, endosperm rupture, and radicle protrusion; that is, the completion of germination *sensu stricto* (Fig. 4C). Phase III water uptake continues during the transition to seedling growth (Figs 1C, 3). Endospermless species such as pea and *Brassica napus* (*Brassica*) enter phase III after testa rupture ('one-step germination', Fig. 2). In non-dormant seeds, exogenous ABA inhibits the transition from water uptake phase II to III and late embryo cell expansion, but does not affect phase I and II and testa rupture (Figs 2B, 3) (e.g. Schopfer and Plachy, 1984; da Silva *et al.*, 2004; Manz *et al.*, 2005; Müller *et al.*, 2006). The presence of endosperm in mature seeds provides an additional target tissue for regulating the completion of germination by ABA and environmental factors. Its visibility as a two-step process appears to be a phylogenetically widespread trait determined by the anatomy of the seed-covering layers (Petruzzelli *et al.*, 2003; Linkies *et al.*, 2010).

Aquaporins, small membrane proteins that can transport water as well as non-polar small molecules, facilitate cell–cell water transport and may contribute to spatial distribution of water within seed tissues during imbibition as well as to the timing of testa rupture in tobacco (Schuurmans *et al.*, 2003; Maurel *et al.*, 2009). Vander Willingen *et al.* (2006) analysed the involvement of aquaporins in the germination of cold-stratified *Arabidopsis* seeds and found that tonoplast intrinsic proteins (TIPs) show a germination-related shift of protein accumulation from TIP3 to TIP1 at the later stages of germination and found a similar pattern for RNA transcription levels. In contrast to TIPs, they did not find strong indications for the involvement of plasma membrane intrinsic proteins (PIP) as neither protein nor transcript presence and changes were detected. A different conclusion for the PIP-type aquaporins comes from evidence obtained with knock-down tobacco mutants of the plasma membrane aquaporins PIP1 and PIP2, for which testa rupture was affected differentially (Ernst, 2007): testa rupture occurred earlier in *pip2* mutant seeds, while it was delayed in *pip1*. The time period between testa rupture and the completion of germination was not altered in the mutants compared with the wild type. Taken together, these contrasting results in tobacco and *Arabidopsis* clearly show that further research in this area is needed.

While *Arabidopsis* seed coat development including the genes and hormones involved in this process has been studied in detail (Haughn and Chaudhury, 2005), little is known about the changes of the seed coat's mechanical and biochemical properties that ultimately lead to testa rupture. In tobacco, testa rupture starts near the micropylar seed end that covers the radicle and spreads along the ridges on the testa (Leubner-Metzger, 2003). Progression of tobacco testa rupture is facilitated by channel-like structures underlying the ridges, suggesting pre-determined breaking points. *Arabidopsis* testa rupture (Fig. 4B) also starts at the micropylar seed end, but it is unknown if pre-determined breaking points exist. Future experiments concerning a potential enzymatic weakening, spatial water redistribution in connection with cell elongation, and transcriptomic activity before testa rupture are required as they might shed light on how testa rupture is controlled. Tissue-specific transgenic approaches might help to elucidate the role of the embryo, endosperm, and integument layers in testa rupture.

Embryo cells elongate prior to the completion of seed germination of *Arabidopsis*, *Brassica*, *Medicago*, and other species; cell division is not evident in the embryos of these seeds during germination (Barroco *et al.*, 2005; Gimeno-Gilles *et al.*, 2009; Sliwinska *et al.*, 2009). After the initial swelling is completed, all changes in seed size and shape during germination are caused by cell expansion. Expanding plant cells adjust the extensibility of their cell walls by remodelling the major components of the wall, the cellulose microfibrils and/or the pectin/hemicellulose matrix. Loosening of the wall allows water influx which drives cell expansion and generates cellular turgor pressure (Schopfer, 2006). This led to the model that embryo growth during germination depends primarily on changes in cell wall extensibility. These changes are accompanied by progressing vacuolation during late phase II of water uptake. Embryo and endosperm cells are not fully vacuolated during early phase II, but display many small vacuoles (Bethke *et al.*, 2007). Sliwinska *et al.* (2009) proposed that *Arabidopsis* embryo elongation occurs in a distinct and confined elongation zone between the radicle and the lower hypocotyl.

Hormonal and temperature regulation of early gene expression in imbibed seeds

Hormone contents, signalling, and interactions play important roles in determining the physiological state of the seed

and in regulating the germination process (Kucera *et al.*, 2005). The endogenous ABA contents of non-dormant and dormant seeds rapidly decline upon imbibition during the early phase of germination (within 6–12 h, Fig. 5; Chiwocha *et al.*, 2005; Nakabayashi *et al.*, 2005; Hermann *et al.*, 2007; Linkies *et al.*, 2009; Preston *et al.*, 2009). However, in dormant and thermoinhibited seeds (i.e. seeds in which high temperatures inhibit germination) this decrease stops, and *de novo* ABA synthesis in the imbibed state causes higher ABA contents which are required for dormancy

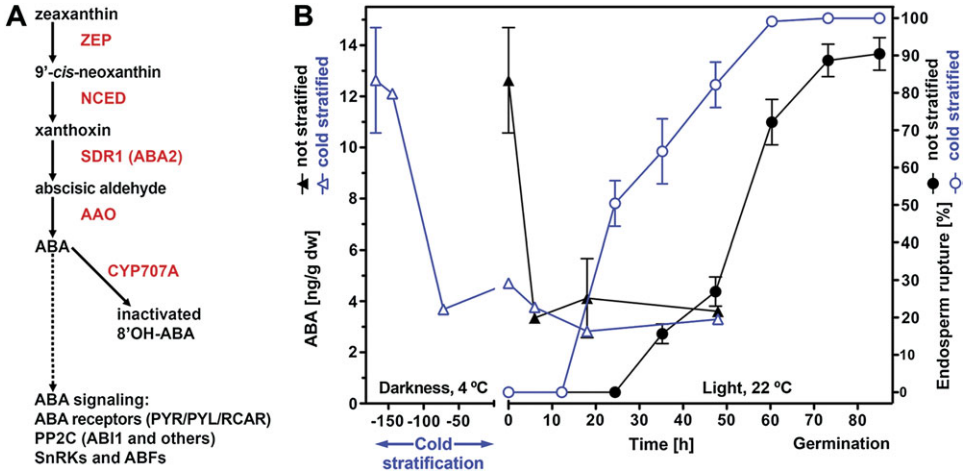

Fig. 5. Abscisic acid (ABA) contents in germinating *Arabidopsis thaliana* seeds and the effect of moist cold stratification. (A) Important steps of ABA biosyntheses, degradation, and signalling; see main text for details. (B) Endogenous contents of ABA in germinating seeds and the effect of moist cold stratification. After-ripened seeds were incubated at 4 °C in the dark which inhibits germination (cold stratification). Germination occurs during subsequent incubation at 22 °C in the light and is completed by endosperm rupture. Data on seed ABA contents used to draw the diagram are from Chiwocha *et al.* (2005).

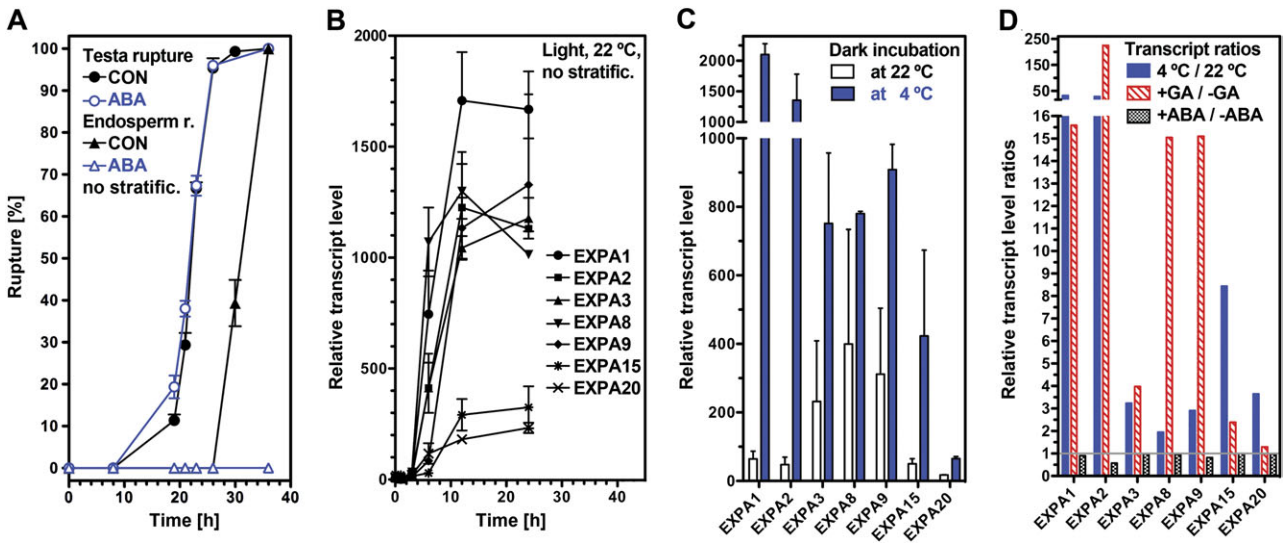

Fig. 6. Gibberellins (GAs), abscisic acid (ABA), and cold stratification as related to α-expansin (EXPA) transcript expression during early seed germination of *Arabidopsis thaliana*. (A) ABA inhibits endosperm rupture, but does not alter the kinetics of testa rupture of after-ripened seeds imbibed in the light without cold stratification. (B) α-Expansin transcript accumulation during early seed germination. (C) The effect of cold stratification on α-expansin transcript accumulation. After-ripened seeds were incubated in the dark which inhibits germination either at 22 °C or at 4 °C. Relative transcript levels were compared at 96 h. (D) The effect of cold stratification, GAs, and ABA on the transcript level ratios (fold induction). For cold stratification the ratios 4 °C/22 °C were calculated from the values in C. For hormones the ratios were calculated by comparison of hormone-treated seeds with the untreated seeds at 6 h and 24 h for GAs and ABA, respectively. Wild-type seeds were used, except for the GA response which was studied in GA-deficient *ga1-3* seeds. Results are from (A) Müller *et al.* (2006), (B–D) *Arabidopsis* transcriptome analysis available via the seed-specific eFP-browser at www.bar.utoronto.ca (Winter *et al.*, 2007; Bassel *et al.*, 2008) based on experiments for non-dormant, non-stratified after-ripened wild-type seeds (Nakabayashi *et al.*, 2005; Preston *et al.*, 2009), cold-stratified wild-type seeds (Yamauchi *et al.*, 2004), ABA-treated wild-type, and GA-treated *ga1-3* seeds (RIKEN transcriptome sets).

maintenance and for inhibiting germination (Nambara *et al.*, 2010). A sufficient decrease in endogenous ABA content during imbibition and early phase II is thus a major prerequisite for the completion of germination. Exogenous treatment of after-ripened *Arabidopsis* or cress seeds with ABA does not affect the kinetics of testa rupture, but inhibits endosperm weakening and rupture (Fig. 6A and Müller *et al.*, 2006). Nine-*cis*-epoxycarotenoid dioxygenaes (NCEDs) and ABA 8'-hydroxylases (CYP707As) are the major key regulatory enzymes for ABA biosynthesis and degradation, respectively (Fig. 5A). NCEDs and CYP707As are encoded by multigene families, and their tissue- and environment-specific regulation determines the ABA contents (Seo *et al.*, 2006; Toh *et al.*, 2008). *CYP707A2* transcripts are expressed in the radicle upon seed imbibition (Okamoto *et al.*, 2006). Changes in hormone contents during the early germination phase are also evident in *Arabidopsis* seeds for jasmonic acid, whose content decreases, and indole acetic acid, whose content increases (Preston *et al.*, 2009).

The germination-inhibiting effect of ABA is counteracted by gibberellins (GAs) and by ethylene. The effects of these hormones on the late germination process have been extensively reviewed (Kucera *et al.*, 2005; Holdsworth *et al.*, 2008; Linkies *et al.*, 2009; North *et al.*, 2010) and their interaction with each other and with light has been studied (e.g. Debeaujon and Koornneef, 2000; Seo *et al.*, 2008; Piskurewicz *et al.*, 2009; North *et al.*, 2010). Ethylene has important roles during the late phase of germination and counteracts the ABA inhibition by interfering with ABA signalling, but it does not affect ABA contents (Linkies *et al.*, 2009). In contrast, GAs are important during the early and the late phase of germination and counteract the ABA inhibition. Bioactive GA_4 was already present in physiologically relevant amounts in the dry, after-ripened seeds that Ogawa *et al.* (2003) used for their transcriptome analysis (Fig. 7B), and a further increase in GA_4 contents occurs during late germination. GA20 and GA3 oxidases (GA20ox and GA3ox) are the major key regulatory enzymes for GA biosynthesis, while GA2 oxidases mediate GA degradation (Fig. 7A). Transcripts of GA20ox and GA3ox accumulate during early germination. Ogawa *et al.* (2003) demonstrated that GA biosynthesis localizes to the radicle, hypocotyl, and micropylar endosperm during germination. Due to the rapid ABA degradation, the GA/ABA ratio increases ~3-fold during early germination and ~10-fold during late germination (compare Figs 5B and 7B). While for the early germination phase Ogawa *et al.* (2003) did not find altered ABA contents upon treatment of GA-deficient *ga1-3 Arabidopsis* seeds with exogenous GA, Yano *et al.* (2009) found that GA_4 contents and *GA3ox1* transcript levels were decreased in ABA-overproducing *cyp707a2 Arabidopsis* seeds. This is most probably due to the increased ABA contents and it therefore seems that ABA can inhibit GA biosynthesis during early germination.

GA20ox and GA3ox are induced by red light and cold stratification (Supplementary Fig. S1 at *JXB* online; Yamauchi *et al.*, 2004; Kucera *et al.*, 2005). Moist cold stratification of *Arabidopsis* (i.e. incubation of imbibed seeds at 4 °C in darkness usually for 1–4 d) is often used to break dormancy and promote subsequent germination in the light. Yamauchi *et al.* (2004) have demonstrated that cold stratification is associated with increased contents of bioactive GAs (Fig. 7C) and by the accumulation of GA20 and GA3 oxidase transcripts (Supplementary Fig. S1B). Furthermore, cold stratification induced a spatial change in *GA3ox1* transcript expression; in addition to the radicle it strongly accumulated in the micropylar endosperm (Yamauchi *et al.*, 2004). The seed-specific eFP-browser and the eNorthern tool at www.bar.utoronto.ca visualize transcript expression patterns based on global transcriptome analyses during *Arabidopsis* seed germination (Winter *et al.*, 2007; Bassel *et al.*, 2008). These tools were used for the purpose of this review to analyse early temporal transcript

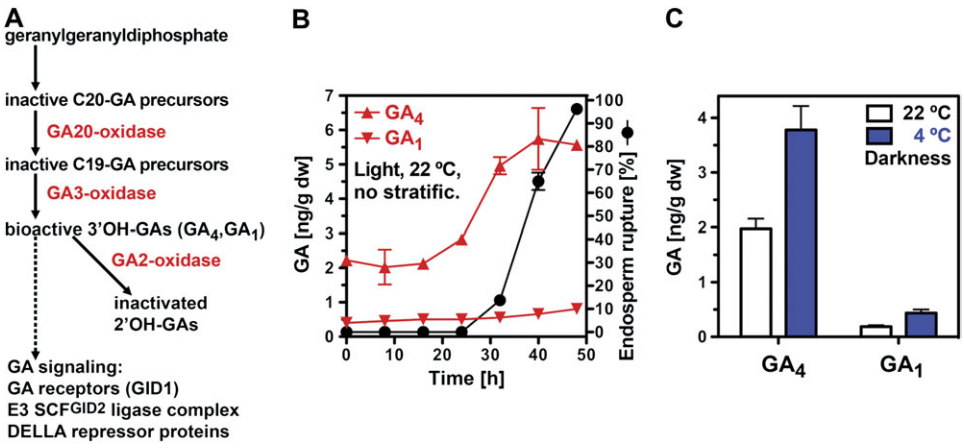

Fig. 7. Gibberellin (GA) contents in germinating *Arabidopsis thaliana* seeds and the effect of moist cold stratification. (A) Important steps of GA biosyntheses, degradation, and signalling. (B) Endogenous contents of bioactive GA_4 and GA_1 in non-stratified germinating seeds incubated in the light at 22 °C. (C) The effect of moist cold stratification on the endogenous contents of bioactive GA_4 and GA_1. GA values of seeds imbibed in darkness (which inhibits germination) for 96 h are compared for 4 °C and 22 °C. Results are from Ogawa *et al.* (2003) (B) and Yamauchi *et al.* (2004) (C).

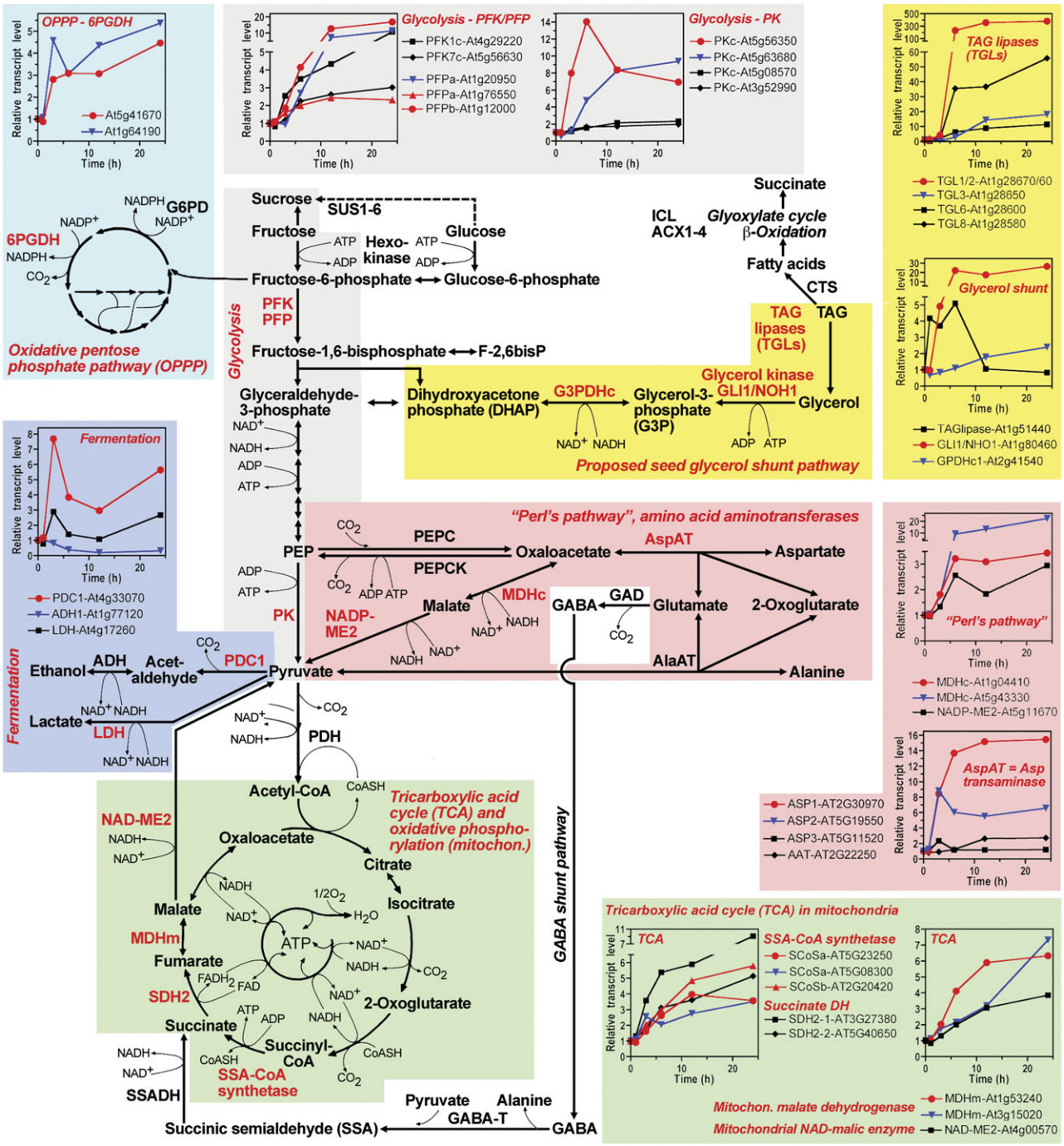

Fig. 8. Reactivation of the primary metabolic pathways and energy production during early seed germination and its possible relationship to early transcriptome changes in *Arabidopsis thaliana*. Glycolysis, aerobic (TCA cycle) and anaerobic (fermentation) respiration are the commonly used pathways for ATP production. The diagrams provide early transcript expression patterns for key genes representing these metabolic pathways as evident from the transcriptome analysis of non-dormant, non-stratified after-ripened *Arabidopsis* Columbia seeds (Nakabayashi *et al.*, 2005; Preston *et al.*, 2009) available via the seed-specific eFP-browser at www.bar.utoronto.ca (Winter *et al.*, 2007; Bassel *et al.*, 2008). Transcript levels are only presented if an early up-regulation of at least 2-fold was evident (0–24 h). Key enzymes and pathways for which transcript levels are up-regulated are labelled in red, while others are labelled in black. Fermentation-related transcripts [pyruvate dehydrogenase (PDC1) and lactate dehydrogenase (LDH)] are transiently up-regulated during early *Arabidopsis* seed germination. The transcript expression patterns in *Arabidopsis* seeds support the early up-regulation of glycolysis [phosphofructokinase (PFK) (irreversible step), pyrophosphate-dependent phosphofructokinase (PFP), and pyruvate kinase (PK)], the TCA cycle [succinate dehydrogenase (SDH), succinyl-CoA ligase (SCoS), and malate dehydrogenase (MDH)], and the OPPP [provides NADPH and precursors, 6-phosphogluconate dehydrogenase (6PGDH)], but not of gluconeogenesis [e.g. phosphoenolpyruvate carboxykinase (PEPCK)], sucrose production [sucrose synthase (SUS1–6)], fatty acid transport [comatose (CTS)], fatty acid β-oxidation [acyl-CoA oxidase (ACX)], the glyoxylate cycle [isocitrate lyase (ICL)], and the γ-aminobutyric acid (GABA) shunt

changes and their regulation by GAs and ABA or upon cold stratification. As discussed later, the transcriptional changes of key metabolic enzymes were analysed during the early phase of germination (0–24 h) of non-dormant, non-stratified *Arabidopsis* seeds (Nakabayashi *et al.*, 2005; Preston *et al.*, 2009) to test and generate hypotheses for the activation of seed metabolism (Fig. 8). Selected transcript changes of non-stratified and stratified seeds were also compared in order to determine the effects of cold stratification (Yamauchi *et al.*, 2004), and GA and ABA treatments (RIKEN transcriptome sets at www.bar.utoronto.ca). This approach is obviously limited as changes in transcript level do not necessarily correspond to similar changes in protein level and/or enzyme activity. The authors are aware of the descriptive nature of this approach and its limitations, but it is believed that this approach is useful to generate hypotheses that can be tested in subsequent experiments on the physiological, protein, and activity level and by mutant approaches.

Here, this approach is applied to investigate the transcript expression patterns of α-expansins and ABA-related genes in *Arabidopsis* seeds. α-Expansins are known to be induced in the endosperm upon imbibition (Penfield *et al.*, 2006; Carrera *et al.*, 2008; Linkies *et al.*, 2009). They are a group of proteins proposed to be involved in cell wall remodelling important for cell expansion growth, and they exhibit extensive regulation during early germination (Fig. 6B–D). During the early phase of *Arabidopsis* seed germination, transcripts of EXPA1, 2, 3, 8, 9, 15, and 20 accumulate 100- to 500-fold from 0 h to 12 h in whole unstratified seeds imbibed in the light (Fig. 6B). This induction upon imbibition is also evident during moist cold stratification (4 °C in the dark), but not if seeds are imbibed at 22 °C in the dark (Fig. 6C). When the 4 °C/22 °C transcript ratios are compared, an ~30-fold cold induction was evident for EXPA1 and EXPA2 (Fig. 6D). Many α-expansin genes are GA inducible, as shown by the +GA/–GA transcript ratios obtained with GA-deficient *ga1-3* mutant seeds; EXPA2 accumulates >200-fold upon GA treatment (Fig. 6D). The cold induction of α-expansin expression could therefore be mediated by GAs as cold stratification is associated with the induction of GA biosynthesis and increased contents of bioactive GAs (Fig. 7C, and Supplementary Fig. S1B at *JXB* online). Cold stratification is also associated with enhanced GA biosynthesis in the micropylar endosperm (Yamauchi *et al.*, 2004) where α-expansin is localized. Cold stratification also promoted the decline in ABA contents and, associated with this, caused earlier completion of germination (Fig. 5B). In contrast to GAs, ABA treatment did not affect the α-expansin transcript expression (+ABA/–ABA ratios, Fig. 6D). ABA also did not affect the kinetics of testa rupture of after-ripened *Arabidopsis* seeds (Fig. 6A). Taken together, these temporal, hormonal, and cold-inducible transcript expression patterns of EXPA2 and other α-expansins in the micropylar endosperm are in agreement with the hypothesis that they could have roles in endosperm-mediated processes during early germination that lead to and control testa rupture.

Recent advances in *Arabidopsis* molecular genetics have revealed the core ABA signalling pathways (Nambara *et al.*, 2010). Group A members of the protein phosphatase 2C (PP2C) family of genes (Supplementary Fig. S1C at *JXB* online), including *ABA-INSENSITIVE1* (*ABI1*), seem to act as negative regulators of seed germination (Kucera *et al.*, 2005; Nishimura *et al.*, 2007). The PYR1/PYL1/RCAR family of START proteins is a family of ABA receptors and may have a prominent function in seed ABA responsiveness through regulation of PP2C activity in an ABA-dependent manner (Nambara *et al.*, 2010; Nishimura *et al.*, 2010). Targets of the PP2C are members of the SNF1-related protein kinase subfamily 2 (SnRK2) that act as positive regulators of ABA signalling in activating ABRE-binding transcription factors such as ABI5 (Nakashima *et al.*, 2009). The SnRK2s become active when they are de-repressed from their inhibition by PP2Cs. Changes in the phosphorylation status of >50 proteins have been demonstrated in 12-day-old *Arabidopsis* plants after the addition of 50 μM ABA to the growth medium (Kline *et al.*, 2010). This included an increase in phosphorylation of four SnRK2s after 30 min of treatment. Transcription is important for seed ABA responsiveness and is mediated, at least in part, by the transcription factors ABI5, ABI4, and ABI3 (Holdsworth *et al.*, 2008; Nambara *et al.*, 2010). ABA degradation (Fig. 5B) combined with a decrease in ABA sensitivity, for example by targeted proteolysis of ABI3 and ABI5 via the N-end rule pathway (Holman *et al.*, 2009), promotes seed germination. Cold stratification not only induces a decline in ABA contents, but also affects the transcript expression of several ABA signalling components including PYL6, ABI4, ABI5, and several PP2Cs and SnRK2s (Supplementary Fig. S1C). In contrast to the group A PP2Cs mentioned above that act as negative regulators of ABA signalling, PP2C5 was found to be a positive regulator of ABA signalling (Brock *et al.*, 2010). ATHB20 is a transcription factor involved in ABA sensitivity that is induced in the micropylar endosperm during early germination (Barrero *et al.*, 2010). The *MFT* (*MOTHER OF FT AND TFL1*) gene serves as a mediator in response to

pathway [glutamate decarboxylase (GAD)]. Seed-specific routes that may contribute to ATP production include (1) the 'seed glycerol shunt pathway' (TAG lipases, GLI1/NOH1, and G3PDHc) for which it is proposed that AtTGL-type TAG lipases are involved and (2) 'Perl's pathway' [PEP carboxylase (PEPC), MDHc, and PK] which includes amino acid aminotransferases (AspAT and AlaAT). The pathways presented describe metabolic routes in the cytosol and mitochondria. Note that transcriptome results of cold-stratified seeds may differ and that early up-regulation of transcript levels provides hypotheses, but is not necessarily associated with the accumulation of protein and activity of the corresponding enzymes.

ABA and GA signals, and regulates seed germination through a negative feedback loop modulating ABA signalling in *Arabidopsis* (Xi *et al.*, 2010). Interestingly, the ABA-inducible expression of *MFT* is confined to the embryo elongation zone identified by Sliwinska *et al.* (2009). Based on these findings and the rapid decline of ABA contents upon imbibition, the seed tissue-specific regulation of ABA signalling is an emerging research field important for early seed germination.

A major decision for seed germination-related experiments is whether or not moist (cold) stratification should be used to release dormancy and achieve fast and uniform germination. The stratification treatment not only releases dormancy, but also promotes germination, and it is often hard to draw a clear line between the two interconnected processes. While a homogenous population is desirable, stratified seeds will already have gone through many processes that are important in early germination, and these will be lost to the subsequent observations. This also implies that early germination is hard to study in deeply dormant seeds such as many conifers which might need multiple months of moist chilling before they are able to germinate (Zeng *et al.*, 2003). The important point is that early germination differs between non-stratified and stratified seeds, and if moist stratification is used it cannot simply be regarded as a technical treatment. It is suggested that if stratification is used, sampling during the stratification period should be included as part of the experimental investigations. In the following sections, the *Arabidopsis* transcriptome will be used to describe the activation of metabolism during early germination of non-dormant, non-stratified seeds. In addition, how ABA, GAs, and cold stratification affect the transcriptome responses regarding metabolism during early seed germination will be addressed.

Reactivation of metabolism: transcription and translation

During water uptake phases I and II, large metabolic changes take place in seeds which set the course for subsequent radicle protrusion. Metabolism is reactivated with enzymes that were stored in the seed during maturation. This has been shown in proteomic approaches in *Arabidopsis*, where a large number of enzymes involved in the major metabolic pathways were found in dry seeds and remained stable or even accumulated further during early germination (www.seed-proteome.com; Gallardo *et al.*, 2001; Rajjou *et al.*, 2004; Fu *et al.*, 2005). Proteomic evidence for this includes enzymes from energy production pathways in dry *Arabidopsis* seeds (Supplementary Table S1 at *JXB* online): glycolysis [6-phosphofructokinase (PFK), phosphoglycerate kinase (PGK)], gluconeogenesis [PEP carboxykinase (PEPCK)], fermentation [alcohol dehydrogenase (ADH)], pyruvate dehydrogenase (PDH), the tricarboxylic acid (TCA) cycle [succinate dehydrogenase, succinyl-CoA ligase, malate dehydrogenase (MDH)], the glyoxylate cycle (isocitrate lyase), and the amino acid aminotransferases. However, the number of proteins detected by proteome analyses of seeds of *Arabidopsis* (see above), cress (Müller *et al.*, 2010), sugar beet (Catusse *et al.*, 2008), *Medicago* (Boudet *et al.*, 2006), barley (Sreenivasulu *et al.*, 2010), and rice (Yang *et al.*, 2007) is limited (<1000) and therefore does not allow a complete comparison with the genome-wide transcriptome analyses. In Supplementary Table S1 the demonstrated presence of these proteins by proteome analysis of dry *Arabidopsis* seeds has been compared with the dry seed transcriptome. In almost all the cases where proteome analyses demonstrated a protein to be present in dry seeds, the corresponding transcript is abundant in the dry seed transcriptome. For example, the proteins of one cytosolic PGK and one cytosolic MDH were detected by proteome analysis. In each of these cases there are three genes, only one of which shows high transcript abundance in the dry seed. The proteins detected correspond to these highly abundant transcripts. It seems clear, therefore, that many of the abundant dry seed transcripts simply reflect translation during seed maturation. This conclusion is further supported by the fact that in many cases the most abundant transcripts in dry seeds are rapidly degraded upon imbibition, whereas several transcripts with lower abundance in the dry state accumulate during the early germination phase (for examples see the next section).

Upon imbibition, dramatic changes in the transcriptome can be observed after as little as 1–3 h, that is in phase I of water uptake (Howell *et al.*, 2009; Preston *et al.*, 2009; Okamoto *et al.*, 2010). Some of these changes have been shown to be tissue specific (Okamoto *et al.*, 2010). Transcripts displaying strong changes in abundance and following similar expression patterns during early germination have been found to share common *cis*-acting elements in their gene promoters. 3' Untranslated regions (UTRs) with motifs associated with RNA stability are enriched in transcripts with a strong regulation between 0 h and 3 h in rice (Nakabayashi *et al.*, 2005; Howell *et al.*, 2009; Preston *et al.*, 2009), possibly indicating that many changes are due to degradation of stored mRNAs. It would therefore be particularly interesting to perpetuate earlier work (Marcus and Feeley, 1964; Dure and Waters, 1965; Comai and Harada, 1990) and investigate polysome-associated transcripts in addition to the general transcriptome in order to get a clearer picture of the actual translational activity of the many RNAs found in seeds during early germination.

A radiolabelling approach in *Arabidopsis* showed that *de novo* protein synthesis already took place in the first 8 h after imbibition and peaked between 8 h and 16 h (Rajjou *et al.*, 2006), which corresponds to early phase II. All components of the transcriptional machinery are stored in dry seeds and are quickly activated upon imbibition, as has been demonstrated by the fact that the addition of the translation inhibitor cycloheximide does not alter early transcript up-regulation in *Arabidopsis* (Kimura and Nambara, 2010). Interestingly, cycloheximide disrupted early down-regulation of transcripts. Translation thus seems to be necessary to activate mRNA degradation mechanisms. In addition, ribosomal proteins are among the first to be

transcribed in *Arabidopsis* (Tatematsu *et al.*, 2008) and maize (Beltran-Pena *et al.*, 1995). Translation is required for successful germination, while inhibition of transcription delays, but does not block, the completion of germination of *Arabidopsis* (Rajjou *et al.*, 2004). Tobacco, on the other hand, can proceed to testa rupture but not to endosperm rupture in the presence of a transcriptional inhibitor (Arcila and Mohapatra, 1992; Leubner-Metzger, 2003). In agreement with a high ABA content during imbibition and early phase II of *Arabidopsis* seeds (i.e. before the ABA levels decline, Fig. 5B), the first 8 h comprise a short window of time in which ABA-induced genes that belong to the set of seed maturation genes that are typically expressed in an ABA-inducible manner during seed maturation are transcribed and translated (Lopez-Molina *et al.*, 2002; Rajjou *et al.*, 2006). From the work of Rajjou *et al.* (2004, 2008) it is clear that translation from stored mRNAs can differ depending on the physiological seed state, but which of the stored transcripts are absolutely required for the completion of germination is still an unsolved question.

Reactivation of metabolism: energy production

Minutes after the start of imbibition, a sharp increase in oxygen uptake and carbon dioxide release can be observed (Botha *et al.*, 1992; Bewley, 1997). Gases may be released from colloidal adsorption or simply pushed out from gas-filled spaces by the water rushing in (Cloetens *et al.*, 2006). Oxygen uptake then stagnates or increases only slowly; this lasts until the end of phase II (Botha *et al.*, 1992; Bewley, 1997).

Dry seeds contain only low amounts of ATP, but a rapid production is initiated upon cellular hydration in association with the gas exchange (Botha *et al.*, 1992; Spoelstra *et al.*, 2002; Benamar *et al.*, 2008). It is clear that respiratory pathways operate in imbibed seeds (Fig. 8), but their relative contribution and the substrates for ATP production during germination are still a matter of debate. Most of our knowledge about metabolic pathways at the level of enzyme activities and their products during the early phase of seed germination comes from studies of species with large seeds such as pea (Obroucheva and Antipova, 1997; Macherel *et al.*, 2007; Benamar *et al.*, 2008; Smiri *et al.*, 2009). Most of the pathways presented in Fig. 8 operate in the cytosol. Mitochondria in dry seeds need repair and differentiation before contributing significantly to ATP production by oxidative phosphorylation. However, substrate oxidation of succinate by mitochondria extracted from dry pea seeds is possible (Morohashi and Bewley, 1980; Botha *et al.*, 1992). Mitochondrial enzymes and membranes in starchy seeds such as pea seem to be protected by LEA proteins, and repair of pre-existing mitochondria takes place upon imbibition (Grelet *et al.*, 2005; Tolleter *et al.*, 2010). In contrast, biogenesis of new mitochondria is more important in oil-seeds (Morohashi *et al.*, 1981; Morohashi, 1986). It is known that many key enzyme activities of the TCA cycle accumulate during early germination.

Oxygen-sensitive microsensors have been used to investigate the spatial and temporal oxygen status of germinating seeds of legumes, cereals, sunflower, and oilseed rape. The results point toward a limitation of oxygen uptake by seed-covering layers leading to hypoxia in seeds. In pea seeds, the internal oxygen content dropped during imbibition to anoxic levels while the respiration rate increased continuously (Obroucheva and Antipova, 1997; Benamar *et al.*, 2008; Rolletschek *et al.*, 2009). The ratio between fermentation and aerobic respiration varies between species and during the progression of germination, as does the sensitivity of seeds to oxygen availability (Benamar *et al.*, 2008; Rolletschek *et al.*, 2009). The adenylate energy charge of pea seeds can be increased and the accumulation of fermentation products decreased by providing the imbibing seeds with additional oxygen, which again points to a limiting role for oxygen rather than enzyme availability. Additional oxygen does not, however, increase the final germination percentage of pea, pointing to the fact that the seeds are adapted to germinate under hypoxic conditions. The outcome of Darwin's experiment described in the Introduction, which imposed salt stress and hypoxia during germination, testifies to the amazing ability of seeds to germinate under conditions of oxygen deprivation.

At the level of enzyme activities far less is known about the early germination of the small seeds of *Arabidopsis*, but the dynamics of the proteome (e.g. Gallardo *et al.*, 2001; Fu *et al.*, 2005) and the transcriptome (e.g. Nakabayashi *et al.*, 2005; Preston *et al.*, 2009) have been investigated. Temporal transcript expression patterns of key metabolic enzymes during the early phase of germination (0–24 h, water phase I and II) of non-dormant, non-stratified *Arabidopsis* seeds (Nakabayashi *et al.*, 2005; Preston *et al.*, 2009) were therefore used to test and generate hypotheses for the activation of seed metabolism (Fig. 4) and to determine the effects of moist cold stratification (incubation of Col seeds in darkness for 96 h at 4 °C compared with 22 °C; Yamauchi *et al.*, 2004), ABA (L*er* seeds at 24 h without and with ABA added), and GA (GA-deficient *ga1-3* seeds at 6 h without and with GA added) treatments (RIKEN transcriptome sets at www.bar.utoronto.ca). It should be kept in mind that while the approach with *ga1-3* has been very informative, the results may have been influenced by the fact that a mutant was used and might not be completely applicable to the wild type. The dry seed transcriptome for energy metabolism was also compared with its counterpart during early germination (imbibed for 6 h) to highlight the similarities and differences in transcript abundances (Fig. 8, and Supplementary Table S1 at *JXB* online). It was presumed that an at least 2-fold increase in transcript abundance is an indication for the activation of the encoded enzyme. As mentioned above, this approach is obviously limited as an effect on the transcript level does not necessarily correspond to a similar change on the protein and activity level. Where possible, information available from proteome and mutant work with *Arabidopsis* is therefore included.

Based on an at least 2-fold increase in transcript levels, the *Arabidopsis* seed transcriptome during early germination supports the view that glycolysis, fermentation, the TCA cycle and the oxidative pentose phosphate pathway (OPPP) are activated during early germination (Fig. 8, Supplementary Fig. S2 at *JXB* online). This is in agreement with evidence from enzyme activity measurements (e.g. Bettey and Finch-Savage, 1996; Wakao *et al.*, 2008; Smiri *et al.*, 2009) and proteome analyses (e.g. Gallardo *et al.*, 2001; Fu *et al.*, 2005; Müller *et al.*, 2010). These activations are, however, not simply evident for each gene of a particular pathway, but seem to be a complex combination of stored proteins, stored transcripts, and *de novo* transcription and translation. Ethanolic fermentation by pyruvate decarboxylase (PDC) and ADH is a good example of this. In the dry seed transcriptome, PDC2 and ADH are the most abundant transcripts of the fermentation pathway, while the abundance of PDC1 and others is low. However, upon imbibition, the PDC2 and ADH transcript levels rapidly decline (3- to 5-fold within 6 h; Fig. 8, and Supplementary Table S1 and Fig. S2). Proteome analysis, however, demonstrated that ADH protein levels remain constant; that is, ADH activity depends on stored ADH protein that accumulated during seed maturation and/or on newly synthesized ADH that replaced stored ADH subjected to protein degradation (Gallardo *et al.*, 2001). The high abundance of ADH transcripts in dry seeds combined with its rapid degradation would then simply be a remnant from seed maturation. On the other hand, *de novo* translation and ADH protein accumulation have also been described (Fu *et al.*, 2005; Rajjou *et al.*, 2006). ADH transcript expression is induced by cold stratification, but not regulated by GA (*ga1-3* seeds ±GA) or ABA (wild-type seeds ±ABA) (Supplementary Fig. S2). While PDC2 is not inducible by these factors, PDC1 transcripts accumulate upon imbibition, cold stratification, and GA treatment (Supplementary Fig. S2). This example demonstrates that activation of energy metabolism during early germination is complex and cannot simply be predicted from the most abundant transcripts in the dry seed transcriptome.

Supplementary Fig. S2 also shows that imbibition itself and cold stratification are the most important factors for up-regulating transcripts of the sugar-related metabolic pathways mentioned above; neither up-regulation by GA nor down-regulation by ABA appears to be of major importance. However, other mechanisms for hormonal regulation exist; for example, ABA induces *AtPirin1* (Lapik and Kaufman, 2003), which is known to regulate pyruvate catabolism by inhibiting the PDH complex (Soo *et al.*, 2007). Cold stratification does not cause an increase in the levels of the TCA cycle metabolites citrate, malate, and succinate, but subsequent incubation in the light causes increases in their levels (Angelovici *et al.*, 2011).

In contrast to the pathways for glycolysis, fermentation, the TCA cycle, and the OPPP mentioned above, transcript expression for key genes of gluconeogenesis, sucrose synthesis, and the peroxisomal pathways (fatty acid β-oxidation and the glyoxylate cycle) is not up-regulated during early germination (Fig. 8, and Supplementary Table S1, Fig. S2 at *JXB* online). Transcripts of the fatty acid β-oxidation enzyme 3-ketoacyl-CoA thiolase (KAT2/PED1) and the glyoxylate cycle enzyme isocitrate lyase (ICL) are among the top 100 most highly expressed transcripts in dry *Arabidopsis* seeds (Kimura and Nambara, 2010). However, the ICL transcript levels decline 25-fold (6 h/dry) upon imbibition and remain low until the late germination phase for which ICL protein accumulation has been shown (Supplementary Table S1, Fig. S2; Gallardo *et al.*, 2001). Gluconeogenesis, sucrose synthesis, and the peroxisomal pathways are most important for post-germinative seedling establishment (e.g. Penfield *et al.*, 2004; Holdsworth *et al.*, 2008; Holman *et al.*, 2009). Seedling arrest, but not germination phenotypes were evident for single-gene knock-out mutants of these pathways (Penfield *et al.*, 2005). In contrast, recent evidence from double mutants for the peroxisomal pathways (the glyoxylate cycle and fatty acid β-oxidation, e.g. Pinfield-Wells *et al.*, 2005; Pracharoenwattana *et al.*, 2005, 2010) and careful consideration of the physiological seed state combined with distinct germination conditions (distinct media, sucrose addition, cold stratification; Footitt *et al.*, 2006) demonstrates that the peroxisomal β-oxidation determines germination potential. This includes the ABC transporter COMATOSE (CTS, also known as PED3) required for the import of substrates for peroxisomal β-oxidation (Russell *et al.*, 2000), for which a complex interaction with ABA has been proposed (Footitt *et al.*, 2006; Kanai *et al.*, 2010). The recent results of Kanai *et al.* (2010) suggest that CTS/PED3 promotes seed germination by suppressing the ABA-mediated inhibition of pectin degradation in the seed-covering layers.

Cleavage of triacylglycerol (TAG, seed oil) by TAG lipases at the water–oil interface of oil bodies provides glycerol and fatty acids. The *Arabidopsis* early germination transcriptome suggests that a glycerol shunt pathway is activated that feeds into glycolysis via dihydroxyacetone phosphate (DHAP) and involves glycerol-3-phosphate dehydrogenase (G3PDc), glycerol kinase (Gycerol-insensitive1/Nonhost1, GLI1/NOH1), and TAG lipases (Fig. 8). *GLI1/NOH1* transcript levels increase >20-fold, the corresponding mutants have a germination phenotype (Eastmond, 2004), and in seedlings a metabolic connection to DHAP involving GLI1/NOH1 and G3PDc is known (Chanda *et al.*, 2008). Transcripts of several *AtTLG*-type TAG lipase genes accumulate dramatically (e.g. *AtTGL1* transcripts >300-fold) (Fig. 8, and Supplementary Table S1, Fig. S2 at *JXB* online). TAG lipase enzyme activity of AtTGL1 has been demonstrated, and the corresponding mutants show delayed germination which could be overcome by sucrose treatment (Körner, 2005). It is therefore hypothesized that glycerol released by AtTLG-type TAG lipases together with a seed glycerol shunt pathway (Fig. 8) could provide energy during early oil-seed germination. In addition, several of the *AtTLG*-type TAG lipase transcripts accumulate upon cold stratification, *AtTGL1* is GA induced, but none of the *AtTLG*-type TAG lipase transcripts is affected by ABA (Supplementary Fig. S2). Other types of

TAG lipase transcripts also accumulate upon imbibition, but none of them as strongly as *AtTGL1* and *AtTGL8*.

Interestingly, fatty acid metabolism is repressed by ABA in the embryo, but not in the endosperm (Manz *et al.*, 2005; Penfield *et al.*, 2006). It has been proposed that ABA inhibits *Arabidopsis* seed germination by limiting the availability of energy and nutrients by preventing seed storage protein degradation (Garciarrubio *et al.*, 1997), but not by inhibiting storage lipid mobilization (Penfield *et al.*, 2005).

Stored proteins in seeds are not only an important source of amino acids during early germination, but are also important for energy production (Angelovici *et al.*, 2011). Their activation is already prepared in the dry seeds: stored proteinases mobilize storage proteins in legume radicles (reviewed by Müntz *et al.*, 2001; Müntz, 2007). Early degradation of protein bodies also occurs in the micropylar endosperm of *Arabidopsis* (Bethke *et al.*, 2007). Aspartate and glutamate are among the most abundant amino acids in seed storage proteins. They are substrates for aspartate and alanine aminotransferases (AspAT and AlaAT) that are activated during imbibition and thought to participate in respiratory pathways (Fig. 8) (Obroucheva and Antipova, 1997; Miyashita *et al.*, 2007; Rocha *et al.*, 2010). AspAT could also contribute with oxaloacetate production to a unique system to explain ATP synthesis in seeds, termed 'Perl's pathway' (Perl, 1986; Botha *et al.*, 1992). It depends on the fact that cytosolic malate dehydrogenase (MDHc) and PEPCK activities are already high in some seeds during the early phase of germination. In this ATP-synthesizing system MDHc provides NADH which is split by NADH-pyrophosphorylase yielding ADP. The latter is converted to ATP by pyruvate kinase (PK; Fig. 8). That PEPCK activity increases in germinating seeds is known from *Arabidopsis* (Penfield *et al.* 2004) and several other species (Botha *et al.*, 1992; Ratajczak *et al.*, 1998). MDHc and PEPCK protein also accumulate in *Arabidopsis* seeds (Supplementary Table S1, and references therein). In support of 'Perl's pathway', transcripts of MDHc, PK, and AspAT accumulate during early germination in *Arabidopsis* seeds (Fig. 8). Further research is needed to elucidate the possible role of this pathway in ATP production during seed germination. Cold stratification induces aspartate accumulation, but accumulation of TCA cycle metabolites derived from it is only evident upon subsequent incubation at 21 °C in the light (Angelovici *et al.*, 2011). ABA induces the expression of glutamate decarboxylase (GAD; Supplementary Table S1 and Fig. S2) which produces γ-aminobutyric acid (GABA) associated with stress responses, and the GABA shunt for energy production is also evident in seeds (Shelp *et al.*, 1995; Bouche *et al.*, 2003).

Seeds store not only protein, oil, and starch, but also essential metals such as iron (Fe). In an innovative approach, Lanquar *et al.* (2005) identified the importance of vacuolar metal storage and activation during early germination in *Arabidopsis* seeds. Metal, in particular Fe, is mobilized during early germination by the redundant broad-range metal transporters NRAMP3 and 4. Germination of the double mutant is inhibited under conditions of Fe deficiency, as the seeds fail to retrieve Fe from the vacuole even though they contain as much Fe as the wild type. Fe, zinc, provitamin A ('Golden Rice'), and folate are the most important micronutrients for which malnutrition can be improved by biofortification (Mayer *et al.*, 2008). Research and breeding programmes are underway to enrich these compounds in crop seeds and depend on understanding seed metabolic engineering.

Novel directions and techniques for studying early seed germination

In the coming years, novel methods will lead to significant advances in our understanding of seed biology and plant evolution. New technologies situated at the interface of biology and disciplines such as material sciences, physical chemistry, and engineering offer the possibility to tackle new questions with interdisciplinary approaches.

Huge advances have been made in the area of imaging. Environmental scanning electron microscopy (eSEM) offers the opportunity to take high magnification images of living seeds (Fig. 4) (Muscariello *et al.*, 2005; Windsor *et al.*, 2000). These tools (eSEM and similar high resolution imaging techniques) can be used to tackle questions surrounding the structure of seeds and changes in these during germination in different seed types. eSEM is an excellent method to show the diversity in seed structures in different species and through the observation link back to questions concerning the evolution of morphologically different seed types.

New tools based on ^{1}H-NMR imaging technology can be used not only to visualize and quantify water uptake (Manz *et al.*, 2005; Wojtyla *et al.*, 2006; Koizumi *et al.*, 2008), but also for non-invasive imaging of seed oils (Neuberger *et al.*, 2009). This enables scientists to assess spatial water distribution and oil content. Microsensors can be used to provide spatial and temporal oxygen maps of seeds (Rolletschek *et al.*, 2009) which can be an important hint to the answer to the question of where and when fermentation processes occur in seeds. Kranner *et al.* (2010*a*) applied non-invasive infrared thermography to seeds and demonstrated that viability and even biochemical processes such as the dissolution of low molecular weight compounds could be assessed. This promising method is able to link biophysical with biochemical parameters and seed viability and could, for example, be used to distinguish different seed types by their thermographic behaviour during germination. MALDI-MS (matrix-assisted laser desorption/ionization mass spectrometry) imaging involves the visualization of the spatial distribution of proteins, peptides, metabolites, biomarkers, or other chemicals within thin tissue sections and might be a powerful tool for exploring the spatial distribution of nutrients in seeds. It has been used to visualize GABA in eggplant fruit sections where it localizes to the seeds (Goto-Inoue *et al.*, 2010). ^{1}H-NMR imaging, microsensors, MALDI-MS imaging, and thermography all

can be used to quantify global changes in seeds in a spatial and temporal manner.

Bringing together confocal microscopy and computer-based image analyses, Sliwinska *et al.* (2009) created informative 3D images of an elongating embryo, which led to the localization of an elongation zone of the embryo. This could be further used for a cross-species approach to investigate conservation and biodiversity of embryo elongation zones in combination with established methods that can identify genes and proteins involved in cell expansion growth during germination. Quantitative phase tomography was used to elucidate structural details of *Arabidopsis* seeds (Cloetens *et al.*, 2006). This technique uses a synchrotron-based approach to generate 3D, high-resolution images of a specimen to the cell level.

There have been great advances in the area of sequencing and epigenetics, which will greatly enhance our knowledge of germination in a wide array of species. Combined epigenetic (ChIP-seq) and transcriptome (RNA-seq) analyses with next-generation sequencing technologies will make it possible to analyse plants without a sequenced genome on a genomic scale (Bräutigam and Gowik, 2010). A combination of high-throughput sequencing with more classical methods can greatly advance our knowledge about developmental processes. Recently such a combined approach was used to study the developmental dynamics in maize leafs and identified 180 transcription factors for which now functional genomics studies would be interesting (Li *et al.*, 2010). To describe dynamic networks from such results 'Systems Biology Graphical Notation' can be used, and corresponds to an engineer's view on regulation as it was published for seed development (Junker *et al.*, 2010).

In addition, new databases and platforms designed specifically to collect and analyse information from high-throughput approaches to seed germination are now in place or being developed. The seed-specific gene ontology system TAGGIT facilitates the identification and visualization of the germination signature (Holdsworth *et al.*, 2008), and the seed-specific eFP-browser and the eNorthern tool at the Toronto bar website (www.bar.utoronto.ca) visualizes *Arabidopsis* transcript expression patterns in seeds (Winter *et al.*, 2007; Bassel *et al.*, 2008). The co-expression tool (CORNET), similar to the Genemania browser from the Toronto bar website, provides an easy to use and helpful tool in finding interactions either in already published experiments or in user-supplied data, and thus helps to handle the generated amount of data better (De Bodt *et al.*, 2010). These tools can help to generate hypotheses by supplying *in silico* data from already published experiments and help curate the massive amount of data generated by high-throughput analyses.

Seeds are starting to be used in cross-species systems biology approaches and interdisciplinary collaborations such as the European 'virtual SEED' network project (www.vseed.eu) where a molecular approach is combined with biophysical and morphological data, enabling the assembly of a more comprehensive model of seed germination. Understanding this process as a whole from the very beginning—from early seed germination to the establishment of the seedling—can help engineer and select for better and more robust crop species, thus increasing crop yield and quality.

Plant species developed a huge morphological and physiological diversity in seed types and states to match local environmental demands for germination timing. Darwin was aware of what he called the 'vitality of seeds' and made a connection to plant evolution: 'The power in seeds of retaining their vitality when buried in damp soil may well be an element in preserving the species, and therefore seeds may be specially endowed with this capacity' (Darwin, 1855*a*). Cross-species approaches in seed science and other areas of plant science will further increase our understanding of the evolution of plants. 'Appropriate germination responses to environmental factors are the first requirement for successful growth and adaptation in any life-history trait; no subsequent life-history trait can even be expressed if the plant does not first survive past the germination stage. As such, germination timing can be a stringent selective sieve, determining which genotypes can establish in particular conditions.' (Donohue, 2005). Seed germination and dormancy are indeed the most important early life-history traits.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Regulation of GA- and ABA-related transcripts by cold stratification of *Arabidopsis thaliana* seeds.

Figure S2. Regulation of transcripts for the energy metabolism of *Arabidopsis thaliana* seeds by imbibition, cold stratification, GA, and ABA.

Table S1. Transcriptome analysis for energy metabolism genes during *Arabidopsis thaliana* germination *sensu strictu* and its regulation by hormones and cold stratification.

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