Review

Tansley review

Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination

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Summary

The transition between dormancy and germination represents a critical stage in the life cycle of higher plants and is an important ecological and commercial trait. In this review we present current knowledge of the molecular control of this trait in *Arabidopsis thaliana*, focussing on important components functioning during the developmental phases of seed maturation, after-ripening and imbibition. Establishment of dormancy during seed maturation is regulated by networks of transcription factors with overlapping and discrete functions. Following desiccation, after-ripening determines germination potential and, surprisingly, recent observations suggest that transcriptional and post-transcriptional processes occur in the dry seed. The single-cell endosperm layer that surrounds the embryo plays a crucial role in the maintenance of dormancy, and transcriptomics approaches are beginning to uncover endosperm-specific genes and processes. Molecular genetic approaches have provided many new components of hormone signalling pathways, but also indicate the importance of hormone-independent pathways and of natural variation in key regulatory loci. The influence of environmental signals (particularly light) following after-ripening, and the effect of moist chilling (stratification) are increasingly being understood at the molecular level. Combined postgenomics, physiology and molecular genetics

approaches are beginning to provide an unparalleled understanding of the molecular processes underlying dormancy and germination.

I. Introduction

The completion of seed germination represents a key ecological and agronomic trait, determining when plants enter ecosystems (Bewley & Black, 1994; Wilkinson *et al.*, 2002). As such it is highly regulated by both internal and external cues that determine the dormancy status and the potential for germination (defined as the final percentage of germination). The definition of seed dormancy has been reviewed by Finch-Savage & Leubner-Metzger (2006) and is generally given as the incapacity of a viable seed to germinate under favourable conditions. Germination is defined as the emergence of the radicle through surrounding structures (Bewley, 1997b; Baskin & Baskin, 2004; Finch-Savage & Leubner-Metzger, 2006). In the plant genetic model, *Arabidopsis thaliana* (hereafter referred to as Arabidopsis) seed dormancy is classified as physiologically nondeep, meaning that embryos released from surrounding structures grow normally and that dormancy is lost through moist chilling (stratification) or after-ripening (Baskin & Baskin, 2004). In Arabidopsis the embryo is surrounded by the single-cell endosperm (also called aleurone) layer, which is also the result of double fertilization (Finch-Savage & Leubner-Metzger, 2006), and the testa (Debeaujon *et al.*, 2000) (Fig. 1). Seed dormancy has been studied in Arabidopsis for many decades, mainly at the genetic and physiological levels. These investigations revealed many components that are required for, or involved with, dormancy induction, maintenance and the completion of germination, including light, temperature and plant hormones. A major conclusion from these studies (as in many other species) is that the plant hormone abscisic acid (ABA) is a positive regulator of dormancy, while gibberellins (GA) release dormancy and promote the completion of germination, counteracting the effects of ABA (Bewley & Black, 1994). The first dormancy and germination loci identified by mutations in Arabidopsis included those acting in GA and ABA biosynthesis and perception (Koornneef, 1978; Koornneef & Van der Veen, 1980; Koornneef *et al.*, 1982, 1984). In the early 1990s the first molecular analyses and cloning of genes that affect dormancy and/or germination were published, including *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), *DIHYDROFLAVONOL-4-REDUCTASE* (*DFR*; *TT3*), *CHALCONE ISOMERASE* (*CHI*; *TT5*) and *FUSCA3* (*FUS3*) (Giraudat *et al.*, 1992; Bäumlein

et al., 1994; Shirley *et al.*, 1995). Recently, the first high-throughput, large scale-omics studies investigating seed dormancy and germination were published, including transcriptomics (Nakabayashi *et al.*, 2005; Cadman *et al.*, 2006; Finch-Savage *et al.*, 2007; Carrera *et al.*, 2008), proteomics (Chibani *et al.*, 2006) and metabolomics (Fait *et al.*, 2006). These new approaches and derived data sets provide an unprecedented level of detail concerning genome expression associated with germination potential.

The various aspects of seed dormancy and germination have been extensively reviewed recently (Bentsink & Koornneef, 2002; Kucera *et al.*, 2005; Finch-Savage & Leubner-Metzger, 2006). In this article we discuss recent findings in Arabidopsis that are associated with the molecular regulation of primary
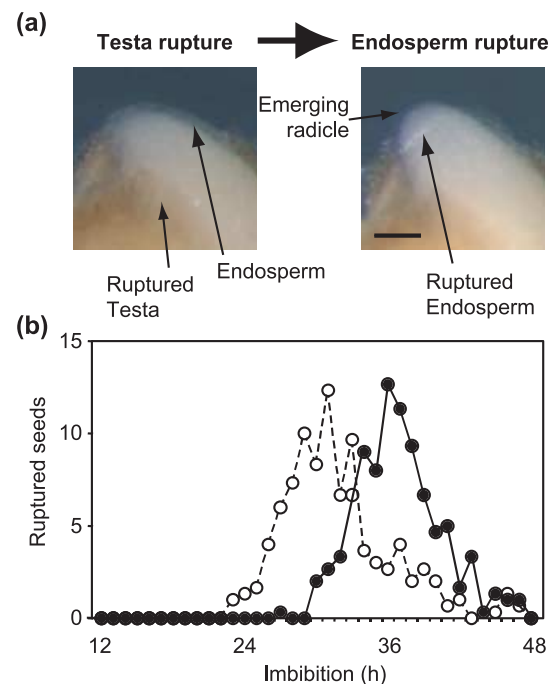
Fig. 1 Arabidopsis seed germination is a two-stage process. (a) Light micrograph showing sequential stages of Arabidopsis accession Col-0 seed germination, testa and endosperm rupture. The position of testa and endosperm tissue is indicated, and following rupture of the endosperm, the emerging radicle is evident. Bar, 25 μm. (b) Time course of testa (open circles) and endosperm (closed circles) rupture in Arabidopsis accession Col-0 seeds.
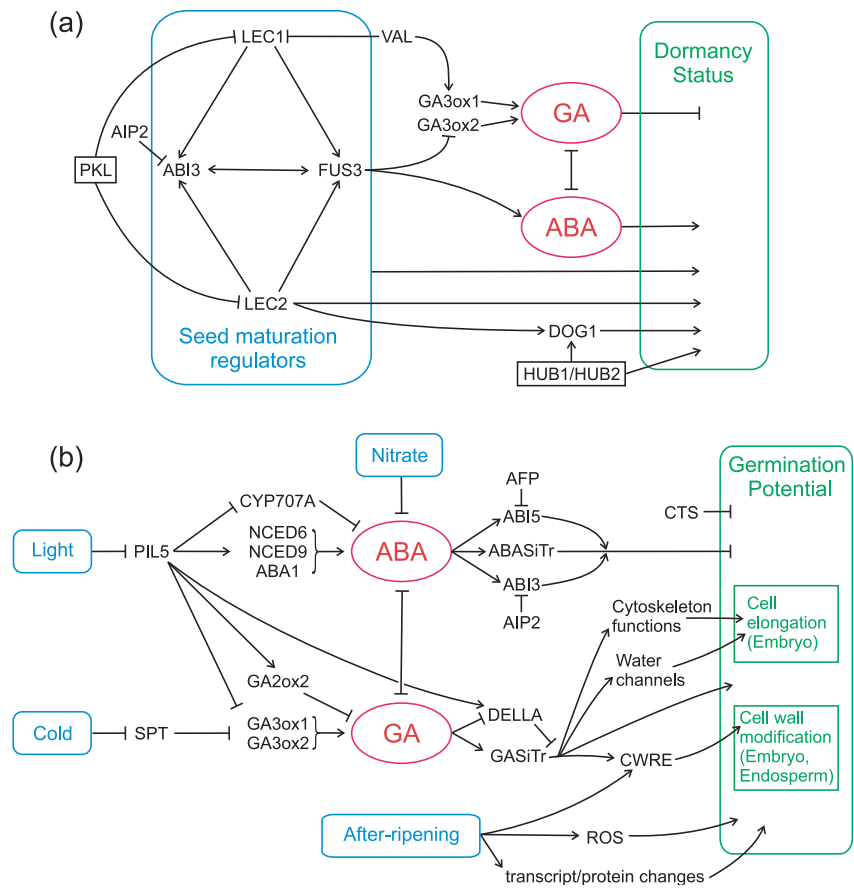
Fig. 2 Regulation of dormancy status during seed maturation and germination potential in imbibed mature seeds in Arabidopsis, and proposed interactions between some of the genes involved. (a) Interactions during seed maturation. Seed maturation factors are located in the blue box, dormancy status is shown in green, and the hormones abscisic acid (ABA) and gibberellins (GA) are shown in red. The boxed genes represent chromatin remodelling factors. An arrow indicates a promotive effect and a bar a repressive effect. (b) Interactions in imbibed mature seeds. Factors that influence germination are shown in blue, germination potential is shown in green, and the hormones ABA and GA are shown in red. ABASiTr, ABA signal transduction; CWRE, cell wall remodeling enzymes; GASiTr, GA signal transduction; ROS, reactive oxygen species. An arrow indicates a promotive effect and a bar a repressive effect. The interactions between regulators, environmental factors, hormones and different genes are described in the main text.

seed dormancy and germination, focusing on the three developmentally associated processes, seed maturation, after-ripening and imbibition.

II. Molecular regulation of seed maturation

Higher-plant seed development is divided into two major phases: embryo and endosperm development (or morphogenesis); and seed maturation (West & Harada, 1993; Gutierrez *et al.*, 2007). Embryogenesis starts with the formation of a single-cell zygote and ends at the heart stage, when all embryo structures have been formed. This is followed by a growth phase during which the embryo fills the embryo sac. At the end of the embryo growth phase, cell division in the embryo arrests (Raz *et al.*, 2001). This is the moment at which the seed enters the maturation phase. Maturation begins with a transition phase during which the switch from maternal control to filial control occurs (Weber *et al.*, 2005). The seed then undergoes a period of embryo growth and filling. Dormancy is initiated early during seed maturation and increases until the seed is fully developed (Raz *et al.*, 2001). Seed maturation is completed when storage compounds have accumulated, water content has decreased, ABA levels have

increased, and desiccation tolerance and primary dormancy are established.

A leading role for ABA in the regulation of dormancy induction during seed maturation

Abscisic acid is an important positive regulator of both the induction of dormancy during seed maturation and the maintenance of the dormant state in imbibed seeds following shedding (Fig. 2a). The role of ABA in seed dormancy and germination has been extensively discussed in several reviews (including Kucera *et al.*, 2005; Finch-Savage & Leubner-Metzger, 2006) and therefore only a very brief review of recent publications is provided here.

In many plant species ABA is involved in the induction and maintenance of the dormant state. Deficiency of ABA during seed development is associated with the absence of primary dormancy in the mature seed, whereas the over-expression of ABA biosynthesis genes can increase seed ABA content and enhance seed dormancy or delay germination (e.g. Finkelstein *et al.*, 2002; Nambara & Marion-Poll, 2003; Kushiro *et al.*, 2004). Abscisic acid produced by the seed itself during seed development can impose a lasting dormancy, whereas

maternal ABA, or ABA application during seed development, fails to induce lasting seed dormancy (Karssen *et al.*, 1983; Groot & Karssen, 1992; Koornneef & Karssen, 1994).

Most seed ABA biosynthetic genes have now been identified (reviewed by Nambara & Marion-Poll, 2003). Genes associated with the regulation of ABA biosynthesis, which have been described in more detail in seeds, include *ABA1*, the 9-*cis*-epoxycarotenoid dioxygenase (NCEDs) and *ABA2/GIN1/SDR1* (Nambara & Marion-Poll, 2003). Overall, the knowledge of the regulation of the seed ABA biosynthetic pathway is very limited; however, it is clear that synthesis is spatially and temporally regulated in seeds, at least at the level of transcript abundance (Nambara & Marion-Poll, 2003).

Abscisic acid response mutants, of which several dozens have been identified to date (Kucera *et al.*, 2005), have been useful tools for using to dissect ABA signal transduction pathways. Analyses of these mutants revealed that the ABA signal transduction pathway associated with dormancy induction involves protein kinases (e.g. ABA-activated protein kinase), phosphatases (e.g. ABI1 and ABI2, and serine/theonine phosphatases) and transcription factors (e.g. ABI3, ABI4 and ABI5; Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003).

A genetic framework for seed maturation

In Arabidopsis, seed maturation and dormancy induction are genetically controlled by at least four major regulators, namely *FUS3*, *ABI3*, *LEAFY COTYLEDON 1* (*LEC1*) and *LEC2* (Raz *et al.*, 2001) (Fig. 2a). *ABI3*, *FUS3* and *LEC2* encode related plant-specific transcription factors containing the conserved B3-binding domain (Giraudat *et al.*, 1992; Luerssen *et al.*, 1998; Stone *et al.*, 2001), whereas *LEC1* encodes a HAP3 subunit of the CCAAT-binding transcription factor (CBF, also known as NF-Y; Lotan *et al.*, 1998). All four *abi3*, *lec1*, *lec2* and *fus3* mutants are severely affected in seed maturation and share some common phenotypes, such as decreased dormancy at maturation (Raz *et al.*, 2001) and reduced expression of seed storage proteins (Gutierrez *et al.*, 2007). However, they also show specific phenotypes, such as the absence of chlorophyll degradation in the dry seed (*abi3*), a reduced sensitivity to ABA (*abi3* and, to a lesser extent, *lec1*), the accumulation of anthocyanins (*fus3*, *lec1* and, to a lesser extent, *lec2*), an intolerance to desiccation (*abi3*, *fus3* and *lec1*), or defects in cotyledon identity (*lec1*, *fus3* and *lec2*) (Bäumlein *et al.*, 1994; Keith *et al.*, 1994; Meinke *et al.*, 1994; Parcy *et al.*, 1994; Parcy & Giraudat, 1997; Luerssen *et al.*, 1998; Vicient *et al.*, 2000; Raz *et al.*, 2001; Stone *et al.*, 2001; Kroj *et al.*, 2003). It was recently shown that several of the *fus3* phenotypes occur as a result of pleiotropic effects caused by truncation gene products of the mutant alleles. The direct effects of *FUS3* are probably restricted to embryo-derived dormancy and determination of cotyledon epidermis cell identity (Tiedemann *et al.*, 2008).

The *LEC1* gene is required for normal development during early and late phases of embryogenesis and is sufficient to induce embryonic development in vegetative cells (Lotan *et al.*, 1998). Loss of function of *LEC1* leads to germination of excised embryos at a similar stage (8–10 d after pollination) as in *lec2* and *fus3* mutants, but earlier during embryo development than in *abi3* mutants (Raz *et al.*, 2001). Ten HAP3 (AHAP3) subunits have been identified in Arabidopsis, which can be divided into two classes based on sequence identity in their central, conserved, B domain (Kwong *et al.*, 2003). *LEC1* and the closely related subunit, *LEC1-LIKE* (*L1L*), constitute *LEC1*-type AHAP3 subunits, whereas the remaining eight are designated non-*LEC1* type. Similarly to *LEC1*, *L1L* is expressed primarily during seed development. However, suppression of *L1L* gene expression induced defects in embryo development that differed from those of *lec1* mutants, suggesting that *LEC1* and *L1L* play different roles in embryogenesis (Kwong *et al.*, 2003).

LEC2 directly controls a transcriptional program involved in the maturation phase of seed development. Induction of *LEC2* activity in seedlings causes rapid accumulation of RNAs normally present primarily during the maturation phase, including seed storage and lipid-body proteins. Promoters of genes encoding these maturation RNAs all possess RY motifs (cis-elements bound by B3 domain transcription factors) (Braybrook *et al.*, 2006). This provides strong evidence that these genes represent transcriptional targets of *LEC2*. One of these genes is *DELAY OF GERMINATION 1* (*DOG1*), which is the first seed dormancy gene accounting for variation occurring in natural populations that has been identified at the molecular level (Bentsink *et al.*, 2006).

It has been postulated that *ABI3*, *FUS3*, *LEC1* and *LEC2* interact as a network to control various aspects of seed maturation; however, this was proven only recently. *LEC1* was shown to regulate the expression of both *ABI3* and *FUS3* (Kagaya *et al.*, 2005); *FUS3* and *LEC2* have been shown to act in a partially redundant manner to control the gene expression of seed-specific proteins; and *LEC2* was shown to regulate *FUS3* expression locally in regions of the cotyledons (Kroj *et al.*, 2003). The indication of redundant regulation within this group of genes was recently shown (To *et al.*, 2006). By analyzing *ABI3* and *FUS3* expression in various single-, double-, and triple-maturation mutants, multiple regulatory links among all four genes were identified. It was found that one of the major roles of *LEC2* was to up-regulate *FUS3* and *ABI3*. The *lec2* mutation is responsible for a dramatic decrease in *ABI3* and *FUS3* expression, and most *lec2* phenotypes can be rescued by constitutive expression of *ABI3* or *FUS3*. In addition, *ABI3* and *FUS3* were shown to regulate themselves and each other positively, thereby forming feedback loops essential for their sustained and uniform expression in the embryo. Finally, *LEC1* also positively regulates *ABI3* and *FUS3* in the cotyledons (To *et al.*, 2006). Although multiple regulatory links were identified amongst these four genes, molecular mechanisms underlying this network, and the downstream targets of the network associated

with dormancy induction, still require further investigation (Fig. 2a).

Gibberellins are required for plant embryogenesis (Singh *et al.*, 2002); however, in later phases of embryogenesis their synthesis must be down-regulated as dormancy is initiated. It is thought that cross-talk with other plant hormone pathways, such as ABA, ethylene or auxin, are involved in this regulation of GA biosynthesis (Curaba *et al.*, 2004). Gibberellin biosynthesis was also shown to be regulated by *LEC2* and *FUS3* pathways (Curaba *et al.*, 2004). *FUS3* represses the expression of *AtGA3ox2*, a key enzyme that catalyzes the conversion of inactive to bioactive GAs, mainly in epidermal cells of the embryo axis. It was also shown that the FUS3 protein physically interacts with two RY cis-elements present in the *AtGA3ox2* promoter. In addition, both LEC2 and FUS3 proteins bind to the RY motif present in seed-specific promoters such as *At2S3* (Reidt *et al.*, 2000; Kroj *et al.*, 2003) (Fig. 2a).

During seed development, different programs of gene expression have been identified, comprising distinct classes that are co-ordinately regulated (Hughes & Galau, 1989; Parcy *et al.*, 1994). The maturation class, expressed at early and mid-maturation phases, includes major seed storage protein (*SSP*) genes (including 2S albumins and 12S globulins). The late embryogenesis abundant (*LEA*) class, which includes primarily genes involved in the acquisition of desiccation tolerance, is expressed during the later stages of seed maturation (Wobus & Weber, 1999; Hoekstra *et al.*, 2001). Regulatory cis-acting elements required for specific expression of *SSP* genes in Arabidopsis and closely related species include the ABA-responsive element (ABRE), and the RY/Sph element (Ellerstrom *et al.*, 1996; Ezcurra *et al.*, 1999; Ezcurra *et al.*, 2000).

The ABRE is one of the best studied components of ABA signalling (Shen *et al.*, 1996; Busk & Pages, 1998). A typical ABRE contains an ACGT nucleotide core motif recognized by bZIP transcription factors (Hobo *et al.*, 1999; Choi *et al.*, 2000; Finkelstein & Lynch, 2000; Uno *et al.*, 2000). Two such factors that participate in the regulation of *SSP* genes are AtbZIP10 and AtbZIP25. These are structurally very closely related to OPAQUE2-like factors involved in the regulation of *SSP* genes in cereal species (Lara *et al.*, 2003). Interestingly, none of the major regulators of seed maturation (ABI3, FUS3, LEC1 and 2) correspond to a transcription factor that interacts with the ABRE boxes. However, the B3 domain, which is present in ABI3, FUS3 and LEC2 proteins, binds the RY element (Ezcurra *et al.*, 1999; Reidt *et al.*, 2000; Monke *et al.*, 2004). In combination with closely associated ABRE-like elements, this module acts as an enhancer of seed-specific transcription (Dickinson *et al.*, 1988; Suzuki *et al.*, 1997).

The Arabidopsis ABI5 bZIP transcription factor binds to the ABRE and activates ABA-mediated transcription in seeds. Genes down-regulated in *abi5* dry seeds showed an over-representation of the ABRE, suggesting that ABI5 contributes to the activation of ABRE-mediated transcription

(Nakabayashi *et al.*, 2005). ABI3, which activates RY element-mediated transcription, interacts physically with ABI5 (Hill *et al.*, 1996; Hobo *et al.*, 1999; Nakamura *et al.*, 2001), and this physical interaction appears to be important for the synergistic activation of gene expression.

III. After-ripening, transcription and translation during dry seed storage

Seed dormancy status increases during seed maturation and reaches a maximum in harvest-ripe seeds (Karssen *et al.*, 1983; Ooms *et al.*, 1993). During subsequent dry storage of the seeds (after-ripening), the dormancy status reduces until seeds are able to complete germination when imbibed under favourable conditions. The speed of after-ripening and dormancy status can vary, depending on environmental conditions during seed maturation, seed storage and germination conditions (Donohue *et al.*, 2005). The molecular mechanisms that decrease the dormancy status during after-ripening are not well understood, but several recent studies provide evidence of function at various levels, from RNA transcription to enzymic and nonenzymic reactions.

Dry mature seeds contain a large number of mRNA species. Stored mRNAs in mature dry seeds were first observed in cotton (Dure & Waters, 1965) and are apparently universal in flowering plant species (Ishibashi *et al.*, 1990; Kuligowski *et al.*, 1991; Almoguera & Jordano, 1992). The function of stored mRNAs remains unknown; however, it is thought that these are not only remnants from embryogenesis and seed maturation but also provide important RNA species for protein synthesis during the early stages of germination. It was shown that mRNAs of more than half of all genes (more than 12 000) are present in dry mature seeds (Nakabayashi *et al.*, 2005). Transcripts of all ontological categories were observed; however, the 2–3% (500 genes) highest expressed genes encode functions mainly associated with metabolism, and with protein synthesis and degradation. The ABRE was identified as being over-represented in the promoters of genes highly expressed in the dry seed transcriptome (Nakabayashi *et al.*, 2005). These authors also showed that genes representing mRNA stored in the seed are clustered on chromosomes rather than being dispersed randomly.

In an early study it was shown that genes in dry seeds are transcriptionally competent, but argued that they are probably not actively transcribed *in vivo* in a quiescent dry seed, as a result of the low overall moisture level (Comai & Harada, 1990). However, transient low-level transcription and translation of the beta-1,3-glucanase gene during tobacco seed after-ripening has been observed, leading to the release of dormancy (Leubner-Metzger, 2005). Further support for transcription in dry seeds came from transcriptomics and proteomics studies that compared freshly harvested dormant and after-ripened nondormant dry seeds. cDNA-AFLP analyses in *Nicotiana plumbaginifolia* (Bove *et al.*, 2005) and

barley (Leymarie *et al.*, 2007) identified several transcripts that increased in amount during after-ripening in dry seeds. These studies also showed that a higher number of transcripts decreased in abundance during after-ripening compared with the number that increased (Bove *et al.*, 2005; Leymarie *et al.*, 2007). Global transcript analysis in Arabidopsis using microarrays also showed that the expression level of 30 genes, including *DOG1*, decreased during after-ripening (Finch-Savage *et al.*, 2007). A proteomic analysis in Arabidopsis also identified several proteins that accumulated during the after-ripening of dry seeds (Chibani *et al.*, 2006). Most of these up-accumulated species corresponded to storage proteins or to proteins with metabolic functions, which may prepare the seed for germination but do not have an obvious role in the release of dormancy. In contrast to transcriptomic studies, proteomic analysis showed fewer proteins reduced in level during after-ripening in comparison to those that were increased (Chibani *et al.*, 2006). The observed decrease in abundance of specific transcripts and proteins in these studies is probably a result of specific processes occurring in the dry seed because the majority of transcripts and proteins do not show changes in level during after-ripening. Mechanisms responsible for these changes in gene expression are unknown but could play an important role in the release of dormancy. These results suggest that active transcription and translation are possible under low hydration conditions in seeds. In accordance with this, it has been shown for seeds from *Bromus tectorum* (Bair *et al.*, 2006) and *Lolium rigidum* (Steadman *et al.*, 2003) that dormancy loss by after-ripening slows down or is absent under very-low-humidity conditions. Under normal conditions transcription and translation probably do not take place throughout the whole dry seed but might be localized to specific areas with higher humidity content. [1]H-magnetic nuclear resonance micro-imaging showed that regions of local higher water content can exist in after-ripened tobacco seeds (Leubner-Metzger, 2005). An important goal of future research should be to investigate whether transcription and translation can indeed take place in such regions of the 'dry' seed. In fact, the use of the term 'dry' seed is problematic in studies of physiology following shedding, because seeds can exist in many states of partial imbibition, and therefore 'dry' may be better replaced with the term 'low-hydrated'. The observation of changes in gene expression in low-hydrated seeds is surprising, and associated data sets require careful analysis and interpretation. However, this intriguing possibility merits further investigation, particularly the relationship between hydration state and capacity for complex cellular reactions.

Nonenzymatic reactions are assumed to play a role in dormancy release by after-ripening, and recently Oracz *et al.* (2007) proposed a causal link between reactive oxygen species (ROS) production and after-ripening (Oracz *et al.*, 2007). They measured a doubling in the amount of H_2O_2 and a 50% increase of O_2^- during dormancy release in sunflower.

Furthermore, incubation of dormant seeds in methylviologen (a ROS-generating compound) released dormancy. Two-dimensional analysis of protein abundance, and analysis of oxidation status, showed that ROS caused carbonylation of a specific set of proteins (Oracz *et al.*, 2007). Oxidation of proteins can result in a modification of enzymatic or binding mechanisms, which can lead to a change in function. Therefore, the analysis of the function of the specifically carbonylated proteins in dry seeds may reveal roles in dormancy release.

IV. Regulation of germination potential following seed imbibition

The single-cell endosperm layer is an important regulator of germination potential

The Arabidopsis seed is composed of the embryo surrounded by a single layer of endosperm cells and a testa (Fig. 1a). The maternally derived testa surrounds the endosperm, and is dead following seed maturation (Debeaujon & Koornneef, 2000; Debeaujon *et al.*, 2000). Analysis of mutants defective in testa pigmentation or structure showed that these features are required for correct aging of seeds and for primary dormancy. It has been well established in several systems (including tomato, tobacco and lettuce) that the endosperm is an important regulator of germination potential (Hilhorst & Karssen, 1992; Bewley, 1997a,b; Kucera *et al.*, 2005), and in these species the endosperm must be breached for germination completion. Recently, it has also become clear that the endosperm in Arabidopsis is an important regulation point for germination potential. Arabidopsis germination consists of the two sequential phases of testa rupture and endosperm rupture (Fig. 1b) (Mansfield & Briarty, 1996; Liu *et al.*, 2005a; Müller *et al.*, 2006; Carrera *et al.*, 2007). The influence of the living endosperm layer on testa rupture is not yet clear, but might involve secretion of hydrolytic enzymes to weaken structures within the testa, or purely physical rupture caused by expansion of the endosperm following imbibition of after-ripened seeds. The endosperm has been proposed to be the primary determinant of seed dormancy in Arabidopsis (Bethke *et al.*, 2007). Bethke *et al.* (2007) showed that seeds remained dormant when the testa layer was removed from imbibed freshly harvested seeds of the dormant accession C24. Investigation of the influence of several hormones on endosperm-regulated dormancy showed that nitric oxide (NO) increased the germination potential and that NO acts upstream of GA signalling. It has also been shown that a signal originating from the embryo contributes to endosperm weakening, that this signal can be replaced by exogenous GA application and that endosperm weakening is controlled by the interaction between GA and ABA levels (Müller *et al.*, 2006). Other work has shown that GA is synthesized *de novo* in the embryo, and the key biosynthetic genes *GA3ox1* and *GA3ox2* are strongly

preferentially expressed in the axis of the imbibed embryo (Mitchum *et al.*, 2006).

A major feature associated with endosperm rupture by the expanding radicle is endosperm weakening. In several species endosperm weakening has been associated with the induction of cell wall remodeling enzymes (CWRE) (for overview see Kucera *et al.*, 2005), including endo-ß-mannanase (Bewley, 1997a), ß-1,3-glucanases (Leubner-Metzger, 2003), expansins (Chen & Bradford, 2000), xyloglucan endo-transglycosylase (Chen *et al.*, 2002), pectin methylesterase (Ren & Kermode, 2000), polygalacturonase (Sitrit *et al.*, 1999), arabinogalactans (van Hengel & Roberts, 2003) and others. In Arabidopsis an extensin-like gene (*AtEPR1*) was shown to be expressed at the micropylar end of the germinating seed, and expression was induced by the application of exogenous GA (Dubreucq *et al.*, 2000). It was postulated that expression of this gene may influence the strength of the micropylar endosperm cell walls, thus facilitating rupture by the embryo. Use of enhancer trap technologies (Liu *et al.*, 2005a) allowed the identification of Blue Micropylar End 3 (BME3, a GATA zinc finger transcription factor) as a positive regulator of germination (Liu *et al.*, 2005b). This gene is expressed in the embryo at the radicle tip, and loss of function resulted in seeds lacking endosperm rupture that could be restored by exogenous GA treatment, suggesting that BME3 function is required to facilitate endosperm rupture.

Analysis of endosperm-specific transcriptome data sets can provide valuable information about the regulation of expression of CWREs. The provision of transcriptome data sets specific to either imbibed embryo or endosperm is a valuable resource for seed researchers (Penfield *et al.*, 2006b). These data sets could be used to define genes showing enhanced expression in either the endosperm or embryo, providing clues as to functional categories expressed exclusively in either seed compartment (Penfield *et al.*, 2006b; Holdsworth *et al.*, 2008). For example, analysis of expression data sets derived from isolated embryo and endosperm tissues can be used to reveal which cell wall-associated genes are preferentially expressed in the endosperm (Supplementary material Table S1, Fig. 3, derived from Penfield *et al.*, 2006b). The majority of the 51 cell wall-related genes (as defined by the Genome Ontology (GO) cellular component 'cell wall') showing twofold or more higher expression in the endosperm than embryo, encode xyloglucan endotransglycosylase-related proteins ($n = 14$), pectin methylesterase-related proteins ($n = 6$) or expansins ($n = 2$) (Supplementary material Table S1, Fig. 3). In general, treatment of seeds with ABA or paclobutrazol (PAC, an inhibitor of GA synthesis) resulted in the down-regulation of expression of these 51 genes, particularly in those enriched 10-fold or more in endosperm compared with embryo (Fig. 3) (Penfield *et al.*, 2006b). These types of analysis can provide a useful indication of which CWREs should be targeted for further investigation, both in relation to the development of hormonal networks regulating
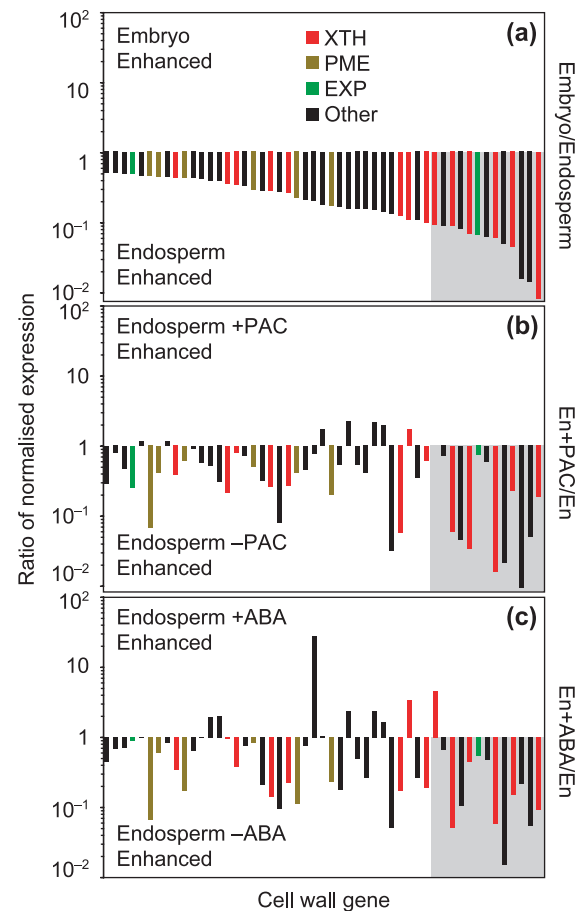

Fig. 3 Expression of endosperm-enhanced cell wall-associated genes during after-ripening and in response to exogenous abscisic acid (ABA) and paclobutrazol (PAC). Graphs show those genes classified as <GO-annotation of cellular component 'cell wall'>, that are twofold or more endosperm enriched (51 genes). (GO, Genome Ontology.) The ratio of normalized expression is shown for the following comparisons. The grey area represents those genes expressed at levels more than 10-fold higher in untreated endosperm than in embryo. Transcriptome data was obtained from seeds imbibed for 3 d at 4°C, followed by transfer to 22°C for 24 h. In this case the seeds had competed radicle emergence from the endosperm: (a) (Embryo/Endosperm) ratio of expression in the embryo compared with the endosperm. (b) (En+PAC/En) ratio of expression in endosperm from seeds treated with 20 µM PAC compared with expression in endosperm from untreated seeds. (c) (En+ABA/En) ratio of expression in endosperm treated with 20 µM ABA compared with endosperm from untreated seeds. Data modified from (Penfield *et al.*, 2006b). EXP, expansion; PME, pectin methylesterase; XTH, xyloglucan endotransglycosylase.

endosperm weakening and in defining those essential activities required for endosperm weakening. Many biochemical changes also occur in the endosperm of after-ripened seeds or moist chilled seeds (Mansfield & Briarty, 1996; Penfield *et al.*, 2006b; Bethke *et al.*, 2007). The ultrastructure of the mature endosperm of imbibed after-ripened seeds has been compared with that of the cereal aleurone (Bethke *et al.*, 2007), in that numerous oil bodies and protein storage vacuoles pack cells,

and it was shown that storage lipid differs in constitution from that of the embryo (Penfield *et al.*, 2004). Following imbibition, endosperm cells undergo defined changes in structure related to the use of stored reserves. Stored lipids and protein reserves are mobilized following imbibition first from endosperm cells adjacent to the radicle tip (Mansfield & Briarty, 1996), which may provide the energy and molecular building blocks for subsequent endosperm function. Ultrastructural changes (particularly vacuolation) were shown to be inhibited in seeds imbibed in the presence of very low levels of ABA (Bethke *et al.*, 2007), although in another study ABA was shown not to inhibit endosperm storage lipid breakdown (Penfield *et al.*, 2004). These studies may indicate the existence of both ABA-dependent and independent networks for endosperm-specific biochemical pathways in Arabidopsis. The transcription factor ABI5 was shown to be expressed in the micropylar endosperm, indicating that this positive regulator of ABA signalling may be involved in aspects of ABA-regulated endosperm function, although not in the breakdown of stored lipid (Penfield *et al.*, 2006b).

Repression of embryonic traits

Genetic evidence suggests that embryonic traits are actively repressed during and after germination. One gene involved in this process is *PICKLE* (*PKL*). *PKL* encodes a CHD3 chromatin remodelling factor that is suggested to repress genes which promote embryonic identity by acting in a GA-dependent manner (Fig. 2a) (Ogas *et al.*, 1997, 1999; Eshed *et al.*, 1999; Rider *et al.*, 2003). In *pkl* mutant plants, expression of *LEC1* and *LEC2* is substantially derepressed during germination (Ogas *et al.*, 1999; Rider *et al.*, 2003). In addition, *PKL* expression during germination was sufficient to repress expression of embryonic traits in the primary roots of mutant seedlings, whereas activation after germination had little effect (Li *et al.*, 2005), and *PKL* has been suggested to regulate stress responses following germination (Perruc *et al.*, 2007). However, *PKL* also acts at multiple points during embryo development as it is required for the repression of *PHERES1*, a type I MADS box gene that is normally expressed during early embryogenesis (Kohler *et al.*, 2003).

The *VP1/ABI3-LIKE* (*VAL*) family of B3-domain transcription factors, which form a sister clade to the *ABI3/FUS3/LEC2* family, is required for repression of the *LEC1*/B3 transcription factor network in germinating seedlings (Fig. 2a). It has been suggested that *VAL* factors target RY cis-element-containing genes in the network for chromatin-mediated repression in conjunction with the *PKL*-related CHD3 chromatin-remodelling factors. *LEC1* and *L1L* may be direct targets of *VAL* (Suzuki *et al.*, 2007). The *val* monogenic mutants are phenotypically similar to wild type; however, *val1/val2* double-mutant seedlings develop embryo-like proliferations in root and apical meristem regions. Like *pkl* (Ogas *et al.*, 1997), the *val1* embryonic seedling phenotype is

conditioned by inhibition of GA synthesis. When there is less GA, as was assessed in experiments using PAC, the *val1* monogenic seedlings exhibit embryogenic characteristics. This suggests that *PKL* and *VAL* function in a common pathway. The conditional embryonic seedling phenotypes of *val1* and *pkl* mutants implicate GA signalling in the repression of embryonic pathways. The *VAL* genes may indirectly regulate GA synthesis during seed development, as *AtGA3ox1*, one of the key 3-oxidase genes expressed during seed germination (Yamaguchi *et al.*, 1998; Mitchum *et al.*, 2006), is down-regulated more than 10-fold in the *val1/2* double mutant compared with wild type (Suzuki *et al.*, 2007). Simultaneous strong up-regulation in the double mutant of genes encoding earlier steps in GA biosynthesis is consistent with the loss of negative feedback regulation of the pathway by GA (Mitchum *et al.*, 2006). Two recently identified histone deacetylases (HDAC; *HDA6* and *HDA19*), which are suggested to redundantly regulate the repression of embryonic properties, show that chromatin remodelling is important for the control of seed development (Tanaka *et al.*, 2008). Histone deacetylase catalyses histone deacetylation, a phenomenon associated with transcriptional repression (Kadosh & Struhl, 1998; Rundlett *et al.*, 1998).

The role of hormones in the regulation of germination completion

Over the last few years many new molecular components associated with hormonal regulation of germination completion have been identified. In particular, many of the components associated with hormone signalling and synthesis or catabolism have been defined for GA and ABA, allowing a far greater understanding of the roles and interactions of these hormones in the imbibed seed. In addition, exciting new information is starting to emerge as to the role of auxin, previously not thought of as an essential hormone for germination in Arabidopsis. In this section the possible functions and interactions of these novel regulators are discussed.

The role of GA signalling and biosynthesis in germination completion Our understanding of GA function in relation to germination has been aided by the recent determination of key components of signalling, including the GID GA receptors (Nakajima *et al.*, 2006) and their interactions with DELLA-domain proteins (Sun & Gubler, 2004) and SCF[SLY1] (McGinnis *et al.*, 2003). All these components have been shown to influence germination potential. The family of Arabidopsis proteins containing a characteristic 'DELLA'-domain was originally defined following the cloning of GAI (GA insensitive) (Peng *et al.*, 1999), and is now known to consist of five members (GAI, RGA, RGL1, RGL2 and RGL3; Sun & Gubler, 2004) that are characterized by a conserved DELLA amino-acid motif. It is likely that they function as transcription-associated factors because they are

located in the nucleus and they repress GA responses during development (Sun & Gubler, 2004). Therefore, the removal of DELLA-domain function may be a requirement for the activation of GA-associated germination. RGL2 has been shown to have a major role in regulating germination potential, and *rgl2* mutant seeds are both insensitive to PAC repression of germination (Lee *et al.*, 2002) and nondormant (Carrera *et al.*, 2007). In addition, the removal of RGL2 function in a *ga1* mutant background restores germination, demonstrating that RGL2 is a repressor of germination in the absence of GA biosynthesis (Lee *et al.*, 2002; Tyler *et al.*, 2004). The expression of RGL2 RNA was shown to increase during imbibition (Lee *et al.*, 2002), although there is no correlation between germination time and level of RGL2 RNA (Lee *et al.*, 2002; Bassel *et al.*, 2004). RGL2 protein was shown to disappear rapidly following GA application to seeds (Tyler *et al.*, 2004), suggesting a discontinuity between RNA and protein levels. In *sly1* mutant seeds, RGL2 protein levels were not correlated with germination potential (Ariizumi & Steber, 2007), leading to the suggestion that RGL2 is inactivated by after-ripening. It is possible that a lack of correlation between germination and RGL2 levels in *sly1* mutant seeds reveals the window of function of GA during after-ripening. Once this window is passed, the requirement for RGL2 destruction no longer exists, highlighting the functionality of after-ripening as a process of changing 'windows' of sensitivity to external and internal signals (Finch-Savage & Leubner-Metzger, 2006). Other DELLA proteins have a minor influence on germination (Koornneef *et al.*, 1985; Cao *et al.*, 2005). A role for DELLA proteins in integrating environmental signals was also proposed, as seeds lacking all four functional DELLAs show light-independent and cold-independent germination (Cao *et al.*, 2005). In addition, it was shown that a correlation exists between the final size of seedling cotyledons and seed dormancy in DELLA mutants, leading to the suggestion that cotyledon size may be a major factor in determining germination potential (Penfield *et al.*, 2006a). As expected, seeds lacking GA receptors (with GID1A, GID1B and GID1C functions removed) fail to germinate (Griffiths *et al.*, 2006; Willige *et al.*, 2007).

Analysis of the function of GA biosynthesis in the regulation of germination completion has identified the key role of the enzyme gibberellin 3-oxidase (GA3OX). This enzyme is encoded by four genes (*GA3OX1–4*), and *GA3OX1* and *GA3OX2* have been shown to be important for GA-regulated germination (Yamauchi *et al.*, 2004; Mitchum *et al.*, 2006). Deletion of the function of both genes results in a marked reduction in germination potential, and both genes are expressed in the embryo during initial imbibition before germination, mainly in the axis of the embryo (Mitchum *et al.*, 2006).

The role of ABA signalling and biosynthesis in germination completion The role of ABA has classically been defined as that of a repressor of germination completion (Finch-Savage

& Leubner-Metzger, 2006). The function of ABA is intimately associated with dormancy induction and maintenance, but distinct from after-ripening induction (Carrera *et al.*, 2008). It has been shown that exogenous application of ABA to seeds does not result in seed phenotypes that mimic dormancy (Penfield *et al.*, 2004; Chibani *et al.*, 2006; Müller *et al.*, 2006; Carrera *et al.*, 2008). Nevertheless, the application of exogenous ABA to imbibed seeds can provide useful information concerning the function of removal of sensitivity to ABA in after-ripening. Thus, genetic screens have been used to identify components of ABA signalling associated with germination. Many loci have recently been identified that are involved in the removal of sensitivity to ABA function, which, when mutated, lead to ABA hypersensitivity of imbibed after-ripened or moist-chilled seeds (Hugouvieux *et al.*, 2001; Xiong *et al.*, 2001; Nishimura *et al.*, 2004, 2007; Katagiri *et al.*, 2005; Zhang *et al.*, 2005; Pandey *et al.*, 2006; Saez *et al.*, 2006; Yoine *et al.*, 2006). These loci encode diverse functions, including those associated with RNA translation and metabolism, protein-degradation pathways and phosphatase components of signalling pathways. The identification of loci encoding components of RNA translation is intriguing, given the potential importance of translation as a control mechanism for germination, and may indicate a relationship between ABA function and the regulation of translation of specific RNAs for controlling the completion of germination. The importance of targeted degradation of proteins is well documented for auxin and GA biology, and recently components associated with the specific degradation of ABA signalling proteins have started to be uncovered. An Arabidopsis mutant expressing altered RPN10 function (a component of the 26S proteasome) exhibited hypersensitive inhibition of seedling establishment in the presence of ABA (Smalle *et al.*, 2003). The E3 ligase ABI3-interacting protein 2 (AIP2; Kurup *et al.*, 2000) was shown to act as a regulator of germination and seedling establishment through the promotion of ABI3 degradation (Zhang *et al.*, 2005). Similarly, ABI5-binding protein (AFP) was shown to regulate the degradation of ABI5 (Lopez-Molina *et al.*, 2003) (Fig. 2b). Loss of function of both AIP2 and AFP led to a slight ABA hypersensitive germination phenotype. Several novel loci that contribute to the positive components of ABA signalling (i.e. those involved in the enhancement of dormancy and repression of germination completion) have recently been described. Loss of function of these components leads to ABA insensitivity of germination and nondormant seed phenotypes. Classic examples of this category include the ABA-insensitive *ABI1-8* loci (Rock, 2000). Novel functions recently identified include microRNA (miRNA) 159 (Reyes & Chua, 2007), and two protein kinases, namely SNF1-RELATED PROTEIN KINASE2.2 (SnRK2.2) and SnRK2.3 (Fujii *et al.*, 2007). The *snrk2.2/snrk2.3* double mutant showed strong ABA insensitivity for germination completion in comparison to wild-type and single-mutant

seeds. The discovery that genes activated by ABA and which contain the ABRE were down-regulated in the double mutant suggested that the mechanism of action of these kinases involved activation of ABRE-driven gene expression through the phosphorylation of ABRE-binding factors (ABFs) (Fujii *et al.*, 2007). A regulator of G-protein signalling (RGS) has been shown to have a role in Arabidopsis germination, and loss of function of this component of G-protein coupled signalling led to a slight reduction in the requirement for after-ripening and a small reduction in the sensitivity of germination completion to ABA (Chen *et al.*, 2006). Analysis of the influence of exogenously applied ABA on germination completion by mutant seeds has therefore allowed the definition of a large number of loci related to either activation or removal of ABA function. Further analysis of the relationship between the function of these loci and the regulation of dormancy loss, either through after-ripening or environment changes in the imbibed state, are required. These will allow a determination of how ABA function fits within the molecular networks integrating internal and external signals, resulting in commitment to complete germination or dormancy.

Recently, three publications have provided evidence for the existence of multiple ABA receptors (Razem *et al.*, 2006; Shen *et al.*, 2006; Liu, XG *et al.*, 2007a). In one case, although the FCA protein was shown to function as a receptor, no phenotype associated with germination was reported (Razem *et al.*, 2006). There is evidence that germination may be regulated by the heterotrimeric guanine nucleotide-binding protein (G protein) signalling pathway, and lack of G alpha and beta subunit (GPA1 and AGB1 respectively) and G-protein coupled receptor1 (GCR1) functions lead to very slight hypersensitivity to ABA and reduced seed dormancy, respectively (Colucci *et al.*, 2002; Ullah *et al.*, 2002; Pandey *et al.*, 2006). A protein identified as GCR2 was suggested to function as an ABA receptor in mature seeds (Liu, XG *et al.*, 2007a), although *gcr2* mutant seeds did not differ greatly in responsiveness to ABA in comparison to wild-type seeds, and subsequent reports suggest that this protein may not encode a receptor associated with germination responses (Gao *et al.*, 2007; Johnston *et al.*, 2007; Liu, XG *et al.*, 2007b). Similarly, seeds lacking the Mg-chelatase H subunit ABAR/CHLH showed some increased insensitivity to ABA repression of germination (Shen *et al.*, 2006), although perhaps not as great as would be expected if perception of ABA was lacking (for example in comparison to the level of ABA sensitivity shown by *abi1-1* mutant seeds Leung *et al.*, 1997). These data do not therefore provide unequivocal evidence that an ABA receptor responsible for the majority of imbibed seed ABA perception has been identified, and further work will be required to define the role of the identified receptors in germination.

The catabolism of ABA has been shown to be important in regulating germination potential (Kushiro *et al.*, 2004; Saito

et al., 2004; Okamoto *et al.*, 2006; Seo *et al.*, 2006). ABA 8′-hydroxylation was shown to play the predominant role in ABA catabolism encoded by the *CYP707A* gene family (*CYP707A1*–*CYP707A4*). The product of hydroxylation, 8′-hydroxy ABA, is converted to phaseic acid (PA) and subsequently inactivated. Therefore, the *CYP707A* family represents a key regulatory point for the removal of ABA. The *CYP707A2* gene was shown to be up-regulated following imbibition of after-ripened seeds before radicle emergence, and expression was increased in the presence of exogenous ABA during imbibition (Kushiro *et al.*, 2004). Freshly harvested seeds of two mutant alleles of *CYP707A2* showed reduced germination potential, whereas *cyp707a3* mutants did not. In addition, *cyp707a2* mutant seeds accumulated six times as much ABA during imbibition as wild-type seeds, indicating that this gene encodes the major function for ABA catabolism during imbibition (Kushiro *et al.*, 2004). A further study showed that the *CYP707A1* gene is responsible for reducing ABA accumulation during mid-seed maturation, whereas *CYP707A2* is responsible at both late maturation and seed imbibition time-points (Okamoto *et al.*, 2006). Localization of *CYP707A2* transcripts by *in situ* hybridization showed that this gene is expressed in both embryo and endosperm tissues during late maturation (Okamoto *et al.*, 2006), suggesting the importance of ABA breakdown in both tissues for the removal of dormancy. These studies provide important evidence demonstrating that the removal of ABA, in addition to removal of perception, is an essential component of after-ripening and the transition from dormancy to germination completion. Interestingly, an analysis of the expression and function of two genes encoding 9-cis-epoxycarotenoid dioxygenases (the key regulatory step in ABA biosynthesis; namely *NCED6* and *NCED9*) showed that one (*AtNCED6*) was expressed exclusively in the endosperm, whilst the other was expressed in both endosperm and embryo (Lefebvre *et al.*, 2006). Reduced dormancy was observed in *nced6/nced9* double-mutant seeds, but not in single mutants (Lefebvre *et al.*, 2006).

Newly identified functions for auxin in germination? Phytohormones other than ABA and GA have been shown to influence germination potential in Arabidopsis. For example, ethylene enhances germination completion (Kepczynski & Kepczynska, 1997; Leubner-Metzger *et al.*, 1998; Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000), and roles for brassinosteroids and cytokinin have been proposed (Steber & McCourt, 2001; Riefler *et al.*, 2006). Until recently the role of auxin in the regulation of germination remained obscure (Kucera *et al.*, 2005), but both genetic and transcriptomics approaches have provided evidence of the involvement of this hormone in both germination completion and seedling establishment (Ogawa *et al.*, 2003; Carrera *et al.*, 2007; Liu, PP *et al.*, 2007). Analysis of the expression of the DR5:GUS auxin reporter (GUS expression driven by the artificial DR5 auxin-responsive cis-element, used extensively to report auxin

distribution during development) indicated that auxin accumulates during embryogenesis and is present in the seed following imbibition. Expression was observed at the radicle tip before germination in one study (Liu, PP *et al.*, 2007) and throughout the embryo at the end of embryogenesis in another study (Ni *et al.*, 2001). Analysis of transcriptome expression showed that RNAs encoding the auxin transporters AUX1, PIN2 and PIN7 were highly up-regulated in response to treatment of *ga1* mutant seeds with GA (Ogawa *et al.*, 2003), and that both efflux and influx transporters are up-regulated in after-ripened seeds compared with dormant seeds (Carrera *et al.*, 2007). This may indicate a role for these transporters in germination per se, or with the establishment of the root apex and gravitropism following radicle emergence. Clearer genetic evidence of a role for auxin in germination has been obtained from an analysis of the regulation of *Auxin Response Factor10* (*ARF10*) by miR160 (Liu, PP *et al.*, 2007). miRNAs have been shown to down-regulate target genes at the post-transcriptional level and they play crucial roles in a broad range of developmental processes (Dugas & Bartel, 2004). It was shown that transgenic seeds expressing an miR160-resistant form of *ARF10* (*mARF10*) were hypersensitive to germination inhibition by exogenous ABA, whereas ectopic expression of miR160 resulted in a reduced sensitivity to ABA (Liu, PP *et al.*, 2007).

These results indicate a role of auxin in germination-associated pathways and suggest that interactions between auxin and ABA signalling pathways may contribute to the germination potential of seeds. An analysis of the function of key components of auxin signalling in relation to after-ripening and germination potential and vigour may reveal novel roles for auxin in these processes.

Interactions between hormones and the environment

Seed dormancy or germination outcomes are determined by a balance between pathways associated with ABA and GA, external environmental signals and internal developmental signals (Fig. 2). The signalling pathways of these hormones are interconnected at several levels and also interact with other hormones, such as ethylene and brassinosteroids, which both influence the ABA–GA balance by counteracting ABA effects and promoting germination. Several recent reviews have dealt extensively with these interactions (Brady & McCourt, 2003; Kucera *et al.*, 2005; Weiss & Ori, 2007). Interactions between ABA and auxin have recently also been identified (Liu, PP *et al.*, 2007). It is likely that the crosstalk between different hormone signalling pathways contributes to the flexibility of seeds in their responses to developmental and environmental factors. (Brady & McCourt, 2003; Chiwocha *et al.*, 2005; Kucera *et al.*, 2005; Weiss & Ori, 2007). Low temperatures and exposure to light are the major environmental factors that release seed dormancy and enable the completion of germination. In the last few years progress has been made

concerning the mechanisms of these environmental influences on the ABA and GA biosynthesis and catabolism pathways. Furthermore, nitrate has also been suggested to affect GA and ABA pathways (Alboresi *et al.*, 2005) and was shown to accelerate the decrease in ABA levels that occurs during seed imbibition (Ali-Rachedi *et al.*, 2004). However, unlike temperature and light, the role of nitrate has not yet been studied at the molecular level.

The GA biosynthesis gene *AtGA3ox1* is induced by stratification at 4°C in dark-imbibed seeds, and plants with a mutation in this gene (*ga4-2*) do not respond to cold treatment (Yamauchi *et al.*, 2004). By contrast, *AtGA2ox2*, which is involved in GA degradation, has decreased transcript levels at low temperatures. These changes in gene expression coincide with an increase in the level of GA at 4°C. In addition, low temperatures cause an increase in the number of cell types in which the *AtGA3ox1* transcript can be detected. The transcript could be detected in the entire embryonic axes and in the aleurone layer when seeds were cold treated, but not in the provasculature and aleurone at 22°C (Yamauchi *et al.*, 2004). Furthermore, Yamauchi *et al.* (2004) showed that red light and GA deficiency were also able to increase the amount of *AtGA3ox1* transcript, suggesting that multiple signals are integrated at this level. The up-regulation of GA biosynthesis genes by red light and reversion by far red light has previously been shown for both lettuce and Arabidopsis, indicating a photo-reversible regulation of GA biosynthesis by phytochrome (Toyomasu *et al.*, 1998; Yamaguchi *et al.*, 1998). Temperature and light also interact at the phytochrome level because the relative roles of the individual phytochromes are dependent on temperature. At higher temperatures (above 25°C), phyA is the most important for germination, at low temperatures (below 10°C) phyE is mainly important, whereas phyB is important for germination across a range of temperatures (Heschel *et al.*, 2007).

A role for the basic helix-loop-helix (bHLH) transcription factors SPATULA (SPT) and PIF3-like 5 (PIL5) was demonstrated in the regulation of *AtGA3ox1* and *AtGA3ox2* expression by the synergistic interaction of light and stratification (Penfield *et al.*, 2005) (Fig. 2b). SPATULA is required in dormant seed to maintain the repression of *AtGa3ox1* and *AtGa3ox2*, is stable in the light and mediates germination in response to temperature. PIF3-like 5 represses seed germination and *AtGA3ox1* and *AtGA3ox2* expression in the dark after stratification, but has no role in the stratification response itself (Penfield *et al.*, 2005). PIF3-like 5 interacts with the Pfr forms of PhyA and PhyB (Oh *et al.*, 2004), and red and far-red light signals promote seed germination by degradation of PIL5 by the proteasome (Oh *et al.*, 2006). In addition to repression of *GA3ox1* and *GA3ox2* expression, PIL5 also activates expression of the GA catabolic gene *GA2ox2* (Oh *et al.*, 2006). Recently it was shown that PIL5 also regulates the responsiveness to GA by increasing the expression of two DELLA genes – *GAI* and *RGA*/*RGA1* – in the dark by directly

binding to their promoters, but interestingly not that of *RGL2* (Oh *et al.*, 2007).

It has been shown that red light decreases endogenous ABA levels (Seo *et al.*, 2006). Expression of the ABA biosynthesis gene *AtNCED6* was suppressed by red light, and expression of the ABA inactivation gene *CYP707A2* was slightly increased by red light. Abscisic acid and GA metabolism are interconnected during seed germination because GA biosynthesis increased in the *aba2-2* mutant and ABA suppresses the biosynthesis of GA during seed germination (Seo *et al.*, 2006). Furthermore, ABA is synthesized more, and degraded less, in the *ga1* mutant compared with wild type, and the opposite is observed after application of GA. PIF3-like 5 has a role in ABA metabolism because it activates ABA biosynthetic genes and represses an ABA catabolic gene, irrespective of *de novo* GA biosynthesis. Environmental factors affect GA and ABA simultaneously as a result of their antagonistic regulation, regardless of the direct target hormone of the given environmental factor (Oh *et al.*, 2007).

V. Other factors influencing dormancy and germination

Most of the identified genes with a role in seed dormancy have a role in seed maturation or influence hormone metabolism or perception. During the last few years several genes have been identified for which this is not the case. These genes could represent downstream or upstream elements in the signal transduction pathways leading to the induction or release of seed dormancy. Natural variation for seed dormancy between the low dormant accession Landsberg *erecta* and the highly dormant accession from the Cape Verde Islands (Cvi) has been exploited in a quantitative trait locus (QTL) analysis for seed dormancy, which yielded several *DELAY OF GERMINATION* (*DOG*) QTLs (Alonso-Blanco *et al.*, 2003). The first of these, *DOG1*, has been cloned and encodes a protein with unknown function (Bentsink *et al.*, 2006). The expression of *DOG1* is seed-specific and disappears after imbibition of the seed. Loss-of-function mutant alleles of *DOG1* are completely nondormant and do not show obvious pleiotropic phenotypes, indicating that *DOG1* plays a crucial role in dormancy. The *dog1* mutant still requires GA for germination and has a normal sensitivity to applied ABA (Bentsink *et al.*, 2006). Elucidation of the DOG1 function should reveal novel aspects about the mechanisms underlying after-ripening and seed dormancy.

A screen for reduced dormancy has yielded four mutants, which are probably not involved in hormone perception or sensitivity (Léon-Kloosterziel *et al.*, 1996; Peeters *et al.*, 2002). The first of these, *HISTONE MONOUBIQUITINATION 1* (*HUB1*) was cloned and encodes a C3HC4 RING finger protein, involved in monoubiquitination of histone H2B (Liu, Y *et al.*, 2007). This histone modification is associated with actively transcribed genes, and transcription of several dormancy-related genes was reduced in *hub1* seeds (including *DOG1*). The Arabidopsis genome contains a homologue of *HUB1*, named *HUB2*. The *hub1* and *hub2* mutants are not redundant and show reduced dormancy and weak pleiotropic phenotypes (Liu, Y *et al.*, 2007). The identification of these genes has revealed a role for chromatin remodelling in seed dormancy. Interestingly PKL, also a chromatin remodelling factor, does not influence dormancy status when mutated (W. J. J. Soppe, unpublished).

The importance of the peroxisome for germination potential has been highlighted through the observation of the critical role of the ABC transporter COMATOSE (CTS) for the completion of germination (Footitt *et al.*, 2002, 2006). Loss of function of this protein, which is located on the peroxisomal membrane, results in a lack of germination that can only be overcome by removal of the surrounding endosperm/testa layers. Loss of function of other components of the fatty acid beta-oxidation pathway (including *kat2*) do not have such a severe effect on germination, although they do result in a lack of capacity of seedlings to establish in the absence of exogenously applied sugar (Footitt *et al.*, 2006). The mechanism through which CTS regulates germination potential is not known, although it has been shown to function after the requirement for GA (Russell *et al.*, 2000; Carrera *et al.*, 2007), and transcriptome analysis revealed genes whose expression was influenced by CTS function (Carrera *et al.*, 2007). It is possible that CTS facilitates the transport of an unknown molecule into the peroxisome that is required for either the activation or repression of germination. Further work is required to determine the full spectrum of endogenous compounds whose transport into the peroxisome is facilitated by CTS; this already includes 12-oxophytodienoic acid (OPDA; a precursor of jasmonic acid), fatty acids or Acyl-CoA derivates, acetate and indole butyric acid (IBA) (Theodoulou *et al.*, 2005; Baker *et al.*, 2006; Hooks *et al.*, 2007).

It is not clear for Arabidopsis whether activation of the cell cycle is important for germination, and two recent publications have reached contrasting conclusions in this respect (Barroco *et al.*, 2005; Masubelele *et al.*, 2005). In one study, transcriptome analysis was carried out in constant light at specific time-points following moist chilling, revealing increased expression of both A-type and D-type cyclins (*CYCA*, *CYCD*) before radicle emergence (Masubelele *et al.*, 2005). Loss-of-function alleles of *CYCD* loci activated early during germination resulted in reduced division activation and much reduced germination velocity. A second study that did not prechill seeds before analysis and assayed germination under a light–dark regime reached the opposite conclusion; DNA replication measured by flow cytometry of germinating seeds was shown to initiate at the onset of root protrusion, following the completion of germination. In addition, several methods used to analyse expression of cell cycle-related genes indicated that transcription was observed only after germination was

completed (Barroco *et al.*, 2005). Although these are apparently incompatible observations, differences in the treatment of seeds before and during the germination assay may have contributed to the observed differences.

VI. Regulation of genome expression in relation to dormancy and germination

Large changes in genome expression occur following imbibition

Several recent publications have studied expression of the Arabidopsis genome following imbibition of mature seeds, using genechip technologies that allow simultaneous monitoring of all genes within the genome, and using proteomics approaches. From these studies it has been possible to obtain important information about the timing of gene expression (Gallardo *et al.*, 2001; Masubelele *et al.*, 2005; Nakabayashi *et al.*, 2005), influence of environment (Cadman *et al.*, 2006; Finch-Savage *et al.*, 2007; Carrera *et al.*, 2008) and function of regulators (Gallardo *et al.*, 2002; Ogawa *et al.*, 2003; Rajjou *et al.*, 2004; Nakabayashi *et al.*, 2005; Cao *et al.*, 2006; Carrera *et al.*, 2007, 2008) associated with germination potential. These studies all indicate that for wild-type seeds there are very large changes in genome expression that are associated with the transition from dormancy to germination. A novel approach for annotating seed transcriptome data sets (termed 'TAGGIT') was developed to aid interpretation of genome expression in wild-type and mutant Arabidopsis seeds (Carrera *et al.*, 2007). Using the TAGGIT workflow it is possible to annotate transcriptome data sets by ascribing, to individual genes, functional categories (as tags) that have previously been associated with seed maturation, dormancy and germination (Fig. 4). This has an advantage over the commonly used GO-based annotation in that the associated 'tags' are much more biologically informative. In addition, the combined information regarding numbers of tagged genes in particular categories provides a 'developmental signature' for the particular transcriptome data sets under analysis. Signatures obtained from differential gene lists of dormant or after-ripened seeds are easily distinguishable and can be used in conjunction with principal component analysis (PCA) as a guide when interpreting the developmental status of the transcriptome in mutant or pharmacologically treated seeds (Carrera *et al.*, 2008).

Time course analysis of transcriptomes of seeds during imbibition before germination showed that gene-expression patterns could be grouped into six separate categories (Nakabayashi *et al.*, 2005). These groups included those genes that were either highly down-regulated (1414 genes) or up-regulated (133 genes) during the initial 24 h of imbibition. Major germination-associated changes in the transcriptome were evident within 6 h of the initiation of imbibition (Nakabayashi *et al.*, 2005; Holdsworth *et al.*, 2008). Specific
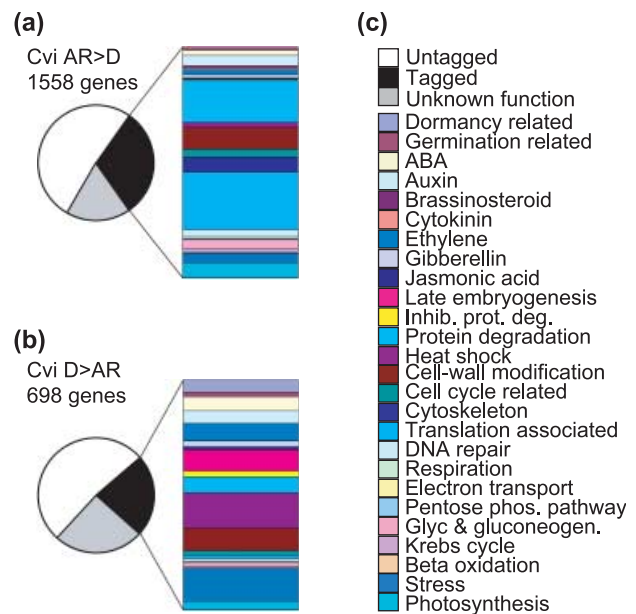

Fig. 4 Analysis of representation of functional categories in transcriptome data sets associated with dormant or after-ripened accession Cape Verde Islands (Cvi) seeds. Representation of functional groups is demonstrated using the TAGGIT approach (Carrera *et al.*, 2007), where proportional representation of functional groups, previously shown to be important for dormancy or germination, are tagged within differentially expressed gene sets. (a) Proportional representation of genes in the set D>AR (i.e. genes up-regulated by twofold or more in dormant compared with after-ripened seeds). (b) Proportional representation of genes in the set AR>D (i.e. genes up-regulated by twofold or more in after-ripened seeds compared with dormant seeds). (c) Key to colour representation of different functional groups. AR, after-ripened; D, dormant.

cohorts of transcripts have been shown to be up-regulated in seeds that will complete germination, when compared with seeds that will remain dormant (Cadman *et al.*, 2006; Carrera *et al.*, 2007, 2008). For example, using the TAGGIT approach it was shown that groups of genes encoding functions associated with cell wall remodelling, RNA translation and protein degradation are all more highly represented in seeds that will complete germination rather than dormant seeds (Fig. 4). Conversely, functional categories associated with embryo maturation (storage proteins, heat shock protein, dehydrins, ABA-induced proteins) are all preferentially expressed in dormant seeds (Fig. 4). Cadman *et al.* (2006) used transcriptomics approaches to assay genome expression following manipulation of dormancy levels of the Arabidopsis accession Cvi. Principal component analysis of data sets derived from seeds of different dormancy statuses demonstrated that whilst transcriptomes from all dormant categories were more similar to each other than to those of after-ripened seeds, each dormant category maintained its own individual characteristics. In particular, the transcriptome of seeds maintained in a primary dormant state for 30 d, and seeds in

a secondary dormancy status, were dissimilar to those of dormant seeds incubated for only 24 or 48 h. This suggests that prolonged imbibition of dormant seeds may induce a different transcriptional state that may be related to the conservation of energy or the expression of specific functions associated with prolonged or secondary dormancy states. A subsequent study (Finch-Savage *et al.*, 2007) has defined more closely the interactions between dormancy status and environmental factors that influence dormancy (including temperature, nitrate and light). This study showed that whereas expression of transcripts linked with ABA declined in association with dormancy relief, higher expression was observed for genes associated with nitrate accumulation and reduction, and higher expression of *GA3ox1* occurred in association with germination completion in the presence of light. A complementary approach was used to define gene networks regulated by the process of after-ripening per se (Carrera *et al.*, 2008). In this study, changes in genome expression were analysed in imbibed nondormant mutants (*aba1* and *abi1*) and compared with those changes in wild-type seeds (before and after storage of seeds) that were related to the length of time required for wild type after-ripening. The results obtained indicate that after-ripening acts as a developmental pathway that can be separated from dormancy of the imbibed seed. Thus, after-ripening was shown to downregulate *ABA1* (involved in ABA biosynthesis) and to upregulate *LPP2* (involved in removal of sensitivity to ABA Katagiri *et al.*, 2005). In addition, it was shown that exogenous addition of ABA did not re-impose a pre-after-ripening type genome expression, and that both *aba1* and *abi1* mutant seeds demonstrated changes in genome expression as a result of dry storage characteristic of after-ripening. Both observations indicate that ABA is not a major regulator of after-ripening in the dry seed.

The influence of germination regulators on the seed transcriptome

Hormonal and genetic regulators have been shown to influence genome expression (Ogawa *et al.*, 2003; Cao *et al.*, 2006; Carrera *et al.*, 2008). Identification of common and unique gene sets associated with regulators should provide an understanding of interactions between regulatory networks and may provide clues about how functions of regulators are controlled. Analysis of the effect of exogenous GA on the transcriptome of imbibed seeds of the nongerminating *ga1-3* mutant identified a set of 230 genes that were up-regulated in response to this hormone (which also results in germination of the mutant seeds) (Ogawa *et al.*, 2003). This gene set includes many of the genes up-regulated in after-ripened wild-type seeds, indicating that expression of these genes in the wild-type situation is regulated by endogenous GA. The influence of DELLA-domain-containing proteins in relation to GA was analysed by comparing transcriptomes of *ga1-3*

seeds with that of seeds of the *ga1-3/gai-t6/rga-t2/rgl1-1/rgl2-1* mutant (that do not require GA for germination) (Cao *et al.*, 2006). This study indicated that half of the GA-regulated genes were regulated in a DELLA-dependent manner, suggesting the existence of a DELLA-independent or DELLA-partially dependent component of GA-dependent germination-associated gene expression. Interestingly, proteomic analysis of germination showed that at this level GA does not appear to participate in processes associated with endosperm rupture (Gallardo *et al.*, 2002). This study analysed the influence of genetic (*ga1* mutant) and pharmacological (exogenous application of PAC) perturbation of GA synthesis following the initiation of imbibition. It was observed that only one protein, corresponding to the cytoskeleton component alpha-2,4 tubulin, required GA for expression. However, the abundance of several proteins was increased in the presence of GA in association with radicle protrusion (i.e. after germination *sensu stricto*). Genetic analysis of GA function has shown that in combination with testa mutants (such as *tt4* and *ttg1*) the requirement of *ga1* for GA is overcome (Debeaujon & Koornneef, 2000). However, double-mutant seeds still required light and chilling for radicle emergence, suggesting that GA is required to overcome the constraints of the testa, but also that under specific conditions, for example when the testa layer has a compromised function, GA is not essential for germination.

Analysis of the influence of GA and the ABC transporter, CTS, on the seed transcriptome identified transcripts that were both coregulated and independently regulated (Carrera *et al.*, 2007). This study also demonstrated the independent requirement for CTS function for the induction of structural and regulatory genes of the flavonoid biosynthesis pathway before germination. This investigation revealed that although *cts-1* mutant seeds do not germinate, transcriptome expression following the after-ripening of mutant seeds is very similar to that of wild-type seeds. Transcriptome analysis, in conjunction with genetic investigation of the epistasis of CTS and GA signalling, suggests that CTS functions later in pathways leading to germination. In addition, it was shown that CTS expression is required for the activation of expression of both *AtGA3ox1* and *AtGA3ox2* (Carrera *et al.*, 2007). However, analysis of protein profiles of imbibed *cts* seeds demonstrated that these do not change substantially over 7 d of imbibition (Russell *et al.*, 2000). This may indicate that these mutant seeds are stalled at the point of translation of the transcriptome, and therefore even though normal after-ripening occurs at this level, this does not result in seed germination because transcriptome changes are not reflected in changes in protein expression and function.

Comparison of the seed transcriptome and proteome

Extrapolating from transcriptomics experiments with regard to changes in proteome function should be viewed with

caution because it has been shown that whereas RNA translation is required for the completion of germination, transcription is not (Rajjou *et al.*, 2004) (see below). In addition, comparison of the several transcriptome and proteome studies using common sampling points and environmental conditions have indicated little correlation between functional classes of genes induced at the transcriptome and proteome levels (Gallardo *et al.*, 2002; Ogawa *et al.*, 2003; Chibani *et al.*, 2006; Carrera *et al.*, 2007). It is possible that changes in RNA abundance in seeds that will complete germination may be associated with vigour of germination, with subsequent functions associated with seedling establishment (i.e. translation occurs after germination completion), or that proteomics approaches are not yet sensitive enough to allow observation of changing protein profiles. It is not yet clear whether changes in dormancy status also principally rely on translational or post-translational control (Holdsworth *et al.*, 2008). Interestingly, there was little commonality between lists of genes shown to be regulated in imbibed seeds by after-ripening obtained from either transcriptomics (Cadman *et al.*, 2006) or proteomics (Chibani *et al.*, 2006) approaches, indicating the importance of study of gene expression at all levels in understanding molecular networks regulating germination. Clearly a key objective of future post-genomics studies will be to integrate information coming from transcriptomic and proteomic studies. Fortunately, for some studies analyses at proteome and transcriptome levels have been carried out under similar growth conditions and assay times, greatly helping an integrated interpretation of results from different studies (Cadman *et al.*, 2006; Chibani *et al.*, 2006; Carrera *et al.*, 2008).

Proteomics studies of germination have provided detailed information about the abundance of specific functional classes of proteins that are associated with after-ripening, dormancy and germination. Several studies have analysed proteome changes associated with germination and dormancy status (Gallardo *et al.*, 2001, 2002; Job *et al.*, 2005; Chibani *et al.*, 2006; Oracz *et al.*, 2007), and in this respect seed biology is at the forefront of plant proteomics. These studies demonstrate changes in the abundance of many proteins in both the dry seed during storage and following imbibition, but also demonstrate the importance of post-translational modifications for expression of the proteome. Studies have demonstrated change in abundance of proteins associated with imbibition before germination and radicle emergence (Gallardo *et al.*, 2001), and changes related to after-ripening status of imbibed seeds (Chibani *et al.*, 2006). An important analysis of the role of transcription and translation in germination (Rajjou *et al.*, 2004) concluded that only translation was essential for germination, as treatment of transparent testa (*tt*) mutant seeds with alpha-amanitin did not block germination potential. By contrast, cycloheximide was shown to be a potent inhibitor of radicle emergence. However, although alpha-amanitin-treated seeds showed a high

germination potential, germination was slower and showed a reduced sensitivity to exogenous GA, indicating that transcription may be important for the expression of genes related to the speed and synchronicity of germination completion. In addition the abundance of several key germination/establishment-associated proteins (including malate synthase; Eastmond *et al.*, 2000) was reduced in treated seeds.

Protein profiles differed greatly between imbibed dormant and after-ripened seeds of the accession Cvi (Chibani *et al.*, 2006). Quantification of [^{35}S]methionine incorporation in dormant and after-ripened imbibed seeds showed that the lack of germination of dormant seeds was not caused by the lack of *de novo* protein synthesis because the levels of incorporation were similar in both seed states. As shown at the transcriptome level (Carrera *et al.*, 2008), the application of exogenous ABA to imbibed after-ripened seeds greatly reduced the germination potential (Chibani *et al.*, 2006), but did not mimic dormancy at the proteome level. Incorporation of [^{35}S]methionine was higher in imbibed after-ripened seeds incubated in the presence of ABA than in dormant or untreated after-ripened seeds, suggesting that ABA treatment does not repress RNA translation, and the abundance of several proteins (including some seed storage proteins) was increased following treatment. At both -omics levels these results show that ABA treatment inhibits germination by a separate pathway to that of dormancy, an observation that is unsurprising because at the physiological level ABA has been shown only to delay germination, not reduce potential (Müller *et al.*, 2006). It is likely that ABA treatment of ungerminated seeds mimics stress responses associated with increased ABA synthesis (such as drought or salinity), and these responses have been studied in seedlings following germination (Lopez-Molina *et al.*, 2001; Lopez-Molina *et al.*, 2002).

VII. Conclusions

As with many areas of plant science, seed biology is at a crossroads, as genetic and physiological information are integrated with novel high-throughput genomics-based approaches. With the advent of genomics and, more recently, postgenomics technologies, the underlying molecular and biochemical processes contributing to the control of germination potential are becoming increasingly uncovered and understood. In particular, the provision of 'benchmark' transcriptomics and proteomics data sets associated with dormancy, germination and after-ripening (Nakabayashi *et al.*, 2005; Cadman *et al.*, 2006; Chibani *et al.*, 2006; Carrera *et al.*, 2008) provide a framework for the initiation of 'systems' approaches, linking developmental outcome (different dormancy statuses or completion of germination) with gene networks. However, before these types of approaches can be seriously undertaken, many important unresolved questions, associated with gene expression and function, remain. For example, it has been

observed that translation may be the major level of control of germination completion (Rajjou *et al.*, 2004).

In relation to the sequential developmental timing of functions required for the induction of dormancy, networks of transcription factors controlling aspects of seed maturation are now well understood (To *et al.*, 2006). Future investigations focussed on downstream processes resulting from these networks, including identification of target genes, should help to define how these networks influence the establishment of dormancy. The role and mechanism of after-ripening remains perhaps the most poorly understood phenomenon associated with seed behaviour, although several recent publications have started to reveal important new information about this process (Chibani *et al.*, 2006; Oracz *et al.*, 2007; Carrera *et al.*, 2008). It appears possible that after-ripening is distinct from ABA function in the establishment of dormancy during maturation, and therefore identification of the molecular components associated with after-ripening will be an important goal. In this respect it will be important to establish the relationship between after-ripening and loci identified through QTL analysis of the dormancy status in Arabidopsis (Alonso-Blanco *et al.*, 2003).

A seed, of course, represents a whole organism, where both embryo and endosperm (and cell types within these compartments) have very different roles. Therefore, an understanding of thet cell and tissue specificity of gene expression will be of major importance, in particular in relation to the role of the endosperm, which may provide the major determinant of germination completion (Bethke *et al.*, 2007) and speed (Müller *et al.*, 2006). Whereas this is becoming possible at the RNA level (through techniques such as laser micro-dissection), it remains a daunting challenge at the proteome and metabolome levels. In addition to simple analysis of gene expression at whatever level, the relative roles of different seed compartments, and integration of these roles with environmental information through hormone function, are key determinants of germination potential. Development of our understanding of how different internal and external signals provide cues leading to different responses of the molecular networks within seed compartments will be the focus for seed researchers for the foreseeable future.

Acknowledgements

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Supplementary Material

The following supplementary material is available for this article online:

Table S1 Normalized expression values and ratios of expression of Genome Ontology (GO) annotation <cell wall> genes, that were enhanced twofold or more in the endosperm compared with the embryo

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/ j.1469-8137.2008.02437.x
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