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Reserve Mobilization in the Arabidopsis Endosperm Fuels Hypocotyl Elongation in the Dark, Is Independent of Abscisic Acid, and Requires *PHOSPHOENOLPYRUVATE CARBOXYKINASE1*

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Arabidopsis thaliana **is used as a model system to study triacylglycerol (TAG) accumulation and seed germination in oilseeds. Here, we consider the partitioning of these lipid reserves between embryo and endosperm tissues in the mature seed. The Arabidopsis endosperm accumulates significant quantities of storage lipid, and this is effectively catabolized upon germination. This lipid differs in composition from that in the embryo and has a specific function during germination. Removing the endosperm from the wild-type seeds resulted in a reduction in hypocotyl elongation in the dark, demonstrating a role for endospermic TAG reserves in fueling skotomorphogenesis. Seedlings of two allelic gluconeogenically compromised *phosphoenolpyruvate carboxykinase1* (*pck1*) mutants show a reduction in hypocotyl length in the dark compared with the wild type, but this is not further reduced by removing the endosperm. The short hypocotyl phenotypes were completely reversed by the provision of an exogenous supply of sucrose. The *PCK1* gene is expressed in both embryo and endosperm, and the induction of PCK1:β-glucuronidase at radicle emergence occurs in a robust, wave-like manner around the embryo suggestive of the action of a diffusing signal. Strikingly, the induction of *PCK1* promoter reporter constructs and measurements of lipid breakdown demonstrate that whereas lipid mobilization in the embryo is inhibited by abscisic acid (ABA), no effect is seen in the endosperm. This insensitivity of endosperm tissues is not specific to lipid breakdown because hydrolysis of the seed coat cell walls also proceeded in the presence of concentrations of ABA that effectively inhibit radicle emergence. Both processes still required gibberellins, however. These results suggest a model whereby the breakdown of seed carbon reserves is regulated in a tissue-specific manner and shed new light on phytohormonal regulation of the germination process.**

INTRODUCTION

Plant seeds store carbon to fuel postgerminative seedling establishment in the form of lipid, starch, and protein (Bewley and Black, 1994). Early seed plants, such as conifers, cycads, and ginkgo, store fatty acids in the form of triacylglycerol (TAG) in maternal tissues surrounding the embryo, whereas angiosperms can also store carbon reserves either in the endosperm or the embryo itself. In *Arabidopsis thaliana* and other members of the family Brassicaceae, carbon is stored in the form of TAG primarily in embryonic tissues.

The development of the Arabidopsis seed requires the coordination of three distinct tissues: the embryo, endosperm, and seed coat. During embryo development, the endosperm undergoes complete cellularization and forms a cell layer similar to the cereal aleurone surrounding the mature embryo (Mansfield and Briarty, 1990; Berger, 1999; Olsen, 2001). By contrast, the maternal tissues of the seed coat undergo programmed cell death and are not physiologically active in the mature seed.

The endosperm has two known roles during germination in angiosperm seeds. In endospermic seeds, such as cereals and castor bean (*Ricinus communis*), hydrolytic enzymes are secreted from the aleurone layer into the free endosperm to mobilize starch or lipid reserves. Carbon from endospermic reserves is transported to the embryo to fuel postgerminative growth in the form of sucrose (Kornberg and Beevers, 1957). In barley (*Hordeum vulgare*) aleurone cells, gibberellic acid (GA) promotes the expression of these genes through the proteasome-mediated degradation of the SLENDER protein, and the activation of transcription factors, including GAMYB (Gubler et al., 1995, 2002). Abscisic acid (ABA) antagonizes the action of GA, represses amylase gene expression, and acts through an ABA-induced protein kinase (Gomez-Cadenas et al., 2001).

Secondly, the endosperm secretes hydrolytic enzymes whose function is to degrade the cell walls of the endosperm and seed coat, thus removing mechanical barriers to radicle emergence. These include a β-1,3-glucanase, an endomannanase, a polygalacturonase, and expansin isoforms (Leubner-Metzger et al., 1995; Nonagaki and Morohashi, 1996; Sitrit et al., 1999; Chen and Bradford, 2000). In Arabidopsis, tobacco (*Nicotiana tabacum*), and tomato (*Lycopersicon esculentum*), it has been

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demonstrated that the cell walls of the seed coat and endosperm constitute an important component of seed dormancy and that the regulation of seed coat breakdown is a critical factor in the regulation of germination itself (Debeaujon et al., 2000; Leubner-Metzger and Meins, 2000; Downie et al., 2003). Despite a wealth of knowledge relating to the development of the endosperm, the importance of the endosperm to the germinating Arabidopsis seed has received comparatively little attention. Moreover, the mechanisms governing the regulation of endosperm activity in germinating Arabidopsis seeds are not well understood.

Arabidopsis has been used as a model system to elucidate the biochemistry and regulation of lipid mobilization during germination. This takes place in the peroxisome and requires a fatty acid transporter, two redundant acyl-CoA synthetase isoforms, and peroxisomal β-oxidation (Hayashi et al., 1998; Germain et al., 2001; Zolman et al., 2001; Footitt et al., 2002; Fulda et al., 2004). One isoform of the key β-oxidation enzyme, 3-KETOACYL-CoA THIOLASE, predominates during germination, and mutants lacking this protein have clarified the role of lipid mobilization in germination. The *PEROXISOME DEFECTIVE1* (*PED1*)*/3-KETOACYL-CoA THIOLASE2* (*KAT2*) gene is required for seedling establishment, yet not for germination defined as radicle emergence (Hayashi et al., 1998; Germain et al., 2001). Characteristically, seedling establishment in the *ped1/kat2* mutant can be restored by the provision of an alternative carbon source.

By contrast, Arabidopsis seedlings lacking the glyoxylate cycle enzyme ISOCITRATE LYASE (ICL) establish in the light but are defective in hypocotyl elongation in dark in a sucrose-dependent manner (Eastmond et al., 2000). A similar phenotype has been observed for plants, in which the expression of the *PHOSPHOENOLPYRUVATE CARBOXYKINASE1* (*PCK1*) gene has been downregulated through antisense (Rylott et al., 2003). The difference between Arabidopsis and earlier endospermic model oilseeds such as castor bean has led to doubts about the role of the glyoxylate cycle and gluconeogenesis in embryonic oilseeds where alternative fates for the β-oxidation–derived acetyl-CoA exist within a cell (Smith, 2002).

Very little is known about the regulation of seed storage mobilization in Arabidopsis. The provision of exogenous sucrose retards lipid catabolism, and this effect can be further enhanced by reducing the availability of nitrate (Martin et al., 2002). The phytohormone ABA also inhibits lipid breakdown, but even superphysiological levels of ABA cannot completely prevent fatty acid catabolism despite the fact that germination is not proceeding (Pritchard et al., 2002).

Here, we examine the importance of storage reserves contained within the endosperm of Arabidopsis. We show that Arabidopsis stores significant amounts of lipid in the endosperm and that carbohydrates derived from this lipid are required for postgerminative seedling growth in the dark. The export of carbon from the endosperm to the embryo is found to be dependent on the *PCK1* gene. Moreover, in contrast with the embryo, the regulation of the mobilization of endosperm reserves and other endosperm-controlled processes is independent of ABA, demonstrating tissue-specific variation in ABA sensitivity and action in germinating Arabidopsis seeds.

RESULTS

The Separation of the Arabidopsis Seed into Embryo and Endosperm

To investigate lipid breakdown in the embryo and endosperm independently, we dissected imbibed Arabidopsis seeds and subjected the dissected tissues to subsequent analyses. The separation of Arabidopsis seeds into two fractions, embryo and endosperm/seed coat, is easy to achieve, and any seeds damaged in the process were discarded (Figure 1A). All analyses on endosperm tissue were undertaken with the seed coat attached. This is possible because the Arabidopsis seed coat undergoes programmed cell death at maturity and contains no storage lipid (Beeckman et al., 2000). The treatment of the Arabidopsis seed in this way mirrors work in cereal experimental systems such as the barley aleurone, in which the aleurone layer is not separated from the seed coat before analysis.

To verify the purity of the two fractions, we isolated RNA from the embryo and endosperm fractions and analyzed cDNA for embryo and endosperm specific marker genes using real-time RT-PCR (see Methods; Figure 1B). Transcripts for the *GLABRA2* (*GL2*) gene are present in both the embryo epidermis and developing seed coat but have not been reported in the endosperm (Szymanski et al., 1998; Costa and Dolan, 2003). These were accordingly detected in embryo cDNA but not from the endosperm fraction. Hence, there was no contamination of the endosperm sample with embryo RNA, and as expected, no *GL2* cDNA from the seed coat was detected. To confirm the purity of the embryo fraction, we assayed the transcript abundance of an endosperm-specific extensin-like gene from both embryo and endosperm cDNA (Dubreucq et al., 2000). This was detected in the endosperm cDNA but only at low levels in the embryo. Thus, these results validate the separation and demonstrate that no significant contamination of each fraction occurs from the other.

The Arabidopsis Endosperm Contains Significant Amounts of Storage Lipid

Looking at cross sections of Arabidopsis seeds under a light microscope, it is clear that they contain numerous oil bodies in the endosperm (Eastmond et al., 2002). The total lipid present in isolated Arabidopsis embryo and endosperm tissues was analyzed by gas chromatography. The quantity of fatty acids detected in isolated embryos and endosperm tissues accounted for the total amount of lipid present in whole seeds (Figure 1C), demonstrating that no loss of material occurred in the dissection process. Fatty acids in endosperm tissues composed approximately one-tenth of those present in whole seeds. Eicosenoic acid (20:1) is used as a marker for fatty acids contained wholly within TAG (Lemieux et al., 1990). Eicosenoic acid was found in embryo and endosperm and thus can be used as a marker for the presence of TAG in both tissues. To examine differences in the distribution of individual fatty acids between the embryo and endosperm, we compared the fatty acid profile from both tissues (Figure 1D). All the fatty acids present in the embryo were also detected in the endosperm; however, the endosperm contained proportionally high levels of 16:1n7, 18:1n7, and 20:1n7 long

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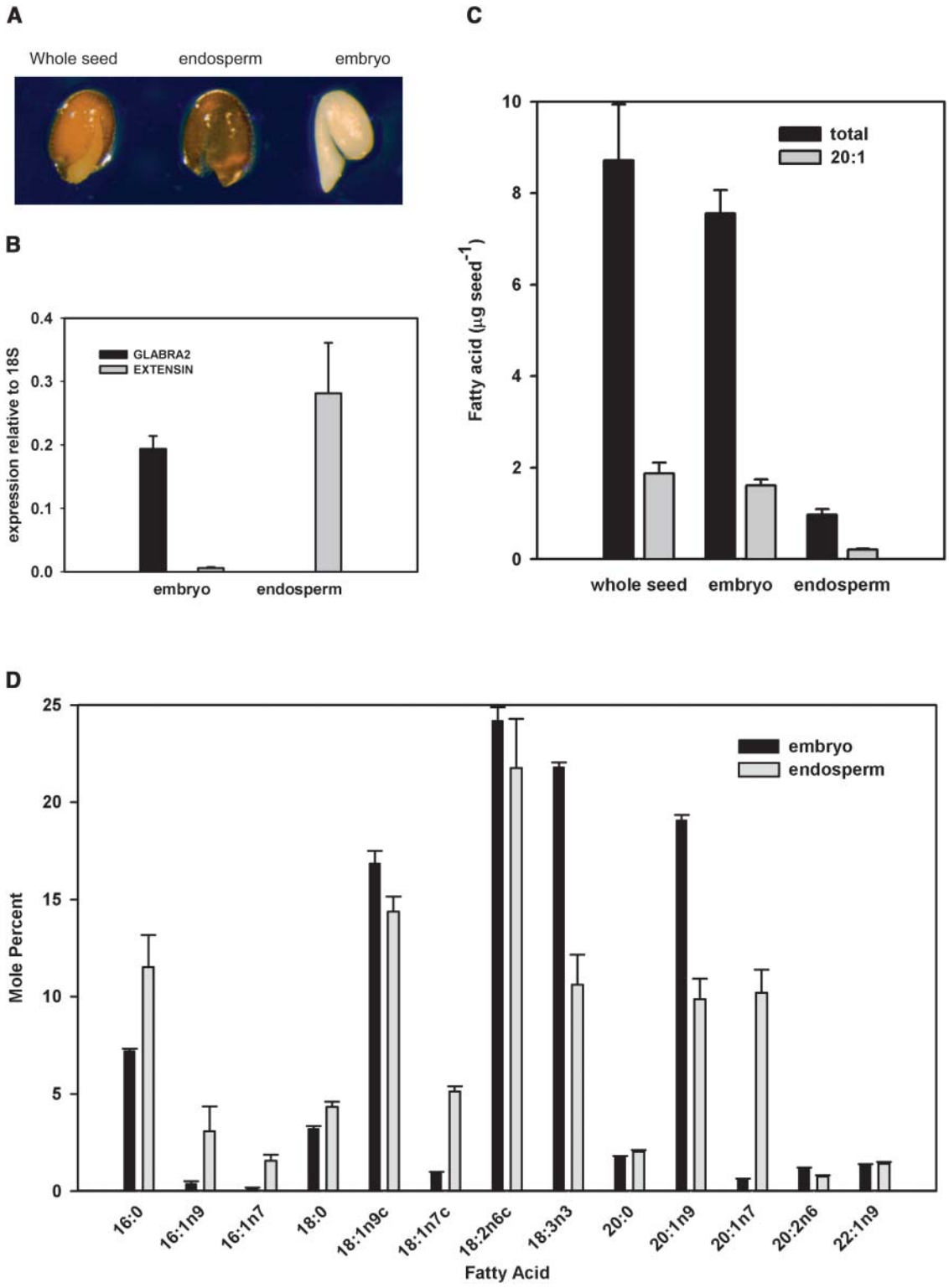

Figure 1. Analysis of the Fatty Acid Content of Arabidopsis Embryo and Endosperm by Gas Chromatography.

Values indicate the mean and standard deviation of four replicates.

(A) A germinating Arabidopsis seed and a germinating seed after dissection into the embryo and endosperm/seed coat fractions.

(B) Real-time RT-PCR analysis of *GL2* and endosperm-specific *EXTENSIN-LIKE* gene transcript abundance in cDNA from isolated embryo and endosperm tissues at radicle emergence.

(C) Analysis of fatty acids from imbibed Arabidopsis seeds by gas chromatography.

(D) Comparison of the fatty acid profile of embryo and endosperm.

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chain fatty acids (LCFAs), accounting for more than 50% of the total n7 monounsaturated fatty acid present in the whole seed. In fact, ~20% of endosperm fatty acids were n7 monounsaturated compared with only 2% in the embryo. Correspondingly, levels of 20:1n9 were proportionally decreased in the endosperm. These observations suggest that alternate pathways for fatty acid biosynthesis predominate in the developing Arabidopsis seed and that these are regulated in a tissue-specific manner.

Gluconeogenesis in the Endosperm Is Required for Skotomorphogenesis and Is Blocked in *pck1* Mutants

Carbon derived from endospermic TAG reserves must be converted into sucrose through gluconeogenesis for export to the embryo (Kornberg and Beevers, 1957). The critical first step in gluconeogenesis is catalyzed by PCK, of which there are two isoforms in Arabidopsis. *PCK1* (At4g38750) is the predominantly expressed form during germination (Rylott et al., 2003). To further elucidate the role of *PCK1* in reserve mobilization, we isolated two T-DNA insertion alleles (Figures 2A and 2B). The first, *pck1-1*, is a T-DNA insertion in the ninth intron and was isolated from the Wisconsin T-DNA insertion collection (Sussman et al., 2000). The second, *pck1-2*, is a T-DNA insertion in the second exon and was obtained from the SALK collection (Alonso et al., 2003). No *PCK1* transcript was detected in germinating seeds of either mutant (data not shown). The role of *PCK1* as the predominant isoform of *PCK* present during seedling establishment was confirmed by comparing PCK activity in the *pck1-1* and *pck1-2* mutants with that of the corresponding wild-type seedlings (Figure 2B). PCK activity in both alleles was reduced to less than one-tenth of the wild-type levels when assayed 2 d after germination (Figure 2C). We have previously reported that antisense *PCK1* lines exhibit a reduction in sucrose content and a short hypocotyl in the dark when germinated on media without a carbohydrate supplement (Rylott et al., 2003). In accordance with this observation, both *pck1-1* and *pck1-2* exhibit a similar short hypocotyl phenotype in the dark, and this can be rescued by the addition of exogenous supply of sucrose, even though they accumulate similar quantities of storage lipid to the wild type (Figure 2D; data not shown). The establishment of *pck1* mutant seedlings in the light is significantly delayed compared with the wild type but not inhibited altogether (data not shown).

To investigate the role of sucrose exported from the endosperm during postgerminative growth, we measured hypocotyl elongation in the dark in the wild-type and the *pck1* mutants, with or without the presence of the endosperm and seed coat tissues (Figure 3). Removal of the endosperm from the wild-type Columbia or Wassilewskija embryos resulted in a reduction in hypocotyl elongation by one-third compared with seeds germinated intact; however, *pck1-1* and *pck1-2* mutants, both impaired in gluconeogenesis and unable to export carbon from the endosperm, showed reduced hypocotyl elongation compared with the wild types but no additional reduction when the endosperms were removed. This is consistent with the hypothesis that the reduction in hypocotyl length observed upon removal of the endosperm from the wild-type seeds is a result of the lack of sucrose arriving in the embryo from the endosperm. To add further support to this hypothesis, the wild-type hypocotyls were

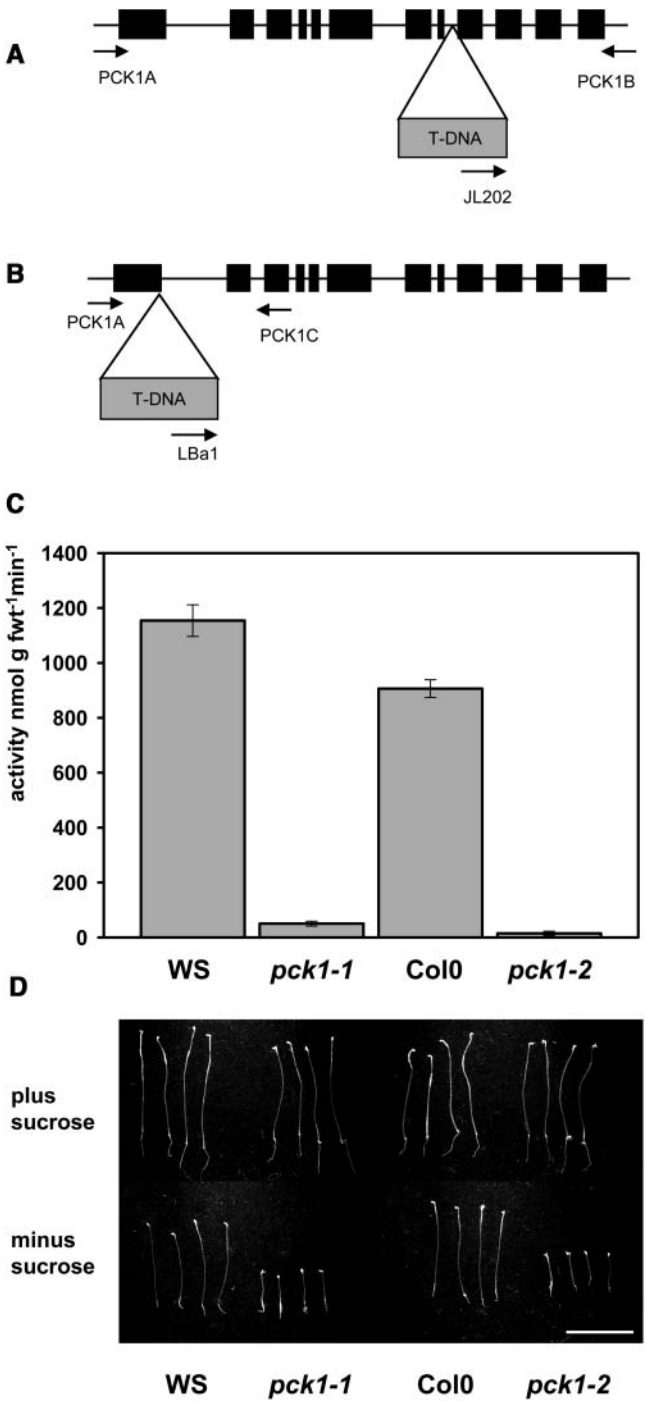

Figure 2. The Isolation of *pck1* T-DNA Insertion Alleles.

(A) and **(B)** Genomic structure of the *pck1-1* and *pck1-2* loci, respectively. Exons are indicated as black bars and primers as arrows.

(C) PCK activity in 2-d-old seedlings. Error bars indicate mean and standard error of five replicate samples. fwt, fresh weight; WS, Wassilewskija ecotype.

(D) Dark-grown seedlings of the wild-type and *pck1* mutants. Bar = 5 mm.

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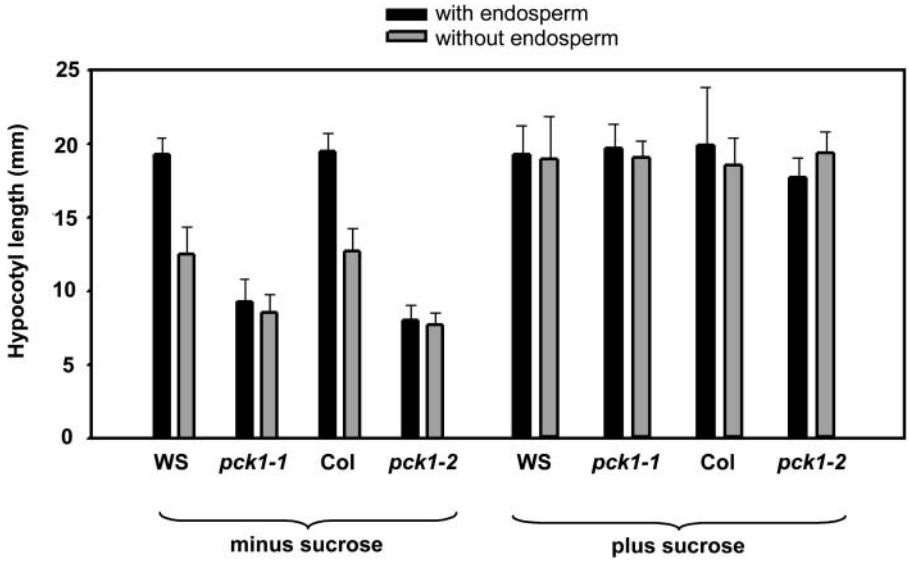

Figure 3. Measurement of Dark-Grown Hypocotyl Length of Wild-Type and *pck1* Mutants Germinated with or without the Endosperm.

Values are mean ± SD of 20 hypocotyls.

germinated without endosperm on growth medium containing 0.5% sucrose (Figure 3). Under these conditions, hypocotyl elongation of both the wild-type and *pck1* mutant seedlings was unaffected by endosperm removal. This demonstrates that the reduction in hypocotyl length observed upon endosperm removal on MS medium alone was because of the lack of sucrose, rather than a second substance provided by the endosperm. Hence, carbon exported from the endosperm contributes significantly to the elongating hypocotyl in the dark, and this export is compromised in the *pck1-1* and *pck1-2* mutants.

PCK1:β-Glucuronidase Is Induced in a Wave-Like Manner upon Radicle Emergence

The expression of genes required for lipid mobilization peaks 2 d after germination and is controlled primarily at the level of transcription (Rylott et al., 2001). To further investigate the transcriptional regulation of reserve mobilization, we fused 2044 bp of the *PCK1* promoter to the β-glucuronidase (GUS) reporter gene (Jefferson et al., 1987). More than 30 independent transgenic lines were obtained, and the expression of the GUS enzyme was observed during germination. Little or no GUS expression is present in seeds imbibed at 4°C in the dark (Figure 4A). At the point of radicle emergence, GUS expression first appears in the colet and subsequently extends to the rest of the hypocotyl with those areas close to the colet expressing first (Figures 4B and 4C). At this point, GUS expression is strongly induced in the area of the first cotyledon immediately in contact with the hypocotyl (Figure 4D). GUS expression is then seen to begin throughout the first cotyledon and also in the second. The shoot apical region is the last to express GUS, aside from the radicle itself, from which GUS expression is consistently excluded (Figure 4E). Lower levels of GUS expression also occur in some areas of the cotyledons before the main wave of expression arrives from the hypocotyl. This is suggestive of more than one mechanism of PCK1 induction operating during germination. The induction of PCK1:GUS is complete before the cotyledons begin to expand and the radicle grows. During seedling establishment, PCK1:GUS expresses in all shoot tissues but not the primary root (Figure 4E). From 3 d after germination, expression reduces to isolated patches of cells in the cotyledons, the hydathodes, and colet (Figure 4F). Thus, the induction of PCK1:GUS in embryonic tissues propagates in a predictable, wave-like manner from the colet by a signal that either diffuses from or is transported from the hypocotyl or that first emanates from the hypocotyl before appearing in all tissues.

In addition to embryonic expression, GUS expression is also strongly induced in endosperm tissues during radicle emergence, where it persists until the fourth day after germination (Figures 4I to 4M). Again, no expression is seen in endosperm tissues from seeds imbibed in the dark at 4°C. Induction of GUS occurs during germination. At the third day postimbibition, expression is reduced, and none is seen after the fourth day. At this point, endospermic reserve mobilization is presumably complete (Figure 7). No expression is ever observed in seed coat tissues, which are not metabolically active at this time (Figure 4N), and little variation in expression is seen from cell to cell (Figure 4O). This is consistent with the hypothesis that PCK1 is critical for carbon mobilization from the endosperm.

The primary role of gluconeogenesis during germination is to provide hexoses and glycolytic intermediates to support growth and anabolic metabolism. It has also been shown that high concentrations of exogenous sugars repress lipid mobilization (Martin et al., 2002). Hence, we determined the effect of exogenous glucose on the induction of PCK1:GUS (Figure 4P). No repression of PCK1:GUS was observed by up to 50 mM glucose,

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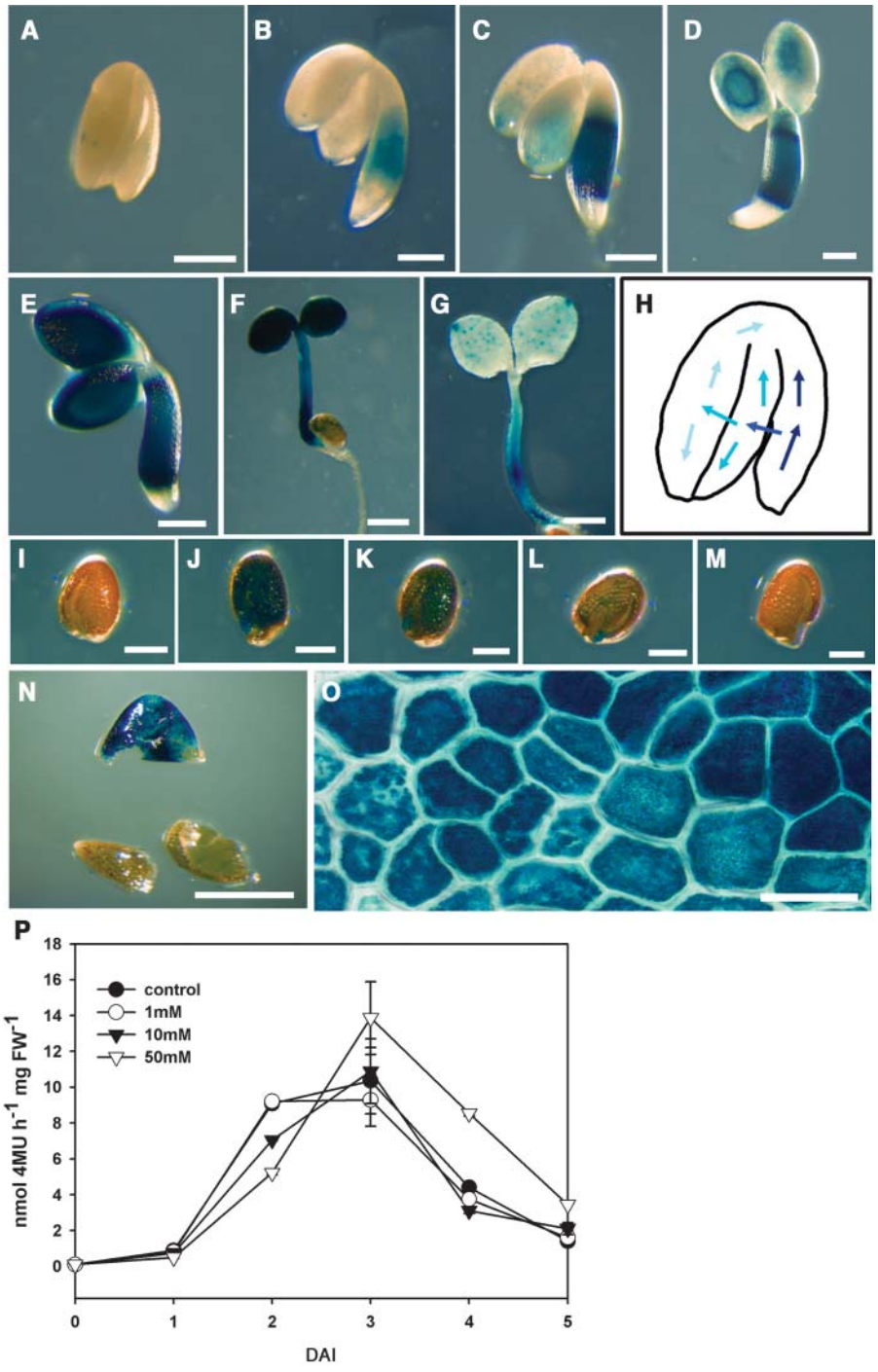

Figure 4. The Expression of PCK1:GUS during Seedling Establishment.

(A) Imbibed seed. Bar = 250 μM.
(B) to **(E)** Radicle emergence. Cotyledons have been dissected away from the hypocotyl to better visualize the expression pattern. Bars = 250 μM.
(F) and **(G)** PCK1:GUS seedling 3 and 5 d postimbibition, respectively. Bars = 1 mm.
(H) Illustration charting the temporal changes in the expression of PCK1:GUS. Darker arrows indicate earlier expression.
(I) to **(M)** PCK1:GUS seeds 0 to 4 d postimbibition from which the embryo has been removed to allow endosperm staining to be visualized. Bars = 250 μM.
(N) PCK1:GUS endosperm from a seed at radicle emergence. The seed coat has been removed to emphasize the specificity of GUS expression in the endosperm. Bar = 250 μM.
(O) Close up of PCK1:GUS endosperm cells. Bar = 10 μM.
(P) Quantitative analysis of PCK1:GUS expression during seedling establishment on 0 to 50 mM glucose. Data points indicate mean and standard error of four samples of 10 plants. DAI, days after imbibition.

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although 50 mM glucose did cause an initial delay in the induction and then a slight increase in activity at 3 to 4 d postimbibition.

The Induction of Reserve Mobilization Genes in the Endosperm Requires GA but Cannot Be Blocked by ABA

To determine the effect of phytohormones on the induction of PCK1:GUS, we assayed GUS activity in the presence of ABA and the GA biosynthesis inhibitor paclobutrazol. No expression was seen in dry or imbibed seeds, yet activity was high at radicle emergence (Figure 5A). Twenty micrometers of ABA could reduce but not eliminate the increase in GUS expression, whereas paclobutrazol effectively prevented GUS expression. To extend the analysis, we constructed plants expressing GUS under the control of a 2014-bp fragment of the Arabidopsis *ICL* promoter (see Methods). ICL:GUS transgenic plants displayed the same wave-like induction of GUS expression at radicle emergence as was observed in PCK1:GUS plants (data not shown). In common with PCK1:GUS, GUS activity in ICL:GUS lines is absent in dry and imbibed seeds and is induced 1 d after imbibition at radicle emergence (Figure 5B). A small induction was observed in the presence of ABA, but no induction was measured in the presence of paclobutrazol. To assess how accurately the GUS reporter lines were reflecting the induction of the endogenous genes, we assayed PCK and ICL activity under the same conditions (Figures 5A and 5B). In all cases, the activity of the endogenous enzyme correlated well with that observed for the corresponding GUS reporter lines, validating the use of the GUS lines to follow the induction of *PCK1* and *ICL* upon germination. To determine the localization of GUS activity under the different conditions, we stained GUS expressing seedlings histochemically. Both ICL:GUS- and PCK1:GUS-expressing plants expressed GUS both in the embryo and endosperm under normal conditions (Figure 5C). Treatment of imbibed seeds with ABA

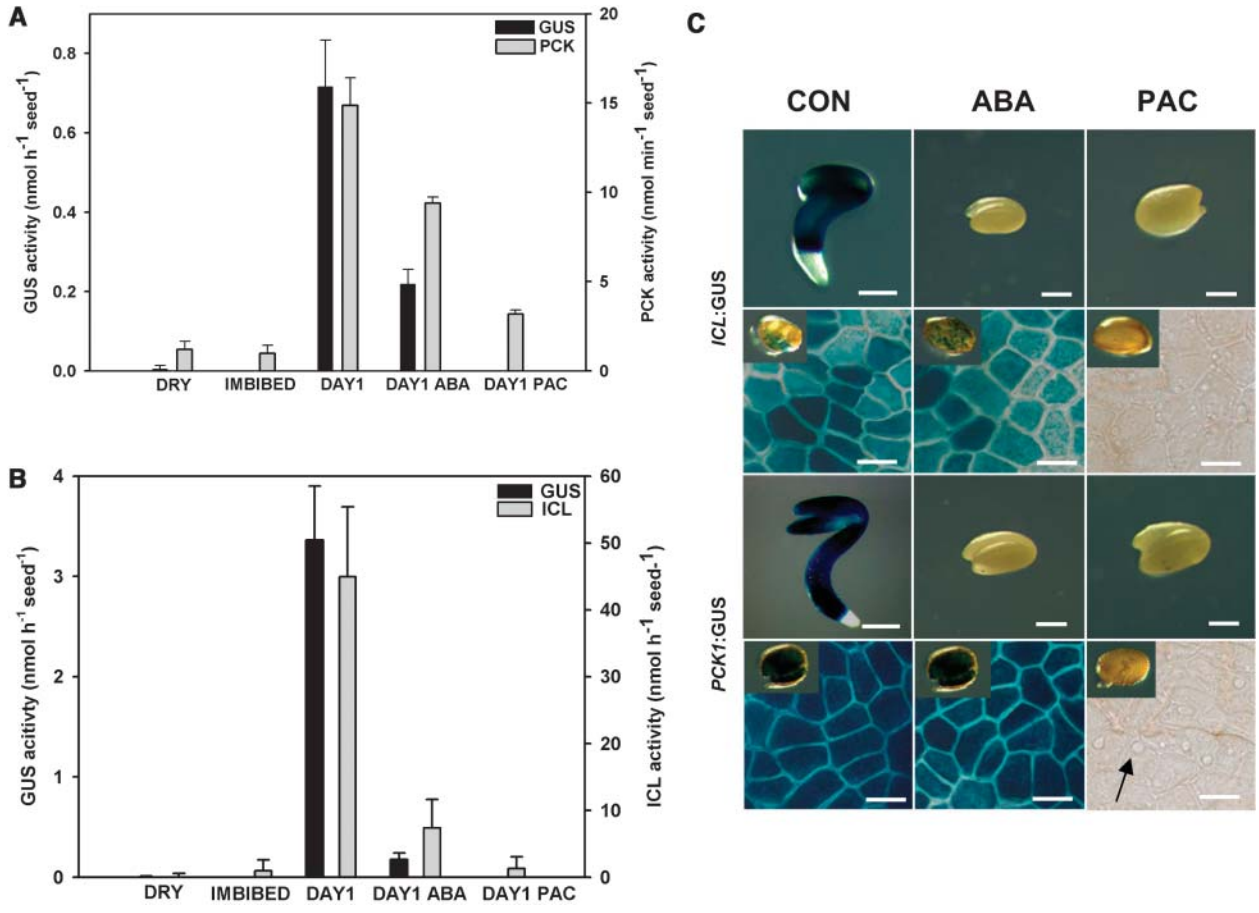

Figure 5. The Regulation of PCK1 and ICL by ABA and GA during Seedling Establishment.

Data points represent mean and standard deviation of four (GUS assays) or five (ICL and PCK assays) samples.

(A) PCK1:GUS activity and PCK1 activity in germinating seeds in the presence of ABA and paclobutrazol (PAC). Imbibed refers to seeds imbibing at 4°C for 72 h.

(B) ICL:GUS activity and ICL activity in germinating seeds in the presence of ABA and paclobutrazol.

(C) Seeds were germinated on control medium or medium supplemented with 20 μM ABA or 20 μM paclobutrazol and dissected into embryo (top panels) and endosperm/seed coat (bottom panels). Representative sections of endosperm cells are shown. Con, control. Bar for embryos = 250 μM; bar for endosperm cells = 10 μM.

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strongly inhibited GUS expression from both promoters in the embryo but had no effect in the endosperm. Imbibition in the presence of paclobutrazol led to the inhibition of GUS expression in both the embryo and endosperm, and large oil bodies were still visible in endosperm cells. Thus, GA is required for the induction of *PCK1* and *ICL* in the embryo and endosperm, yet ABA inhibits this induction only in the embryo.

It has previously been shown that the induction of luciferase (*LUC*) under the control of the Arabidopsis *MALATE SYNTHASE* promoter cannot be prevented by ABA (Pritchard et al., 2002). Hence, we analyzed *LUC* expression in isolated embryo and endosperm tissues in the presence of ABA and paclobutrazol (Figure 6). A low level of *LUC* expression was already present in embryo and endosperm tissue before germination, which reflects translation of *LUC* transcripts before desiccation (data not shown). *LUC* expression was strongly upregulated in both embryo and endosperm tissues upon imbibition and peaked at 2 d after imbibition. ABA treatment delayed the induction of *LUC* in the embryo (Figure 6A) but had no effect on the speed of induction in the endosperm (Figure 6B); however, ABA did prolong *LUC* expression in the endosperm, suggesting that it is not completely insensitive to the phytohormone. No induction was seen in the presence of paclobutrazol, in agreement with the results obtained from PCK1:GUS and ICL:GUS reporter constructs.

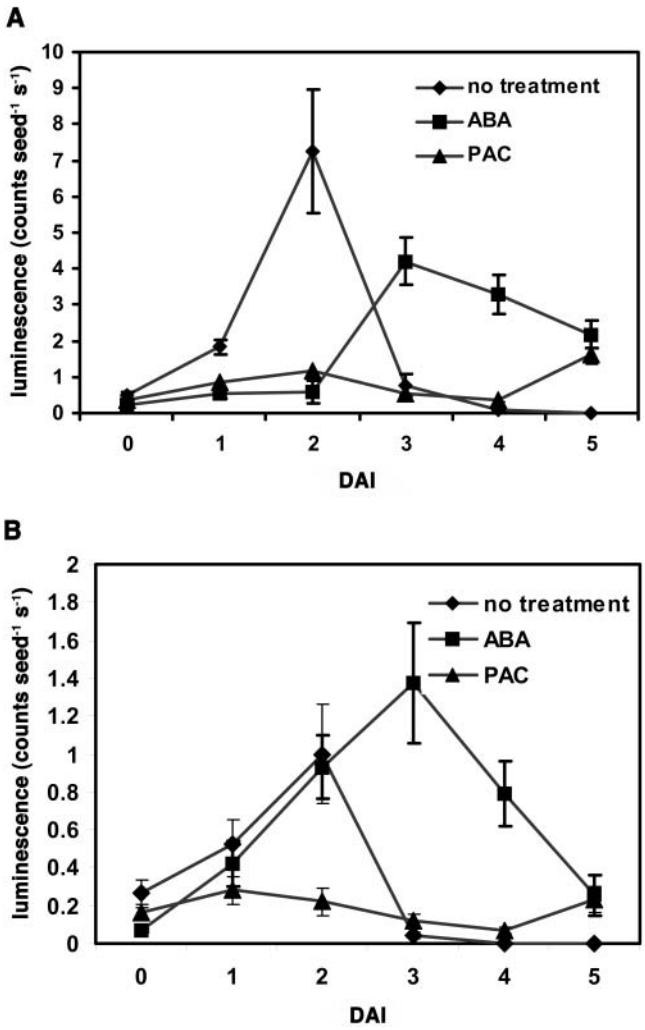

Figure 6. *LUC* Activity from the Malate Synthase Promoter during Imbibition.

Data points represent mean and standard error of 20 seeds/seedlings. DAI, days after imbibition.

(A) Embryo.

(B) Endosperm.

Endosperm Lipid Catabolism Requires GA but Is Not Blocked by ABA

To further test the hypothesis that ABA cannot inhibit lipid breakdown in the endosperm, we measured the fatty acid content of Arabidopsis embryo and endosperm tissues 5 d after imbibition in the presence of ABA and paclobutrazol (Figure 7A). Using 20:1 as a marker for TAG, lipid mobilization was complete in the embryo and endosperm 5 d after imbibition. In ABA-treated seeds, little TAG remained in the endosperm after 5 d, but the embryo still contained more than 70% of the TAG present at day 0. By contrast, paclobutrazol-treated seeds still retained most of their TAG in both the embryo and endosperm tissues. These results support the evidence obtained from the GUS reporter constructs that ABA can inhibit lipid catabolism in the embryo but not the endosperm in Arabidopsis.

The Imbibition-Induced Cell Wall Hydrolysis of the Endosperm and Seed Coat Is Not Blocked by ABA

While dissecting seeds for lipid analysis, it became clear that ABA-treated seeds did not appear equivalent to paclobutrazol-treated seeds under the microscope. ABA-treated seeds exhibited ruptures in the seed coat and endosperm tissues, and this was accompanied by dark discoloration indicative of the oxidation of these tissues (Figure 7B). This occurred even in the presence of high concentrations of ABA that strongly inhibit germination, and the radicle never emerged from the micropylar endosperm under these conditions; however, paclobutrazol-treated seeds never exhibited this phenotype, nor did seeds of the GA biosynthetic mutant, *ga1-3* (Koornneef et al., 1982; data not shown). These remained intact and were not visibly distinguishable after 5 d in the growth cabinet from seeds that had only been imbibed for a few hours.

DISCUSSION

The mature Arabidopsis seed consists of two physiologically active compartments: the embryo and endosperm. We separately considered the role of the two compartments and the regulation of their activity. We have shown that, contrary to expectations, the Arabidopsis endosperm contains significant lipid reserves and that these differ in composition from those in the embryo. We have also demonstrated that these are mobilized via gluconeogenesis upon germination and that their removal has measurable consequences for the germinating seedling. Additionally it is clear from these results that the regulation of lipid

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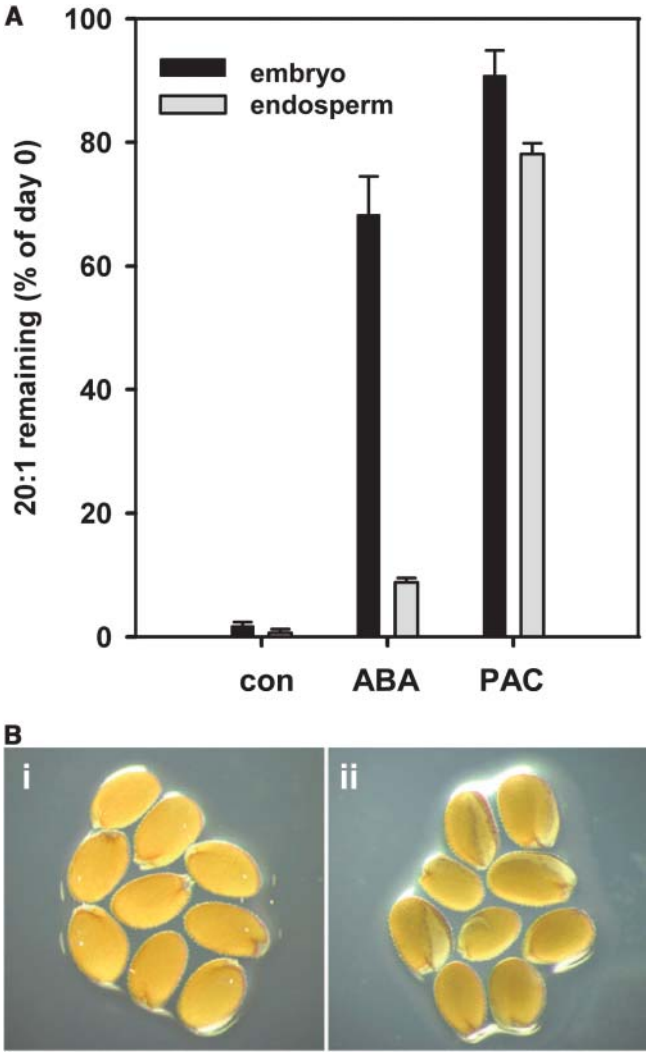

Figure 7. Differential Response of the Embryo and Endosperm in the Presence of ABA.

(A) Fatty acid catabolism 5 d postimbibition. Error bars indicate mean and standard deviation of four samples. con, control; ABA = 20 μM ABA; PAC = 20 μM paclobutrazol.

(B) Appearance of Arabidopsis seeds after 5 d imbibed on 20 μM ABA or 20 μM paclobutrazol. Paclobutrazol (i); ABA(ii).

catabolism in the endosperm differs remarkably from that in the embryo. The mobilization of storage reserves in the endosperm is not blocked by the presence of ABA yet still requires induction by GA, thus separating the actions of these two antagonistic hormones during germination in Arabidopsis.

Fatty Acid Accumulation in the Endosperm

Arabidopsis is an excellent model system for the study of oilseed development and the accumulation of lipid reserves and has been used for biochemical, genetic, and transcriptomic studies of the processes governing TAG accumulation (Katavic et al., 1995; Focks and Benning, 1998; Ruuska et al., 2002). Previous reports have analyzed the fatty acid content of the Arabidopsis seed in its entirety, but in this study, we consider separately the embryo and endosperm. This required the dissection of the seed into two portions: the embryo and endosperm/seed coat. The available evidence suggests that the fatty acids measured in the endosperm/seed coat portion exist exclusively in the endosperm. First, no lipid bodies can be observed in the mature Arabidopsis testa (Beeckman et al., 2000; Penfield et al., 2001). Second, the fatty acids detected in the endosperm/seed coat are effectively catabolized upon germination, suggesting that they exist in a physiologically active tissue. This catabolism can also be blocked by paclobutrazol, showing that it is an active metabolic process rather than a nonenzymatic breakdown of any seed coat lipid.

Significant differences exist between the fatty acid profiles of embryo and endosperm tissues. Specifically, the endosperm synthesizes proportionally less linolenic acid (18:3n3), and this is compensated by a corresponding increase in the n7 LCFAs palmitic acid (16:1n7), vaccenic acid (18:1n7), and paullinic acid (20:1n7). Paullinic acid is rare in the plant kingdom, known predominantly from seed oils in the family Sapindaceae, a family of neotropical shrubs, and may be catabolized to produce compounds with insecticidal properties (Spitzer, 1996). Interestingly the production of linolenic acid requires the activity of a delta15 desaturase, and this has been shown to be induced by ABA in microspore-derived embryos of rape (Zou et al., 1995). Hence, it is possible that decreased sensitivity of the endosperm to ABA during seed development could underlie differences in the fatty acid composition of the embryo and endosperm in mature seeds. ABA is also known to enhance LCFA biosynthesis in rape embryos and to induce the expression of a fatty acid elongase (Finkelstein and Somerville, 1989; Qi et al., 1998). Although this is not completely consistent with the results presented here, it does underline the potential importance of ABA in the regulation of storage lipid accumulation.

Alternatively, tissue-specific isoforms of fatty acid desaturase and elongase enzymes may exist and account for the compositional differences between embryo and endosperm. Increased activity in the endosperm of a fatty acid elongase with specificity for 16:1n7 might produce an increase in vaccinic acid and paullinic acid in these cells. Very little is known about differential gene expression in the embryo and endosperm during seed development, but the Arabidopsis *FATTY ACID ELONGATION1* gene is reported to be expressed exclusively in the embryo, further supporting the idea that endosperm LCFA biosynthesis could involve a distinct set of enzymes (Rossak et al., 2001).

The Role of Gluconeogenesis in Arabidopsis Reserve Mobilization

The importance of gluconeogenesis during germination has remained unclear in an oilseed such as Arabidopsis, which stores lipid predominantly in the embryo. Two allelic mutants at the *PCK1* locus confirm previous results from antisense experiments that gluconeogenesis is vital to germinating Arabidopsis seeds (Rylott et al., 2003) and suggest that the contribution of the second Arabidopsis isoform of PCK is small during this time

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period. The short hypocotyl phenotype of the *pck1-1* and *pck1-2* mutants shows that gluconeogenesis is required for skotomorphogenesis, and this is partly a consequence of the use of TAG stored in the endosperm. These results serve to underline the similarities between Arabidopsis and classic endospermic model oilseeds such as castor bean (Kornberg and Beevers, 1957). Consistent with this view, *PCK1* expression is high in the endosperm, reflecting the absolute requirement for the transport of carbon to the embryo for use. In addition, the removal of the endosperm from *pck1* mutant seeds does not further compromise hypocotyl elongation in the dark, demonstrating the critical role of *PCK1* in gluconeogenesis in the endosperm. *PCK1* is also expressed in elongating hypocotyl cells in the dark, however (data not shown), and hypocotyl elongation in the wild-type seedlings with the endosperm removed still exceeds that of *pck1* mutants. It is possible that sucrose is transported to the elongating hypocotyl cells from a second source, presumably either the cotyledons or even hypocotyl cells with a carbon surplus. Alternatively gluconeogenically derived sugars could be used intracellularly to provide raw materials for cell wall synthesis. Interestingly, endospermic lipid reserves are required for one-third of the total hypocotyl elongation in the dark, despite representing only one-tenth of the total available lipid. This suggests that the ultimate fate of lipid-derived carbon in the embryo is to some extent inflexible and that carbon is committed to fuel other vital processes, even when sucrose is limiting for the expanding hypocotyl. One possible fate of this carbon is for chloroplast development.

The Induction of Lipid Mobilization in the Embryo

During germination, seed carbon reserves are catabolized to fuel the processes required for seedling establishment. Using the PCK1:GUS reporter lines, it can clearly be demonstrated that the signal that induces PCK1 promoter activity appears first in the hypocotyl and then spreads in such a manner that the next cells to strongly induce GUS expression are immediately adjacent to those already expressing. This leads to the impression of a wave of induction spreading around the embryo. The wave of expression also appears to spread across from the hypocotyl to the immediately adjoining cotyledon, showing that this is not a barrier. This induction from hypocotyl to embryo can be blocked by a glass cover slip, in which case GUS expression appears in the cotyledons only after it has progressed to the top of the shoot apex (data not shown). These observations are suggestive of a mobile signal propagating from the base of the hypocotyl around the embryo, inducing reserve mobilization and possibly other metabolic processes. Interestingly, the region at the base of the hypocotyl has been reported to be the site of expression of the *RGA-LIKE2* gene, a critical regulator of germination in Arabidopsis (Lee et al., 2002).

The Role of GA and ABA in the Control of Endosperm Function

ABA action is an important component of dormancy and prevents germination in unfavorable conditions such as low water availability and high salt conditions (Werner and Finkelstein, 1995); however, despite considerable attention to the role of ABA in germination, the site of synthesis and action are largely unknown in Arabidopsis. In barley aleurone layers, GA promotes and ABA inhibits the hydrolysis of starch reserves (Chrispeels and Varner, 1966); however, in Arabidopsis, it has been shown that ABA cannot completely prevent reserve mobilization in even ungerminated seeds and that ABA-treated seeds accumulate sucrose (Pritchard et al., 2002). Here, we demonstrate that lipid catabolism in the endosperm is essentially independent of ABA, whereas ABA effectively inhibits reserve mobilization in embryonic tissues. Surprisingly, GA is still required for lipid breakdown in the endosperm. These conclusions are supported by several lines of evidence. Firstly, promoter reporter constructs for PCK1, ICL, and MS all respond in an embryo-specific manner to ABA. Secondly, quantification of lipid breakdown clearly shows that whereas ABA is effective at inhibiting lipid breakdown in the embryo, little or no effect is seen in the embryo. In the context of the role of GA in reserve mobilization, it is interesting to note that previous work has suggested that lipid bodies in the endosperm disappear as a consequence of germination rather than as a direct effect of GA on lipid breakdown itself (Debeaujon and Koornneef, 2000).

The insensitivity of the endosperm to ABA is not confined to the regulation of lipid mobilization. Visual inspection of Arabidopsis seeds imbibing in the presence of concentrations of ABA far in excess of that required to inhibit germination showed that whereas radicle emergence was blocked, significant enzymatic hydrolysis of the tissues surrounding the embryo was taking place. This process still requires GA, however, because the same phenomenon was not observed in the wild-type seeds imbibing on the GA biosynthesis inhibitor, paclobutrazol, nor in imbibing GA-deficient *ga1-3* mutant seeds (data not shown). This observation is not without precedent. GA is required for the expression of an Arabidopsis extensin-like gene in the endosperm, yet the induction could not be prevented by ABA (Dubreucq et al., 2000). The same has been reported for a xyloglucan endotransglycosidase in germinating tomato seeds. Induction upon imbibition was abolished in the GA-deficient *gib-1* mutant but could not be blocked by ABA in the wild-type seeds (Chen et al., 2002). Interestingly, in tomato the induction of endo-β-mannanase expression and activity cannot be inhibited by ABA in the lateral endosperm but is under ABA control in the micropylar endosperm where radicle emergence actually takes place (Toorop et al., 2000). This suggests further refinements in the cell-specific sensitivity to ABA even within the various endosperm cells themselves. It is tempting to speculate that as seed ABA levels drop, the reduced sensitivity to ABA of the endosperm allows metabolic activity to be induced here before the embryo and that this paves the way for radicle emergence through cell wall hydrolysis and a soluble carbon supply. There is also evidence for differential ABA responses in developing tobacco seeds. Tobacco seeds accumulate protein bodies in both embryo and endosperm during maturation; however, storage protein accumulation is inhibited in transgenic tobacco embryos overexpressing anti-ABA antibodies and hence reduced active ABA, yet no effect is seen in the endosperm (Lubaretz and zur Nieden, 2002).

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We can only speculate as to the mechanism through which this differential sensitivity between embryo and endosperm occurs, while still retaining a requirement for GA. It is possible that GA levels themselves are ordinarily extremely high in the endosperm or that endosperm tissues are so sensitive to GA that even high levels of ABA cannot antagonize the signal. During germination, the role of GA is probably to promote the degradation of the REPRESSOR OF GAI-3-LIKE 2 (RGL2) protein, mutations in which restore the germination of GA-deficient mutants (Lee et al., 2002); however, the expression of RGL2 has only been reported in the embryo (Lee et al., 2002), so it is at present unclear through which genes GA signaling in the endosperm proceeds.

Alternatively, ABA action or catabolism could be altered in the endosperm compared with the embryo. This could involve increased activity of ABA catabolizing enzymes (Kushiro et al., 2004) or reduced expression of one of the *aba insensitive* (*abi*) loci (Koornneef et al., 1984). Arabidopsis *aba* and some *abi* mutants also germinate in the presence of GA biosynthesis inhibitors, however (Nambara et al., 1991), so it is more likely that the cause of ABA insensitivity in the endosperm is more complex. More information on the localization of expression of these key regulatory genes in the endosperm might begin to answer these questions.

The Role of Sugars in the ABA Regulation of Germination and Reserve Mobilization

We did not observe any repression of *PCK1* gene expression by exogenous sugars. Although 50 mM glucose did appear to delay the peak of PCK1:GUS expression, the total expression was generally higher (Figure 4).This slight inhibition may be an indirect effect of the well-characterized retardation of germination itself by the presence of exogenous sugars (Price et al., 2003). Exogenous glucose also has no significant repressive effect on ICL:GUS (data not shown). We have also previously reported that the steady state transcript levels of various β-oxidation, glyoxylate cycle, and PCK1 are not repressed by sugars during germination and seedling establishment (Rylott et al., 2001).

Previous work reported that the ABA block on germination can be rescued by exogenous sugar, raising the possibility that this was a consequence of a block on reserve mobilization (Garciarrubbio et al., 1997; Finkelstein and Lynch, 2000); however, ABA-treated seeds accumulate sucrose as a result of lipid breakdown (Pritchard et al., 2002). In this work, we show that ABA inhibits reserve mobilization in the embryo, raising the possibility that a localized limitation of carbon supply exists in the presence of ABA that can be overcome by exogenous sugar application.

In conclusion, we have shown that clear differences in storage reserve accumulation and use exist between the embryo and endosperm in the Arabidopsis seed. These include the form of TAG stored and the phytohormonal regulation of reserve mobilization in these tissues. This work clearly demonstrates tissue-specific sensitivity to ABA during the germination of Arabidopsis seeds. These results have implications for experiments such as transcriptomics and proteomics that routinely rely on whole seeds for analysis and highlight the need for tissue-specific approaches to data collection when considering germinating seeds.

METHODS

Isolation of *pck1* Mutant Alleles

pck1-1 was obtained by screening the T-DNA–mutagenized *Arabidopsis thaliana* population at University of Wisconsin-Madison Biotechnology Center (http://www.biotech.wisc.edu/Arabidopsis/; Sussman et al., 2000) using primers spanning the *PCK1* gene (At4g37870), PCK1A 5′-CGAGTGTAATCAACTTCGTAACTCTGTCT-3′ and PCK1B 5′-CAGAGAGATTATCACAGCAAAGAAAAACA-3′. Sequence flanking the left border of the T-DNA insert was sequenced using the left border primer JL202 (5′-CATTTTATAATAACGCTGCGGACATCTAC-3′).

pck1-2 was discovered in the SIGnAL database (Alonso et al., 2003) and obtained from the Nottingham Arabidopsis Stock Centre (N532133; http://nasc.nott.ac.uk). Primers PCK1C 5′-CGCCAAAGCTCCATTAGAAGTGATAA-3′ and T-DNA left border primer LBba1 5′-TGGTTCACGTAGTGGGCCATCG-3′ were used to verify the presence and site of the T-DNA insertion.

Germination, Hypocotyl Elongation, and Fatty Acid Assays

Endosperm/seed coat tissues were removed from the embryo using a dissecting microscope, after allowing seeds to soften in water for 6 h at 4°C. Where indicated, seeds were germinated on MS media supplemented with 20 μM ABA (Sigma, Poole, UK) or 20 μM paclobutrazol (Greyhound Chromatography, Birkenhead, UK). Seeds were imbibed for three nights at 4°C before transferring to the growth cabinet at 20°C, 16 h photoperiod with 50 to 70 μM m^2 light from fluorescent bulbs. Dark-grown seedlings were given 20 min of light before wrapping in three layers of foil. Hypocotyl lengths were measured to the nearest millimeter from 7-d-old dark-grown seedlings on MS media with or without the addition of 0.5% sucrose. Before transfer to 20°C, 20 min of light was given to stimulate germination, and the endosperm/seed coat was removed where indicated.

Fatty acids were quantified by gas chromatography, as described previously (Larson and Graham, 2001), from samples consisting of five embryos or five endosperm/seed coats.

RNA Extraction and Real-Time RT-PCR

RNA was isolated from pooled samples of 50 embryos or endosperms using the Absolutely RNA nanoprep kit according to manufacturer's instructions (Stratagene, Cambridge, UK). The total RNA sample from this extraction was used for one round of amplification using the MessageAmp kit from Ambion (Austin, TX), using the manufacturer's instructions. cDNA was made from 1 μg (embryo) or 500 ng (endosperm) of total RNA using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA) in a 20-μL reaction using random hexamer primers according to manufacturer's instructions. The cDNA was diluted to 200 μL, and 4 μL was used for subsequent RT-PCR reactions. For the real-time RT-PCR, 12.5 μL of SYBR-green PCR mix (Applied Biosystems, Foster City, CA), 4 μL of cDNA, 7.5 μL of water, and 0.5 μL of each primer at 10 μM were used in triplicate 25-μL reactions and subjected to the following cycling conditions on an ABI Prism 7000 thermal cycler (Applied Biosystems): 50°C for 2 min, followed by 95°C for 10 min, then 40 cycles of 95°C for 15 s, and 60°C for 1 min. Results were analyzed using the ABI Prism 7000 SDS software (Applied Biosystems). A single amplification product in each reaction was confirmed by a dissociation curve and amplification quantified by comparison to a standard curve. Results for each tissue

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were normalized to the amplification of the 18S control gene. Primer sequences were as follows: 18SF, 5′-TCCTAGTAAGCGCGAGTCATC-3′; 18SR, 5′-CGAACACTTCACCGGATCAT-3′; GL2F, 5′-GCGCAAGCCTC-TTCGTTTC-3′; GL2R, 5′-ATAAACACAATCCCCGCATCTC-3′; Extensin-likeF, 5′-GTCCCACTAATAGATTTCTTGAGATTCTT-3′; Extensin-likeR, 5′-GCGTGACCACTATCAGTCTCATACTT-3′.

Promoter GUS Fusions

The *PEPCK1* and *ICL* promoters were amplified from Arabidopsis genomic DNA using five units of Hotstartaq (Qiagen, Valencia, CA) according to manufacturer's instructions, in the presence of 50 μM dNTPS (Promega, Southampton, UK) and 200-nM primers in a 50-μL reaction. Primers used were as follows: PEPCK1F, 5′-AAGCTTCCTG-TAACCACAACACAAAAAAAAGACA-3; PEPCK1R, 5′-CTCGAGCTTG-CACATGGTTAATCTACAGCGTTG-3; ICLF, 5′-CTCGAGCATCCACGA-GCTAAGCAAGTAGCATC-3; ICLR, 5′-AGAGAAAGATGCAGCCATGG-CTTAAC-3′. These primers contained restriction sites (underlined) to facilitate subcloning. PCR reactions were performed as described (Penfield et al., 2001). PCR products were cloned into the pGEM-T vector (Promega) according to manufacturer's instructions and sequenced to confirm the fidelity of the amplification. The *PCK1* promoter was digested from pGEM with *Xho*I and *Hind*III and subcloned into the *Sal*I and *Hin*dIII sites of the pGREENGUS vector (Penfield et al., 2001) using standard molecular biology techniques (Sambrook et al., 1989). The *ICL* promoter was subcloned in the same way into pGREENGUS using *Nco*I and *Xho*I. Constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 containing the pSOUP vector (Hellens et al., 2000) by electroporation and into Arabidopsis ecotype Columbia by the floral dip method (Clough and Bent, 1998). Transformants containing single T-DNA insertion events were selected for analysis.

Measurement of Enzyme Activities

Histochemical and quantitative GUS assays were performed as described (Jefferson et al., 1987). Quantitative assays were performed with four independent extractions of 10 plants. *LUC* activity was detected as described (Pritchard et al., 2002), with the exception that seeds were dissected into embryo and endosperm/seed coat before application of 5 mM luciferin. Twenty seeds were used per data point. PCK activity was determined by the method of Cooper et al. (1968). ICL activity was determined as described previously (Hooks et al., 1999).

Photography of Seeds and Seedlings

Seeds and GUS-stained seedlings were photographed using a Leica MP6 dissecting microscope using a SPOT RT image capture system (Diagnostic Instruments, Sterling Heights, MI). Images were manipulated using Adobe Photoshop (Mountain View, CA). Close-up images of endosperm pavement cells were photographed using an Olympus BX60 microscope (Tokyo) fitted with an Olympus DP50 image capture system and a ×40 objective.

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