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## ARABIDOPSIS: A RICH HARVEST 10 YEARS AFTER COMPLETION OF THE GENOME SEQUENCE

# Arabidopsis seed secrets unravelled after a decade of genetic and omics-driven research

Helen North<sup>1</sup>, Sébastien Baud<sup>1</sup>, Isabelle Debeaujon<sup>1</sup>, Christian Dubos<sup>1</sup>, Bertrand Dubreucq<sup>1</sup>, Philippe Grappin<sup>2</sup>, Marc Jullien<sup>2</sup>, Loïc Lepiniec<sup>1</sup>, Annie Marion-Poll<sup>1</sup>, Martine Miquel<sup>1</sup>, Loïc Rajjou<sup>2</sup>, Jean-Marc Routaboul<sup>1</sup> and Michel Caboche<sup>1,\*</sup>

<sup>1</sup>INRA, Seed Biology Laboratory, Institut Jean Pierre Bourgin (IJPB), UMR 204 INRA/AgroParisTech, 78026 Versailles CEDEX, France, and

<sup>2</sup>AgroParisTech, Seed Biology Laboratory, Institut Jean Pierre Bourgin (IJPB), UMR 204 INRA/AgroParisTech, 78026 Versailles CEDEX, France

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\*For correspondence (fax +33 1 30 83 31 11; e-mail caboche@versailles.inra.fr).

## SUMMARY

Seeds play a fundamental role in colonization of the environment by spermatophytes, and seeds harvested from crops are the main food source for human beings. Knowledge of seed biology is therefore important for both fundamental and applied issues. This review on seed biology illustrates the important progress made in the field of Arabidopsis seed research over the last decade. Access to 'omics' tools, including the inventory of genes deduced from sequencing of the Arabidopsis genome, has speeded up the analysis of biological functions operating in seeds. This review covers the following processes: seed and seed coat development, seed reserve accumulation, seed dormancy and seed germination. We present new insights in these various fields and describe ongoing biotechnology approaches to improve seed characteristics in crops.

## INTRODUCTION

In the 1970s, Arabidopsis was not considered an attractive plant species for seed biology studies. Monocots such as barley (the model for the aleurone layer that produces  $\alpha$ -amylase) or maize (kernel mutants affected in deposition of storage compounds) were considered more appropriate. In dicots, a number of species were used to study seed biology (azuki beans, cottonseeds, pea, etc.), but none of these were selected as the reference. This often led to the useless duplication of work on different species. Arabidopsis seed research was started by pioneers such as M. Koornneef and A. Muller. Maarten took a genetic approach to study the role of abscisic acid (ABA) and gibberellins (GA) in seed biology, and Andreas studied *EmbFusca* mutants. Nevertheless, a number of seed biologists hesitated to use Arabidopsis for their research, expecting difficulties performing biochemical studies on such small seeds. These difficulties were overcome by the availability of efficient analytical tools such as MS equipment. However, the major force that drove the seed community to work on Arabidopsis was access to 'omics' resources, which enabled molecular genetic approaches on seeds to blossom. These resources

have continued to be exploited over the past decade, despite experimental difficulties resulting from gene duplications/redundancies (e.g. identification of the GA receptor), and will obviously continue to be valuable. However, the study of seed biodiversity, an important topic in terms of evolution, cannot be restricted to the analysis of intra-specific natural variation in Arabidopsis. It is time to look at biological diversity in other seed-producing plants in order to understand what makes them unique in terms of biochemical composition, tolerance to adverse environmental conditions, germination features, competition with other plants, and many other processes. Here we review the present status of Arabidopsis seed research and its biotechnological impact. This research will provide a valuable foundation for studies in other species.

## SEED GENOMICS

Access to the sequence of the Arabidopsis genome has dramatically changed our approach to the study of plant biology. Although the number of genomic resources specifically devoted to seed biology is small, the study of seeds has

been enormously stimulated by the availability of various Arabidopsis genomic resources. The structural annotation of the Arabidopsis genome has provided a basis for gene classification into functional categories. Transcriptome analysis studies have identified which members of these gene families are expressed in seeds (reviewed by Nakabayashi *et al.*, 2005) and meta-analysis tools such as Genevestigator (<http://www.genevestigator.com/>) and BAR eFP (<http://www.bar.utoronto.ca/efp/>; Bassel *et al.*, 2008) have been used to identify co-expressed genes that possibly contribute to the same biological function in seeds. BAR eFP is particularly suitable for the study of seed dormancy and germination. Transcriptome analyses were performed to study various seed developmental processes, such as transition from cell division to cell enlargement in the embryo (Ruuska *et al.*, 2002). Proteomic approaches have provided valuable information on the accumulation of various classes of proteins in seeds (Gallardo *et al.*, 2001). The seed proteome database contains data for mature, dormant, non-dormant and germinating seeds (<http://www.seed-proteome.com/>; Rajjou *et al.*, 2008). Comparison of transcriptome and proteome data has revealed the extent of post-transcriptional regulation during various phases of seed development, as illustrated for biotin metabolism (Chen *et al.*, 2009). Despite dry seeds being in a metabolically quiescent state with relatively low water content, transcriptome and proteome changes have been found to occur (Holdsworth *et al.*, 2008). In addition to seed storage proteins (SSPs), three functional categories of proteins (energy metabolism, protein metabolism and stress responses) were predominant in dry seeds and synthesized during the early stages of seed germination, as well as being induced by seed priming.

Collections of insertion mutants such as SIGNAL, GABI-KAT, FLAGdb, CHSLTrapperdb and RIKEN activation lines have been exploited for functional analysis of seed-expressed genes using reverse genetic approaches. An inventory of seed-defective mutants was performed by the SeedGenes project (<http://seedgenes.org/>; Tzafrir *et al.*, 2004). Tagged mutants in 358 genes and 1400 non-tagged seed mutants have been found, of which a large proportion are embryo-lethal.

Metabolomic approaches have also been used for Arabidopsis seeds as part of more global projects such as the Golm metabolome database (<http://csbdb.mpimp-golm.mpg.de/>) or the PRIME platform from RIKEN (<http://prime.psc.riken.jp/>). Metabolome studies targeted to the seed maturation and desiccation phases (Ruuska *et al.*, 2002; Fait *et al.*, 2006) have been used to build models of metabolic networks operating during seed development and maturation. Other systems biology approaches are being developed for the modelling of seed dormancy and germination (Penfield and King, 2009; vSEED project, <http://www.erapg.org/everyone/16790/18613/19533/19546>).

## SEED DEVELOPMENT

### Control of seed formation and imprinting

Seeds are chimeras. The double fertilization process occurring in angiosperms leads to three genetically distinct seed tissues: the seed coat of maternal origin, the triploid endosperm (with two maternal and one paternal genome equivalents) and the diploid embryo (Berger *et al.*, 2006). During the maturation phase, embryo growth and cell-cycle activities stop. The seed coat or 'testa' arises from the two ovule integuments. Development of the inner integument results in three cell layers, and the outer integument is two-layered. These five cell layers follow specific fates, accumulating various compounds (e.g. mucilage or flavonoids) and/or undergo programmed cell death (Haughn and Chaudhury, 2005; Lepiniec *et al.*, 2006). The triploid endosperm develops initially as a syncytium, followed by cellularization and differentiation events. Interestingly, seed formation from the ovule (i.e. testa and endosperm development) can be triggered in the absence of true fertilization, and therefore in the absence of an embryo, as observed in *fis* (fertilization-independent seed) mutants (Kohler and Makarevich, 2006; Huh *et al.*, 2008).

Imprinting has recently been shown to play an important role in the endosperm. The FIS proteins, namely FIS1/MEA (MEDEA), FIS2, FIS3/FERTILIZATION-INDEPENDENT ENDOSPERM and the WD repeat (WDR) protein MSI1, belong to a Polycomb complex homologue of animal PRC2 (POLYCOMB REPRESSIVE COMPLEX 2) that represses central cell proliferation until fertilization (Huh *et al.*, 2008). This complex is involved in histone methylation and the control of gene imprinting, leading to differential expression of maternal versus paternal alleles in the endosperm. Furthermore, *fis* mutations cause parent-of-origin effects on seed viability, and seeds defective in the maternal allele cannot form normal endosperm or embryos (Chaudhury *et al.*, 1997). However, embryo development can be rescued by fertilization with pollen that has a hypomethylated genome. Taken together, these results suggest that a functional FIS complex is required for normal female gametophyte and seed development.

Imprinting in the endosperm is a sophisticated mechanism (Huh *et al.*, 2008; Jullien and Berger, 2009). In addition to FIS/PRC2-mediated histone methylation, it also involves DNA methylation by MET1 (a DNA methyltransferase) and demethylation by DEMETER (DME), a DNA glycosylase. FIS2 and MEA are themselves imprinted genes, and DME activates maternal alleles of these genes in the central cell. DME is also involved in the demethylation of many other genes, including a set of newly discovered imprinted genes (Hsieh *et al.*, 2009). In addition, the paternal allele of MEA is repressed by PRC2 in the endosperm. Retinoblastoma-related cell-cycle regulators also regulate the expression of MET1 and PRC2 members (Johnston and Gruissem, 2009;

Jullien and Berger, 2009). Finally, a link has been demonstrated between imprinting and siRNA accumulation in the endosperm (Hsieh *et al.*, 2009; Mosher *et al.*, 2009). Interestingly, seeds containing a uniparental diploid endosperm (without paternal fertilization) can produce a viable diploid (fertilized) embryo in a *fis* background (Nowack *et al.*, 2007). This result suggests that endosperm imprinting is necessary to prevent parthenogenic development, thus promoting 'sexualization' of the female gametophyte.

### Seed size control

Seed size control is a rapidly expanding research area with practical applications in crop breeding for yield. Cell number appears mainly to be controlled by maternal factors, whereas cell size is mostly non-maternally controlled (Alonso-Blanco *et al.*, 1999). Characterization of various mutants supports the hypothesis that endosperm development controls seed size through various pathways (Berger *et al.*, 2006). Crosses between *Arabidopsis* accessions with different ploidies have demonstrated that maternal to paternal genome dosage is critical for normal seed development (Dilkes *et al.*, 2008). These observations illustrate the 'parental conflict' hypothesis. Higher paternal ploidy levels result in low viability of  $F_1$  hybrids. Interestingly, *TTG2* (*TRANSPARENT TESTA GLABRA 2*), a gene involved in the regulation of integument cell elongation, contributes to this lethality (Dilkes *et al.*, 2008). Reduced seed size in *ttg2* mutants is also a maternally controlled trait, suggesting that seed size control involves cross-talk between the endosperm and testa. Characterization of *haiku* (*iku*) mutants, which produce viable seeds with a reduced size, supports the hypothesis that endosperm growth controls seed size (Garcia *et al.*, 2005). In addition, genes such as *MINISEEDS3* (*MINI3*) (Luo *et al.*, 2005), *APETALA2* (*AP2*) (Jofuku *et al.*, 2005) and *AUXIN RESPONSE FACTOR 2* (*ARF2*) (Schruff *et al.*, 2006) have been found to control seed size by apparently unrelated mechanisms, suggesting that saturation mutagenesis for seed size has not yet been achieved. Organ size control is therefore a rapidly expanding research area that may also lead to practical applications in crop breeding for yield.

### The genetic control of seed maturation

A few master regulatory genes, namely *LEC1* (*LEAFY COTYLEDON 1*), *LEC2* and *FUS3* (*FUSCA 3*) have been shown to act as key transcriptional regulators of seed maturation, together with *ABI3* (*ABSCISIC ACID INSENSITIVE 3*) (Figure 1) (for reviews, see Braybrook and Harada, 2008; Santos-Mendoza *et al.*, 2008; Suzuki and McCarty, 2008). Accordingly, the corresponding mutants share overlapping and pleiotropic phenotypes, such as a reduced level of storage compounds, a lack of desiccation tolerance, or precocious germination. Interestingly, mutant cotyledons display various similarities with young leaves. Together,

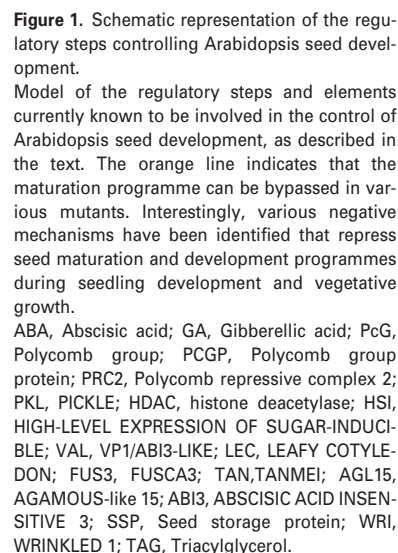
these phenotypes suggest that the regulators act by conferring embryonic competence to egg-derived cells. *TANMEI* (*TAN*), a protein with a WDR domain whose precise function is unknown, has overlapping roles with the proteins encoded by these genes during seed formation (Yamagishi *et al.*, 2005). *LEC2*, *FUS3* and *ABI3* encode plant-specific transcription factors that possess a 'B3' DNA-binding domain, and *LEC1* displays some similarities with a CAAT box-binding factor subunit. In recent years, genetic and molecular analyses have demonstrated that these regulatory proteins belong to a complex 'AFL' (for *ABI3/FUS3/LEC2*) network of local and redundant pathways that partially interact with other factors as well as with sugar and hormonal signalling (Figure 1) (e.g. GA, ABA and auxin; see Gazzarrini *et al.*, 2004). Furthermore, the AFL complex directly triggers the expression of genes encoding SSPs and proteins embedded in the half unit-membrane surrounding oil bodies (Baud and Lepiniec, 2009). Other important regulators of *SSP* genes have been identified including, *ABI4* [an *APETALA 2* (*AP2*) family protein] or various bZIPs (e.g. *ABI5* or *ENHANCED EM LEVEL*) acting in the same signalling pathway, but downstream of *ABI3* (Alonso *et al.*, 2009). Transcriptional activation of the fatty acid biosynthetic network appears to involve additional factors such as *WRINKLED 1* (*WRI1*), a member of the *AP2*-ethylene response element binding factor family (Cernac and Benning, 2004). *WRI1* specifies the regulatory action of *LEC2*, and possibly other master regulators, in this metabolic pathway (Figure 1). Interestingly, the AFL network can be repressed in seedlings through a variety of pathways, for example involving *PICKLE*, an ATP-dependent chromatin remodeller, several histone deacetylases, or the *VP1/ABI3-LIKE* (*VAL*)/*HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE 2* (*HSI2*) family of transcription factors (Zhang and Ogas, 2009).

### SEED COAT DIFFERENTIATION AND FUNCTION

The seed coat protects the embryo against biotic and abiotic stress, and has an impact on seed dormancy and germination. Three types of molecules that contribute to the physiological characteristics of the seed coat have been studied more extensively: flavonoids, mucilage and lipid polyester derivatives. Significant progress has been made over the past decade to elucidate the corresponding biosynthesis pathways.

#### Flavonoid biosynthesis

Flavonoids are plant secondary metabolites derived from the phenylpropanoid pathway (reviewed by Lepiniec *et al.*, 2006). In *Arabidopsis* seeds, they reinforce longevity and coat-imposed dormancy. *Arabidopsis* seeds accumulate two types of flavonoids during their development, namely proanthocyanidins (PAs) or condensed tannins, and flavonols. PAs are located specifically in the seed coat. In contrast, flavonols are present in the seed coat, the embryo and the



shown to be encoded by the *TT15* gene (DeBolt *et al.*, 2009), suggesting a membrane function for sterylglucosides in trafficking lipid polyester precursors.

## Mucilage biosynthesis

Mucilage pectins are synthesized in the Golgi and deposited in a polarized manner in the apoplasm via fusion of Golgi-derived secretory vesicles with the plasma membrane



(Young *et al.*, 2008). Mutants affected in mucilage accumulation or release have enabled identification of a number of genes required for pectin biosynthesis. A UDP-rhamnose synthase is required for synthesis of the rhamnogalacturonan I backbone, which is initially produced with galactan and arabinan ramifications; their trimming modifies mucilage hydroscopic properties (Arsovski *et al.*, 2009 and references therein). Further pectin modifications such as methyl esterification, regulated by a subtilisin-like serine protease, AtSBT1.7, may also modify mucilage properties or the outer primary cell wall to allow mucilage release (Rautengarten *et al.*, 2008).

#### Genetic control of flavonoid and mucilage biosynthesis

Biosynthesis of seed flavonoids and mucilage is tightly regulated and coordinated with cell differentiation at the spatial and developmental levels. Flavonol biosynthesis is controlled by three R2R3-MYBs (i.e. MYB11, MYB12 and MYB111), that specifically regulate expression of early biosynthetic genes (Stracke *et al.*, 2007). Using *tt(g)* mutants, six transcription factors affecting PA biosynthesis have been identified (Lepiniec *et al.*, 2006). TT2/MYB123 (R2R3-MYB), TT8/bHLH42 and TTG1 (WDR protein) form an MYB–BHLH–WDR (MBW) complex that controls expression of the late biosynthetic genes, *TT8* (self-activated feedback loop) and *TTG2* (Gonzalez *et al.*, 2009). Interestingly, in a recent study, MYBL2 (R3-MYB) was identified as a putative negative regulator of PA biosynthesis (Dubos *et al.*, 2008), and was proposed to act at the post-transcriptional level by inhibiting the activity of the MBW complex.

Several regulators have been identified that control seed coat epidermal cell differentiation and certain genes encoding mucilage biosynthesis enzymes. ENHANCER OF GLABRA 3 (EGL3)/bHLH001 and TT8 are partially redundant, and can form MBW complexes with MYB5 and TTG1. These complexes control epidermal cell differentiation through transcriptional regulation of three other genes encoding transcription factors (Gonzalez *et al.*, 2009; Li *et al.*, 2009 and references therein), namely *TT8*, *TTG2* and *GLABRA 2* (HD-Zip protein). MYB61 is also involved in this process, but in a TTG1-independent manner. Recently, a putative WDR transcription factor MUM1 (MUCILAGE MODIFIED 1) has been shown to control expression of *MUM2* (encoding a  $\beta$ -galactosidase) (J. Huang and G. Haughn, Department of Botany, University of British Columbia, Vancouver BC, Canada, personal communication).

#### Suberin and cutin biosynthesis

Two distinct types of insoluble polymers essentially derived from lipid polyester are present in mature seed: cutin and suberin. They contribute to the permeability barriers in the cuticle of the outermost integumentary layer and the chalazal plug formed at the end of seed maturation, respectively (reviewed in Pollard *et al.*, 2008).

Recently, the functions of GPAT5 (acyl CoA:glycerol-3-phosphate acyltransferase), CYP86B1 (fatty acid  $\omega$ -hydroxylase), DAISY (fatty acid elongase) and TT15, which participate in seed suberin synthesis, have been characterized (Compagnon *et al.*, 2009 and references therein; DeBolt *et al.*, 2009).

#### SEED STORAGE RESERVE ACCUMULATION

During the maturation process, Arabidopsis seeds accumulate triacylglycerols (TAGs) and SSPs in zygotic tissues of the seed. The embryo constitutes the major site of reserve deposition. In dry seeds, embryo cells are packed full of protein storage vacuoles (PSVs) and oil bodies. SSPs or TAGs are accumulated in these structures, respectively, and each account for 30–40% of the seed dry weight. While hormone (ABA:GA ratio) and sugar metabolism (glucose:sucrose ratio, trehalose-6-phosphate levels) are known to trigger seed maturation and influence the accumulation of seed storage compounds, the regulatory mechanisms involved remain to be elucidated (Gomez *et al.*, 2006). Seeds, being mostly heterotrophic, are dependent on nutrients supplied by the mother plant in the form of sugars (mainly sucrose) and amino acids for the synthesis of these storage compounds. Although the anatomy of Arabidopsis seeds has been well described, our understanding is still incomplete for phloem unloading and nutrient transport across apoplastic borders separating the phloem from the embryo (Stadler *et al.*, 2005; Suzuki and McCarty, 2008). Isolation and characterization of seed-specific transporters involved in the transport of sucrose (Baud *et al.*, 2005), amino acids (Schmidt *et al.*, 2007) or minerals (Kim *et al.*, 2006) have revealed the complexity of the nutrient transport system operating during the maturation process. Incoming sucrose is cleaved and releases hexose phosphate that can enter either the glycolytic or pentose phosphate pathways to ultimately generate acetyl CoA molecules, precursors for *de novo* fatty acid synthesis that occurs in the plastid. Fatty acid synthesis starts with the formation of malonyl CoA from acetyl CoA by acetyl CoA carboxylase, followed by the sequential condensation of a fatty acyl group with malonyl ACP (acyl carrier protein) by the fatty acid synthase complex, a soluble, dissociable multi-subunit complex (Brown *et al.*, 2006). 16:0-ACP and 18:0-ACP are the final products, and most 18:0-ACP is readily desaturated into 18:1-ACP by the stromal stearyl ACP desaturase. These acyl groups are then hydrolysed by specific acyl ACP thioesterases (Salas and Ohlrogge, 2002) to free fatty acids, which are then activated to CoA esters after crossing the plastid envelope. These long-chain fatty acids can be modified (desaturated and elongated) in the endoplasmic reticulum (ER). Elongation uses malonyl CoA substrates and is catalysed by fatty acid elongase, a membrane-bound multi-enzyme complex. It proceeds via reactions analogous to those of *de novo* fatty acid synthesis (Bach

*et al.*, 2008), and produces very-long-chain fatty acids up to 24 carbons. The mixed pool of acyl CoAs is a source of fatty acids for the acyltransferases involved in TAG synthesis. It has also been shown that the level of glycerol-3-phosphate on which fatty acids are sequentially acylated limits the amount of TAG formed (Gibon *et al.*, 2002).

Once formed, seed oil is sequestered in discrete subcellular organelles called lipid bodies, oil bodies or oleosomes. These spherical structures (0.2–2.0  $\mu\text{m}$ ) comprise a TAG core surrounded by a monolayer of phospholipids. The exact mechanism of oil body biogenesis is still a matter of debate, although it is agreed that they originate from the ER (Herman, 2008 and references therein). Proteins are embedded in the half-unit membrane and their presence at the surface is thought to prevent oil body coalescence by steric hindrance and electrostatic repulsion. The maintenance of oil bodies as individual units until germination provides a high surface-to-volume ratio that favours the action of lipases during TAG mobilization. These oil body-associated proteins (reviewed by Purkrtova *et al.*, 2008) belong to three major groups comprising structural proteins (oleosins and caleosins), enzymes (e.g. steroleosin and lipases) and minor proteins (e.g. aquaporins). Various functions have been described for oil body-associated proteins (Siloto *et al.*, 2006; Purkrtova *et al.*, 2008), suggesting that oil bodies are involved in other cellular processes in addition to TAG storage and mobilization.

Two types of SSPs are predominantly stored in Arabidopsis embryos: 12S globulins (referred to as cruciferins) and 2S albumins (referred to as arabins). Other proteins, such as late embryogenesis-abundant proteins, also accumulate but are less abundant. SSPs are synthesized from amino acids directly taken up by the embryo or obtained after transamination reactions. Precursor forms of SSPs are synthesized on the rough ER and then transported into PSVs by a vesicle-mediated pathway. On arrival, limited proteolysis at specific sites releases mature forms of SSPs into PSVs. Work to isolate the Asn-specific endopeptidases involved in the cleavage of precursor forms of SSPs has led to the identification of vacuolar processing enzymes, a new subclass of the Cys endopeptidase family. Strikingly, removal of vacuolar processing enzyme function results in the successful deposition of alternatively processed forms of SSPs cleaved at sites other than the conserved Asn residues targeted by vacuolar processing enzymes (Gruis *et al.*, 2004). PSVs are electron-dense vacuolar compartments delimited by a lipid bilayer that contain not only the soluble SSPs, but also intra-organellar inclusions called globoids that correspond to spherical inclusions of phytin, a salt of phytic acid, and the cations  $\text{Mg}^{2+}$ ,  $\text{K}^{+}$  and  $\text{Ca}^{2+}$  (Gillespie *et al.*, 2005). Seed iron is also stored in globoids of the PSVs. Study of SSP transport, processing and deposition into PSVs has led to detailed characterization of the Golgi-dependent pathway hosting these processes (Otegui *et al.*, 2006). SSPs and their pro-

cessing enzymes appear to be segregated into different cisternal domains during Golgi trafficking, then into distinct vesicles that ultimately fuse to allow the proteolytic processing of SSP precursors. Vacuolar sorting receptors have been identified that may play a key role in SSP recognition and sorting (Hinz *et al.*, 2007). The precise function of these receptors, namely RING H2 MOTIF PROTEIN 1 (RMR1) (Park *et al.*, 2005) and the type 1 membrane protein VACUOLAR SORTING RECEPTOR 1 (VSR1) (Shimada *et al.*, 2003), is currently a matter of debate.

## SEED DORMANCY AND SEED GERMINATION

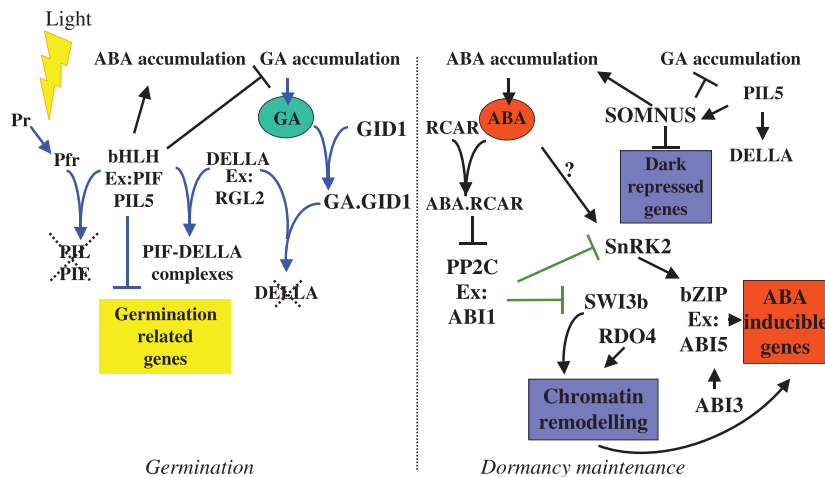
Seed dormancy is considered as the inability of an undamaged and viable seed to undergo germination under optimal conditions. Primary dormancy is induced during seed development and can be released in many plant species, including Arabidopsis, by after-ripening in dry storage or stratification (moist chilling) of mature seeds. Secondary dormancy occurs when non-dormant imbibed seeds encounter unfavourable environmental conditions.

Seed germination starts with seed imbibition, which leads to the initiation of radicle elongation. Seed germination vigour is a measure of the ability of a seed lot to perform fast, homogeneous and synchronized germination under a large range of adverse environmental conditions. Seed vigour decreases during dry storage; however, resistance of the mature seed to ageing can sometime exceed a century.

### ABA is a key regulator of seed dormancy

Genetic and physiological studies have shown that ABA promotes dormancy during seed development. Mutations impairing ABA biosynthesis reduce seed dormancy, while over-expression of biosynthesis genes or mutations in catabolism genes enhance seed dormancy (Finkelstein *et al.*, 2008). Coordinated ABA synthesis and degradation regulate ABA accumulation in developing seeds, and correlate well with dormancy status (Okamoto *et al.*, 2006). In addition, basal ABA levels are fine-tuned during seed imbibition to modulate dormancy maintenance. Importantly, only ABA synthesized in zygotic tissues, and not maternal or exogenously applied ABA, was able to impose dormancy (Finkelstein *et al.*, 2008). A study of expression of *NCED* (9-*cis*-epoxycarotenoid dioxygenase) genes, encoding key enzymes of the ABA biosynthesis pathway, has shown that both embryo and endosperm contribute to dormancy (Lefebvre *et al.*, 2006).

The downstream signalling network that controls the induction of seed dormancy is less well understood, despite identification of a number of loci that affect germination responses to ABA and/or seed dormancy. A major recent breakthrough has been the identification of potential ABA receptors. Although various putative receptors had previously been reported, convincing evidence indicates that the PYR/PYL/RCAR (pyrabactin resistance/PYR-like/



**Figure 2.** Preliminary models for the regulatory networks involved in dormancy maintenance and light-stimulated germination.

Examples of transcription factors and other regulators contributing to these processes are given.

(a) Processes that occur in dormant seeds are indicated by black arrows, and those that promote germination by green arrows. Note the key role of the ABA receptor RCAR in induction of the SnRK2 kinase, leading to the transcription of ABA-inducible genes.

(b) Processes induced by light are indicated by blue arrows, and those maintained in the dark by black arrows. Note the key role of the GA receptor *GID1* in destruction of the DELLA proteins that repress germination.

ABI3, ABA INSENSITIVE3; bHLH, basic HELIX LOOP HELIX; GID, GIBBERELLIN INSENSITIVE DWARF PIF, PHYTOCHROME-INTERACTING FACTOR; PIL, PIF LIKE protein; PP2C, protein phosphatase 2C; RDO, REDUCED DORMANCY; RCAR, REGULATORY COMPONENT OF ABA RECEPTOR; RGA, REPRESSOR OF *ga1-3*; RGL, RGA like. SnRK2, SNF1 Related Kinase; SWI3, SWITCHING DEFECTIVE MUTANT 3.

regulatory components of ABA receptor) family of START proteins are ABA receptors (Ma *et al.*, 2009; Park *et al.*, 2009). In the presence of ABA, these proteins bind type 2C protein phosphatases (PP2C), thus inhibiting their enzymatic activity (Figure 2a) (Miyazono *et al.*, 2009). Members of this PP2C family, which notably includes ABI1, ABI2 and HYPERSENSITIVE TO ABA 1 (HAB1), function as negative regulators in ABA signalling in both seeds and vegetative tissues. They interact with SnRK2 (SNF1-related kinase 2) kinases and inactivate them by dephosphorylation. In the presence of ABA, the RCAR/PYR ABA receptor binds and inactivates these PP2Cs, allowing SnRK2 to phosphorylate downstream substrates (Umezawa *et al.*, 2009). Other proteins have been shown to interact with PP2C, and in particular HAB1 interacts with the chromatin remodelling factor SWI3B to modulate ABA germination responses (Saez *et al.*, 2008). Further evidence of a role for chromatin remodelling in seed dormancy comes from *rdo4* (*reduced dormancy 4*) mutants that are defective for histone mono-ubiquitination (Liu *et al.*, 2007). Downstream transduction of the hormonal signal has been shown to rely largely on protein phosphorylation. In particular, ABA-activated kinases of the SnRK2 family act redundantly as positive regulators of seed dormancy (Figure 2a) (Nakashima *et al.*, 2009). SnRK2 kinases have been demonstrated to phosphorylate bZIP transcription factors of the ABI5/AREB/ABF family, which bind to ABA response elements (ABRE) in promoter sequences of ABA-inducible genes. In accordance, ABA-regulated genes containing ABRE have been shown to be down-regulated in SnRK2 multiple mutants. Transcriptome analysis showed that the

presence of ABRE elements in promoters correlated positively with dormancy induction of the corresponding genes (Cadman *et al.*, 2006). None of these bZIP transcription factors have yet been proven to regulate seed dormancy directly, but dormancy is affected in the B3 class transcription factor mutant *abi3*, and the ABI3 protein can interact with the bZIP factor ABI5 (Finkelstein *et al.*, 2008).

Finally, recent genetic approaches exploiting Arabidopsis natural variation have proven to be useful for the identification of QTLs controlling seed dormancy, and the cloning of new loci such as *DELAY OF GERMINATION 1*, the function of which remains to be established (Bentsink *et al.* 2006).

### Environmental factors act on hormone balance to control dormancy release and germination

The hormonal balance between GA and ABA is widely accepted to regulate dormancy release and germination. This hormonal balance is under the control of environmental factors, including light, day length, cold and nitrate. During imbibition, light and cold treatments act synergistically to break dormancy and promote germination by increasing GA levels through up-regulation of GA 2-oxidation, the last GA biosynthesis step, and down-regulation of GA 3-oxidation, a major route for GA inactivation (Yamauchi *et al.*, 2004; Rieu *et al.*, 2008). In contrast, light has the opposite effect on ABA levels, removing ABA inhibition of germination, i.e. decreased biosynthesis and increased catabolism (Seo *et al.*, 2009). The differential regulation of ABA/GA signalling pathways by light involves the bHLH transcription factor



PIL5 (PIF3-LIKE 5), which is a member of the PIF (phytochrome interacting factor) protein family (Oh *et al.*, 2007). Light promotes the degradation of PIL5 by the 26S proteasome when bound to the active form of phytochrome (Pfr), thus releasing its repressive effect on GA biosynthesis and germination (Figure 2b). In the absence of light, PIL5 repression is mediated by transcriptional activation of a CCCH-type zinc finger protein, SOMNUS, which in turn down-regulates GA and up-regulates ABA levels (Figure 2a) (Seo *et al.*, 2009).

DELLA proteins, which belong to the GRAS family of transcription factors, are major negative regulators of GA signalling. GA binding to the GA receptor GIBBERELLIN INSENSITIVE DWARF 1 (GID1) promotes its interaction with DELLA proteins, which are then subjected to degradation by the 26S proteasome (Daviere *et al.*, 2008). Recent studies have demonstrated that DELLA proteins are key regulators for the integration of light signals into the hormone metabolic/signalling pathways to control seed germination (Figure 2b) (Piskurewicz *et al.*, 2009). DELLA proteins have been shown to regulate PIF protein function in hypocotyl growth in response to light by direct interaction (Daviere *et al.*, 2008; Feng *et al.*, 2008). It is therefore possible that bHLH proteins involved in seed germination, such as PIL5, may also undergo GA-mediated DELLA regulation.

### Emerging features of seed viability and germination

Germination is not an irreversible process. During the early stages of germination, seeds can re-induce part of the late maturation programme (Lopez-Molina *et al.*, 2002). Recent reports have demonstrated that various nitrogenous molecules, including nitrate ( $\text{NO}_3^-$ ) and nitric oxide (NO), are able to break dormancy through the up-regulation of the *CYP707A2* gene involved in ABA catabolism (Liu *et al.*, 2009; Matakias *et al.*, 2009). NO is probably implicated in signalling pathways involved in dormancy release. The target tissue of NO in Arabidopsis seeds is the aleurone layer (Bethke *et al.*, 2007). NO could act via protein S-nitrosylation in this and other tissues.

It has become increasingly accepted that damage resulting from oxidative stress plays an important role in seed deterioration during dry storage and ageing processes. The contribution of the seed coat to protect the seed from ageing is well documented. Vitamin E-derived antioxidant metabolites protect seeds from ageing (Sattler *et al.*, 2004). However, it should be noted that seed ageing and the loss of seed vigour are not only related to irreversible oxidative damage. Using genetic modulation of L-isoaspartyl-O-methyltransferase activity (Oge *et al.*, 2008), it has been clearly established that non-oxidative spontaneous isoaspartate accumulation in proteins is a major source of age-related protein damage in seed and reduces seed vigour during storage. The L-isoaspartyl-O-methyltransferase repair activity significantly improves seed longevity and vigour by

limiting isoaspartate production during the maturation phase and upon seed hydration. The accumulation of DNA mutations in embryos is presumed to contribute to seed ageing. Recent studies showed that correct DNA repair, involving nicotinamide-dependant poly(ADP)ribosylation, is required for proper germination (Hunt *et al.*, 2007).

Oil reserve mobilization is not required for seed germination, but is essential for seedling establishment. This process is severely compromised in Arabidopsis mutants disrupted in either lipolysis of TAGs, transport of fatty acids into the glyoxysomes, activation of fatty acids inside the glyoxysome, or in any of the core reactions of  $\beta$ -oxidation (Graham, 2008). Hydrolytic enzymes degrade SSPs, localized in PSVs, to release amino acids required for seedling growth. These enzymes are synthesized *de novo* in germinating seeds and transported to the PSV through a lytic vacuole pathway. Two observations indicate roles for SSPs and molecular chaperones in seed vigour: reduced seed longevity in *abi3* and *lec2* mutants defective for SSP accumulation, and improved seed longevity in Arabidopsis transgenic lines over-accumulating a heat stress transcription factor involved in accumulation of heat shock proteins. Apart from their role in amino acid supply, the abundance of the SSPs could help to limit deleterious effects of desiccation and age-related injuries.

Important progress has been made over the last 10 years in the field of seed germination and seed ageing. Due to space limitations, certain aspects are only briefly described or not included in this review: for example, FLC regulation of dormancy, cold-induced dormancy release, and effect of brassinosteroids, ethylene and ROS on germination. We expect further major advances to be made in these fields. For instance, chromatin structure in dormant and non-dormant seeds is currently being investigated. It is expected that subtle changes in chromatin structure, induced by changes in the environment, will programme a different profile of permissive germination conditions. New avenues of research have been opened by the discovery of karrikins, molecules that are produced as a result of forest fires that stimulate germination (Nelson *et al.*, 2009). Molecules involved in plant competition for space are also expected to be discovered. In the coming years, some of these discoveries may lead to applications in crop breeding and seed production as described below.

### SEED BIOTECHNOLOGY

Seeds are used both as a starting material for the production of most agriculture commodities and as major nutritional source for humans and livestock. Moreover, as seeds are conveniently stored and transported, they are excellent systems for introducing biotechnological innovations. Arabidopsis genomics has revolutionized the speed of gene discovery for important plant traits and integration of these genomic resources using systems biology approaches. We

describe possible applications drawn from our knowledge of the Arabidopsis seed.

Crop yields depend largely on germination quality. The analysis of Arabidopsis seed germination has been useful for the improvement of seed priming and identification of protein markers for seed vigour. The discovery that protein repair contributes significantly to seed longevity (Oge *et al.*, 2008) is currently being used to develop ageing markers.

The identification of master regulators involved in seed development has potential for a wide range of applications in rapeseed. Identification of the *SHATTERPROOF* MADS box genes controlling fruit dehiscence in Arabidopsis will allow genetic engineering to reduce seed dispersal in crop plants, allow co-existence with other crops and increased yield (Liljegren *et al.*, 2000). Arabidopsis and rapeseed translational genetic approaches are being used to modify seed coat flavonoid composition to improve digestibility and oil content.

The increasing use of plant oils for the production of chemicals and biofuels has stimulated interest in yield improvement and the production of fatty acids in diverse tissues (Durrett *et al.*, 2008; Slocombe *et al.*, 2009). To date, the most promising engineering approaches for the modification of lipid storage compounds concerns the production of high-value fatty acids such as docosahexaenoic acid by synthetic biology approaches (Damude and Kinney, 2008; Ruiz-Lopez *et al.*, 2009). Advances in our understanding of tocopherol synthesis are also providing new opportunities to increase vitamin E content in crop plants for nutritional applications (DellaPenna, 2005). Increased pro-vitamin A content is also a goal for a number of crops, e.g. 'Golden Rice', and should be achievable by exploiting our knowledge of carotenoid biosynthesis. There is no doubt that, in the coming decade, crop seed quality will be improved further, and adapted to various uses (food, feed, green chemistry), based on the knowledge acquired from Arabidopsis seeds.

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