# A Nuclear Gene Encoding the Iron-Sulfur Subunit of Mitochondrial Complex II Is Regulated by B3 Domain Transcription Factors during Seed Development in Arabidopsis[1][W][OA]

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Mitochondrial complex II (succinate dehydrogenase) is part of the tricarboxylic acid cycle and the respiratory chain. Three nuclear genes encode its essential iron-sulfur subunit in Arabidopsis (*Arabidopsis thaliana*). One of them, *SUCCINATE DEHYDROGENASE2-3* (*SDH2-3*), is specifically expressed in the embryo during seed maturation, suggesting that SDH2-3 may have a role as the complex II iron-sulfur subunit during embryo maturation and/or germination. Here, we present data demonstrating that three abscisic acid-responsive elements and one RY-like enhancer element, present in the *SDH2-3* promoter, are involved in embryo-specific *SDH2-3* transcriptional regulation. Furthermore, we show that ABSCISIC ACID INSENSITIVE3 (ABI3), FUSCA3 (FUS3), and LEAFY COTYLEDON2, three key B3 domain transcription factors involved in gene expression during seed maturation, control *SDH2-3* expression. Whereas ABI3 and FUS3 interact with the RY element in the *SDH2-3* promoter, the abscisic acid-responsive elements are shown to be a target for bZIP53, a member of the basic leucine zipper (bZIP) family of transcription factors. We show that group S1 bZIP53 protein binds the promoter as a heterodimer with group C bZIP10 or bZIP25. To the best of our knowledge, the *SDH2-3* promoter is the first embryo-specific promoter characterized for a mitochondrial respiratory complex protein. Characterization of succinate dehydrogenase activity in embryos from two homozygous *sdh2-3* mutant lines permits us to conclude that SDH2-3 is the major iron-sulfur subunit of mature embryo complex II. Finally, the absence of SDH2-3 in mutant seeds slows down their germination, pointing to a role of SDH2-3-containing complex II at an early step of germination.

Succinate:ubiquinone oxidoreductase (succinate dehydrogenase [SDH]; EC 1.3.5.1), commonly referred to as mitochondrial complex II, has a central role in mitochondrial metabolism as a member of both the electron transport chain and the tricarboxylic acid (TCA) cycle. This important membrane-associated complex catalyzes the oxidation of succinate to fumarate and the reduction of ubiquinone to ubiquinol. In bacteria and heterotrophic eukaryotes, complex II is constituted by four subunits: two peripheral membrane proteins, a flavoprotein (SDH1) and an iron-sulfur protein (SDH2), and two small integral membrane proteins (SDH3 and SDH4; Lemire and Oyedotun, 2002; Yankovskaya et al., 2003). The succinate-binding site is formed by the SDH1 protein, and this flavoprotein subunit interacts with the SDH2 subunit, which contains three nonheme iron-sulfur centers acting as conductors of electrons from the flavoprotein to the membrane. The two integral membrane proteins, SDH3 and SDH4, anchor the SDH1-SDH2 subcomplex to the matrix side of the inner mitochondrial membrane and contain a b-type heme and the ubiquinone-binding site (Yankovskaya et al., 2003). Surprisingly, plant complex II may contain additional subunits of unknown function, along with the four classical subunits (Millar et al., 2004).

Complex II subunits are all encoded in the nuclear genome in Arabidopsis (*Arabidopsis thaliana*; Figueroa et al., 2001, 2002; Millar et al., 2004). Surprisingly, we

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found that three nuclear genes, designated *SDH2-1* (At3g27380), *SDH2-2* (At5g40650), and *SDH2-3* (At5g65165), encode the iron-sulfur subunit in Arabidopsis (Figueroa et al., 2001). The three proteins would be functional as complex II iron-sulfur subunits, since they are highly conserved when compared with their homologs in other organisms and contain the Cys motifs involved in binding the three iron-sulfur clusters essential for electron transport (Figueroa et al., 2001). However, only SDH2-1 and SDH2-2 have been identified in the Arabidopsis mitochondrial proteome (Heazlewood et al., 2004). There is only one previous report describing more than one *SDH2* gene in a eukaryotic organism, a sheep nematode, and this fact may be related to a switch in energy metabolism during development (Roos and Tielens, 1994). The unusual presence of three *SDH2* genes in Arabidopsis raises interesting questions about their origin and function.

*SDH2-1* and *SDH2-2* genes likely arose via a relatively recent duplication event, while separation with *SDH2-3* would be more ancient (Figueroa et al., 2001). This is supported by the completely different exon-intron structure of *SDH2-3*, which encodes a protein only 67% similar to SDH2-1 and SDH2-2. Moreover, whereas *SDH2-1* and *SDH2-2* have similar expression patterns, being expressed in all organs from adult plants (Figueroa et al., 2001; Elorza et al., 2004), *SDH2-3* is highly expressed in the embryo during the maturation phase of seed development and *SDH2-3* transcripts are abundant in dry seeds and decline during germination (Elorza et al., 2006). These data suggest that *SDH2-1* and *SDH2-2* are probably redundant and that *SDH2-3* may have a specific role as the complex II iron-sulfur protein-coding gene during embryo maturation and/or germination.

Analysis of Arabidopsis plants carrying *SDH2-3* promoter fusions to the GUS reporter gene allowed us to show that *SDH2-3* expression is regulated at the transcriptional level during seed development (Elorza et al., 2006). In silico analysis of the promoter revealed the presence of three potential abscisic acid (ABA)-responsive elements (ABREs), characterized by the consensus sequence YACGTGGC containing the ACGT core (Busk and Pagès, 1998; Leung and Giraudat, 1998), and a RY-like enhancer element (Nambara and Marion-Poll, 2003). The seed-specific expression of *SDH2-3* overlaps with that of genes encoding abundant seed storage proteins (SSPs; e.g. At2S3) and late embryogenesis abundant proteins (LEAs; e.g. AtEm1; Parcy et al., 1994). Since ABRE and RY elements have been implicated in the expression of these genes (Busk and Pagès, 1998; Nambara and Marion-Poll, 2003), we mutated them in the *SDH2-3* promoter and show here that they are involved in the high embryo expression of *SDH2-3*.

*ABSCISIC ACID INSENSITIVE3* (*ABI3*), *FUSCA3* (*FUS3*), and *LEAFY COTYLEDON2* (*LEC2*) encode related plant-specific transcription factors containing the conserved B3 DNA-binding domain (Giraudat et al., 1992; Luerssen et al., 1998; Stone et al., 2001) and act in concert to regulate key pathways during seed maturation (Santos-Mendoza et al., 2008). They are interlocked in a cross-regulated network, resulting in overlapping or specific functions. For instance, *abi3*, *fus3*, and *lec2* mutants share common phenotypes in reduced expression of SSP but exhibit specific phenotypes, such as ABA insensitivity (*abi3*), early germination of immature embryos (*fus3*), lack of chlorophyll degradation (*abi3* and *lec2*), desiccation intolerance (*abi3* and *fus3*), or leafy cotyledons (*fus3* and *lec2*; To et al., 2006). Here, we demonstrate that ABI3, FUS3, and LEC2 are also involved in the regulation of *SDH2-3* expression.

A wide range of mutant, antisense, or silenced plants with deficient expression of enzymes from the TCA cycle have been described, including citrate synthase (Landschütze et al., 1995), aconitase (Carrari et al., 2003), malate dehydrogenase (Nunes-Nesi et al., 2005), fumarase (Nunes-Nesi et al., 2007), succinyl CoA ligase (Studart-Guimarães et al., 2007), and $NAD^+$-dependent isocitrate dehydrogenase (Lemaitre et al., 2007). These studies have shown that modifications in the TCA cycle can modulate photosynthetic performance and, in the case of potato (*Solanum tuberosum*) citrate synthase, lead to a specific disintegration of the ovary tissues of flower. Nevertheless, they have not analyzed the expression of TCA cycle genes during seed development, nor have phenotypic alterations been reported during seed maturation or germination.

To gain insight into the physiological role of complex II and to explore the function of the multiple genes encoding the same SDH subunit, our group has undertaken a reverse genetic analysis of the *SDH* genes (León et al., 2007). Here, we report the analysis of two insertional mutants in *SDH2-3* and show that SDH2-3 is the major iron-sulfur subunit of embryo complex II and plays a role during germination.

## RESULTS

### ABRE and RY Elements Are Required for *SDH2-3* Promoter Activity

The 223 bp upstream of the *SDH2-3* transcription start site are sufficient to confer high expression of the GUS reporter gene in mature seeds (Elorza et al., 2006) and have three potential ABRE elements (ABRE1, ABRE2, and ABRE3) and a RY-like enhancer element (Fig. 1A). Furthermore, removal of the region between −223 and −65 comprising ABRE2 and ABRE3 drastically reduced GUS expression (Elorza et al., 2006). To evaluate the function of these elements, constructs containing substitution mutations were made (Fig. 1A) and GUS activity was determined in mature T2 seeds from transgenic plants carrying the wild-type or mutated *SDH2-3* promoters fused to GUS. Mutating any of the three ABRE elements or the RY element caused significant reduction of GUS expression, mutation of ABRE2 producing the most drastic reduction of activity (Fig. 1B).

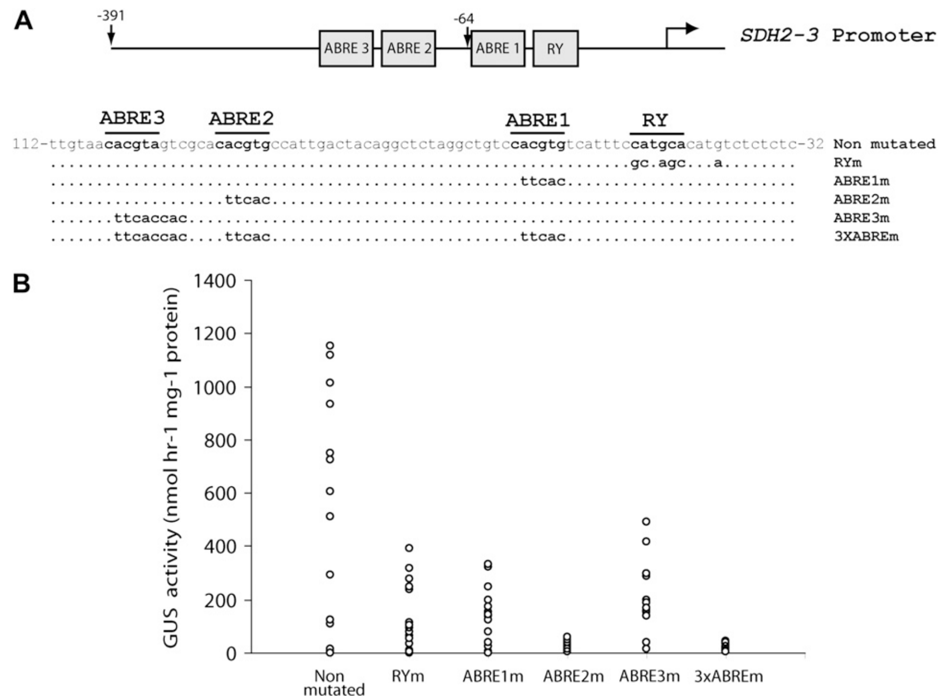


**Figure 1.** Mutation analysis of ABRE and RY elements in the *SDH2-3* promoter. A, Structure of the mutant constructs. Motifs targeted by in vitro mutagenesis are boxed. Numbers are in relation to the transcription initiation site, indicated by a curved arrow. The sequence of the wild-type promoter is shown (Non mutated), and the mutated nucleotides in each construct are indicated below. B, GUS activity was measured in duplicate seed extracts from 12 to 20 independent transgenic lines carrying the wild-type or mutant promoters fused to GUS. Each point represents one transgenic line, and SD values have been omitted for clarity.

**ABI3, FUS3, and LEC2 Are Involved in the Regulation of *SDH2-3* Expression**

ABI3, FUS3, and LEC2 are considered master regulators of seed maturation (Santos-Mendoza et al., 2008). Accordingly, we analyzed *SDH2-3* expression in a severe *abi3-5* mutant allele (Ooms et al., 1993). Homozygous *abi3-5* mutant seeds fail to degrade chlorophyll and are thus identified by their green color (Fig. 2A). The accumulation of *SDH2-3* mRNA was dramatically reduced in *abi3-5* seeds, as was the control LEA gene *AtEm1* (Fig. 2B).

The reduction of *SDH2-3* mRNA levels in *abi3-5* seeds is probably due to a decrease in promoter activity, since *abi3-5* plants crossed with homozygous plants carrying either 1.6 or 0.4 kb of the *SDH2-3* promoter:GUS fusions showed a dramatic reduction of GUS activity (Fig. 2C). Furthermore, no GUS staining was observed in any isolated embryo from ABI3-deficient green seeds, whereas embryos containing wild-type *ABI3* alleles showed strong staining.

Ectopic expression of *ABI3* confers the ability to accumulate seed-specific transcripts in response to ABA in vegetative tissues (Parcy et al., 1994). Therefore, wild-type seedlings and seedlings carrying the *ABI3* cDNA fused to the cauliflower mosaic virus 35S promoter were transferred onto plates with 50 $\mu$M ABA or without hormone. After 48 h, *SDH2-3* transcripts were only observed in ABA-treated *35S*::*ABI3* plants, as were the control *AtEm1* transcripts (Fig. 3A). To determine if this *SDH2-3* mRNA increase is due to promoter activation, *35S*::*ABI3* plants were crossed to *SDH2-3* promoter (0.4 kb)::GUS plants. Two-week-old *35S*::*ABI3*/*pSDH2-3*::*GUS* seedlings were transferred onto plates containing 50 $\mu$M ABA, incubated for 48 h, and then stained for GUS. GUS staining was clearly detected in leaves and roots, while plants not treated with ABA showed no GUS expression (Fig. 3B). Furthermore, GUS activity was quantified in protein extracts from leaves of 2-month-old plants. As shown in Figure 3C, ABA strongly induced GUS expression.

To evaluate the role of FUS3 and LEC2 in *SDH2-3* expression, northern-blot analysis was performed using dry seed RNA from *fus3-3* and *lec2-1* mutants (Fig. 4). *SDH2-3* expression was reduced to a similar extent in both *fus3-3* and control *abi3-5* seeds, as was the *At2S3* storage protein gene (Fig. 4). In contrast, *lec2-1* had a slight effect, if any, on *SDH2-3* and *At2S3* transcript levels in dry seeds. We decided to analyze *SDH2-3* expression during maturation of *lec2-1* seeds, since *LEC2* expression decreases toward the end of seed maturation and *LEC2* transcript levels become undetectable in dry seeds (Kroj et al., 2003). RNA was extracted at different developmental stages from early maturation to desiccation, and expression was examined by northern blot (Fig. 5A). In wild-type seeds, *SDH2-3* transcripts accumulate during the maturation phase of seed development and remain high during the desiccation phase (lane 7) and in dry seeds (lane 8), as reported previously (Elorza et al., 2006). Expression of the *At2S3* control albumin gene is similarly induced during maturation, but transcript levels decrease during desiccation. Interestingly, *SDH2-3* expression is clearly reduced in *lec2-1* seeds before desiccation (lanes 1–6) but not in dry seeds (lane 8) or seeds that have begun to desiccate (lane 7). For comparison, we

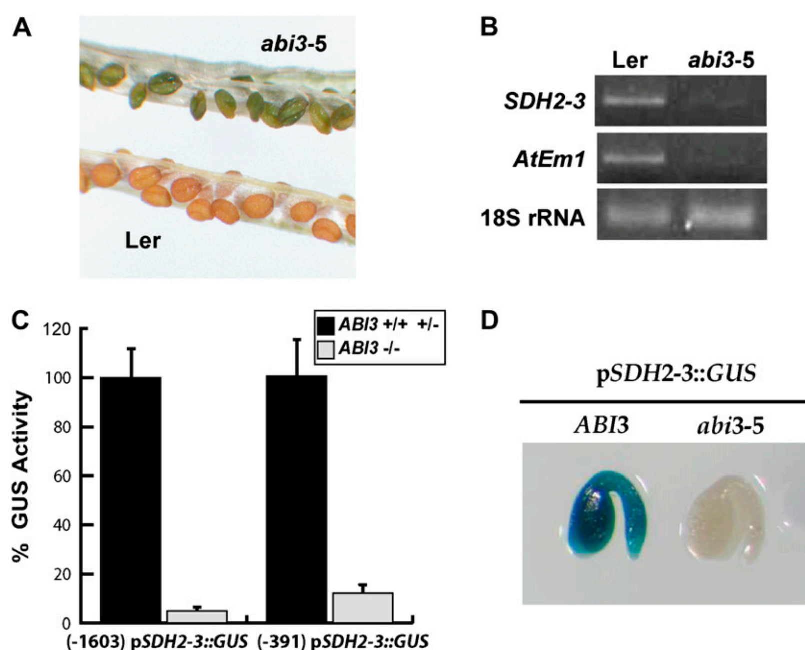


**Figure 2.** ABI3 regulates *SDH2-3* expression in seeds. A, Homozygous *abi3-5* and wild-type (L*er*) siliques. B, *SDH2-3* expression is drastically reduced in *abi3-5* seeds. RT-PCR analyses of *SDH2-3* and *AtEm1* transcripts were performed on total RNA from wild-type and *abi3-5* seeds. Thirty-five amplification cycles were used for *SDH2-3* and *AtEm1*, and 15 cycles were used for the 18S rRNA load control. C, *SDH2-3* promoter activity is reduced in *abi3-5* seeds. GUS activities were measured in protein extracts from brown seeds (black bars), carrying at least one wild-type ABI3 allele, and green homozygous *abi3-5* seeds (gray bars). A value of 100% activity corresponded to 452 ± 63 nmol 4-methylumbelliferone $h^{-1}$ $mg^{-1}$ protein for the long *SDH2-3 promoter::GUS* construct and 141 ± 25 nmol 4-methylumbelliferone $h^{-1}$ $mg^{-1}$ protein for the short *SDH2-3 promoter::GUS* construct. Four biological replicates were performed. D, The *SDH2-3* promoter is inactive in *abi3-5* embryos. GUS staining was performed on embryos extracted from yellow and green seeds prior to complete desiccation.

analyzed *fus3-3* developing seeds, since *FUS3* mRNA is present until the dry seed stage: expression of *SDH2-3* (and *At2S3*) was drastically reduced in *fus3-3* seeds from the beginning of maturation to the dry seed stage (Fig. 5B).

Therefore, our results reveal that ABI3, FUS3, and LEC2 have a profound effect on *SDH2-3* expression and that LEC2 is only necessary before desiccation.

### FUS3 and ABI3 Bind the *SDH2-3* Promoter

RY motifs are putative targets for B3 domain transcription factors (Suzuki et al., 1997; Reidt et al., 2000; Kroj et al., 2003; Mönke et al., 2004). To investigate whether these factors could recognize in vitro the *SDH2-3* RY promoter element, ABI3 and FUS3 proteins expressed in *Escherichia coli* were tested for their ability to bind to an oligonucleotide containing this element in an electrophoretic mobility shift assay (EMSA). No retardation was observed with the ABI3 protein (data not shown). However, FUS3 was able to bind to the RY probe but not to the RYm element, indicating that RY sequence integrity is required for proper recognition by the transcription factor (Fig. 6A).

Mönke et al. (2004) described a sensitive ELISA-type test to analyze protein-DNA interactions using biotinylated DNA fragments fixed to the solid phase and soluble proteins. Binding of recombinant ABI3 containing the T7 tag at the N-terminal end, and of recombinant FUS3-glutathione *S*-transferase (GST) to fixed ABRE1-RY and ABRE2-ABRE3 probes, was detected with anti-T7 tag or anti-GST antibodies. FUS3 recognized only the RY-containing element, confirming the results obtained by EMSA (Fig. 6B). In contrast to the EMSA results, binding of ABI3 to the same probe was demonstrated using this system. Both proteins did not bind or bound poorly to the ABRE2-ABRE3 sequence.

### Basic Leu Zipper Transcription Factors Bind the *SDH2-3* Promoter

ABRE elements (also called G-boxes) are targets for basic Leu zipper (bZIP) transcription factors. Thus, we analyzed the binding properties of bZIP factors to the *SDH2-3* promoter. We chose two members of group C of bZIPs (Jakoby et al., 2002), bZIP10 and bZIP25, functionally related to maize Opaque 2 and reported to induce SSP expression synergistically with ABI3 (Lara et al., 2003). The group S1 bZIP53 is a dimerizing partner of bZIP10 and bZIP25 (Ehlert et al., 2006; Weltmeier et al., 2006) and is the only S1 member with an expression pattern in seed development matching that of *SDH2-3*.

Recombinant proteins were expressed in *E. coli* and tested for their ability to bind to a probe containing ABRE2 and ABRE3 in an EMSA assay. In this system, bZIP53, but not bZIP10 or bZIP25, was able to bind to the ABRE2-3 probe (Fig. 7A). bZIP53 binding occurs specifically through the ABRE sequences, since mutations at these sequences abolished binding. However, when the ELISA binding test was used to analyze the effect of bZIP53 on bZIP10 and bZIP25 binding to a fixed ABRE2-ABRE3 probe, binding of bZIP10 and, to a lesser extent, of bZIP25 could be detected. More importantly, their binding was enhanced in the presence of bZIP53 (Fig. 7B). Interactions between bZIP53 and either bZIP10 or bZIP25 were also observed in EMSA assays (Supplemental Fig. S1; data not shown).

Altogether, these results are consistent with the hypothesis that the *SDH2-3* promoter is a target of

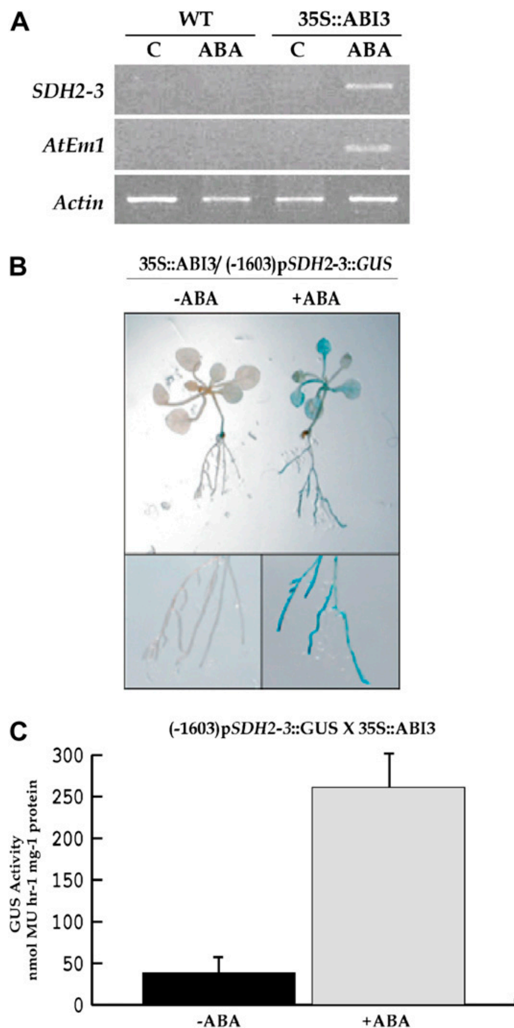


**Figure 3.** *SDH2-3* induction by ABA and ABI3 in vegetative tissue. A, ABA induction of *SDH2-3* in *35S*::*ABI3* plantlets. RT-PCR analyses of *SDH2-3* and *AtEm1* transcripts were performed on RNA prepared from 2-week-old wild-type (WT) and transgenic *35S*::*ABI3* seedlings incubated for 48 h with or without 50 $\mu$M ABA. B, ABA induction of *SDH2-3 promoter*::*GUS* in seedlings ectopically expressing ABI3. Two-week-old (−1,603) *pSDH2-3*::*GUS/35S*::*ABI3* seedlings were incubated for 48 h with or without 50 $\mu$M ABA and then stained for GUS activity. C, GUS activity in protein extracts from leaves of 60-d-old plants infiltrated and then incubated for 40 h with or without 10 $\mu$M ABA. Four biological replicates were performed. MU, 4-Methylumbelliferone.

bZIP transcription factors and that ABRE boxes are involved in promoter recognition by these factors.

### SDH2-3 Is the Main Iron-Sulfur Subunit of Complex II from Mature Seeds

To evaluate the role of *SDH2-3* in complex II biogenesis, two *sdh2-3* mutant lines were identified and characterized. Insertion/*SDH2-3* gene junctions were sequenced, demonstrating that no major deletions or chromosomal rearrangements took place during the insertional events. In the dSpm line, the transposon was confirmed to be in the fourth of five exons, interrupting codon 221, and in the DsLox line, the T-DNA interrupted intron 2 (Fig. 8A). Segregation of Basta resistance and Southern-blot analysis of homozygous *sdh2-3* dSpm and DsLox mutant plants (Fig. 8B) were consistent with one dSpm and two DsLox insertions. Northern-blot (data not shown) and reverse transcription (RT)-PCR (Fig. 8C) analyses showed that no *SDH2-3* mRNA was detected in mutant plants. Altogether, these results indicate that both mutant lines possess knockout alleles of *SDH2-3* and that any phenotypic alteration observed in the dSpm line could be linked to the *sdh2-3* mutated allele.

Mature embryos from wild-type, dSpm, and DsLox seeds were assayed for in situ SDH activity as described by Baud and Graham (2006). SDH was clearly detected in wild-type embryos, being homogenous throughout the embryo, and there was negligible background activity in the absence of succinate (Fig. 9A). Interestingly, this activity was greatly reduced in both dSpm and DsLox homozygous *sdh2-3* knockout mutants (Fig. 9A), clearly indicating that *SDH2-3* codes for most of the iron-sulfur protein of embryo complex II. Nevertheless, SDH activity was detected in the mutant plants, likely resulting from basal expression of *SDH2-1* and/or *SDH2-2*.

*SDH2-1* and *SDH2-2* transcripts are low in dry seeds (Elorza et al., 2006). To analyze their expression during embryo development, we used plants transformed with fusions of the *SDH2-1* and *SDH2-2* promoters to the GUS reporter gene (Elorza et al., 2004). Embryos from the early maturation stage to the desiccation stage were dissected and stained for GUS activity (Fig. 10A). Whereas no or very weak GUS staining was observed for the *SDH2-2* promoter, GUS expression was detected for the *SDH2-1* promoter. Interestingly, *SDH2-1* promoter activity decreased during embryo development (Fig. 10A), but GUS staining was not

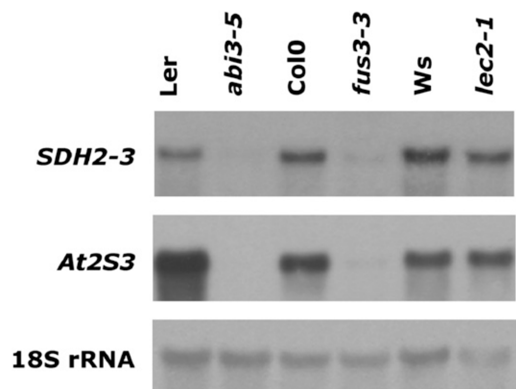


**Figure 4.** *SDH2-3* expression in *fus3-3*, *lec2-1*, and *abi3-5* mutant dry seeds. Northern blot analysis of *SDH2-3* and *At2S3* transcripts was performed on 10 $\mu$g of total RNA from mutant dry seeds and their respective wild-type controls. Ws, Wassilewskija.

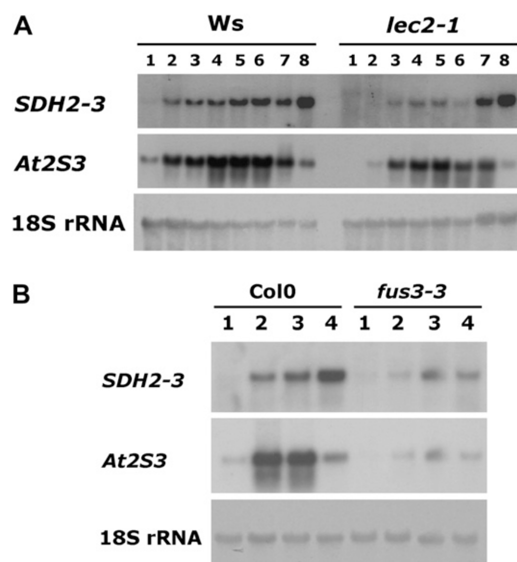


**Figure 5.** *SDH2-3* expression is reduced during maturation of *lec2-1* seeds. A, Northern-blot analysis of *SDH2-3* and *At2S3* transcripts during *lec2-1* and wild-type (Wassilewskija [Ws]) seed development. Total RNA was extracted from maturing green siliques (thin, approximately 8 mm long [lane 1] to thick, approximately 14 mm long [lane 6]), yellowing siliques (lane 7), and yellow siliques (lane 8). B, Analysis of *SDH2-3* and *At2S3* transcripts during *fus3-3* and wild-type (Col-0) seed development. Total RNA was extracted from maturing green siliques (thin, approximately 8–10 mm long [lane 1] and thick, 12–14 mm long [lane 2]), yellowing siliques (lane 3), and yellow siliques (lane 4).

completely eliminated in dry seeds. Furthermore, data on *SDH2-1* and *SDH2-2* expression from public expression databases confirm that seed *SDH2-1* expression decreases from the torpedo to the green cotyledon stage and that *SDH2-2* is expressed at a very low level, if any (Fig. 10B; http://www.bar.utoronto.ca; Schmid et al., 2005).

### Germination Is Retarded in Seeds Lacking a Functional *SDH2-3* Gene

Homozygous *sdh2-3* mutant plants showed no obvious phenotypic defects during vegetative or reproductive growth when compared with wild-type plants, at least under the growth conditions used (Supplemental Fig. S2). These results indicate that *SDH2-3* is not an essential gene for Arabidopsis growth and development. Given its expression pattern, we decided to investigate the germination of mutant *sdh2-3* and wild-type seeds. Germination of *sdh2-3* mutant seeds was retarded compared with that in the wild type (Fig. 9B), suggesting an important role of SDH2-3 for seed germination.

## DISCUSSION

The specific expression pattern of *SDH2-3* during seed maturation raises interesting questions about its regulation and function. A similar pattern has been described only once for a mitochondrial protein, a pea (*Pisum sativum*) LEA protein. This protein may be involved in protecting the inner mitochondrial membrane during seed desiccation (Grellet et al., 2005; Tolleter et al., 2007); however, no data concerning the regulation of its expression are available. Here, we have performed a detailed characterization of the *SDH2-3* promoter and identified key regulatory elements and transcription factors involved in its regula-

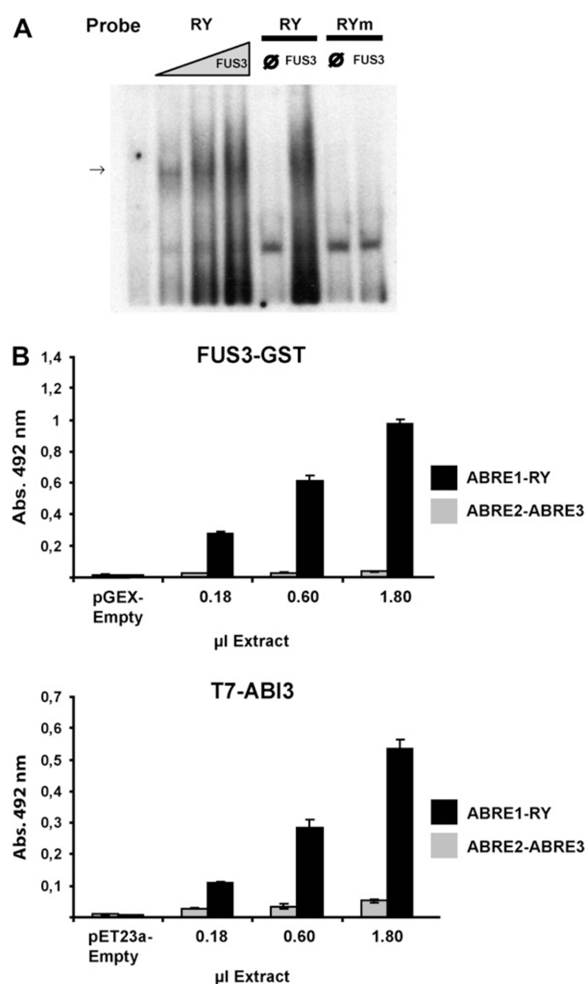


**Figure 6.** FUS3 and ABI3 bind to probes containing the RY element present in the *SDH2-3* promoter. A, EMSA of the native RY probe for increasing concentrations (1:3–1) of a protein extract containing FUS3. Controls were performed with extracts from bacterial cells transformed with the empty pET23a vector (lanes ∅) or the mutated RYm element as probe (lanes RYm). The arrow shows the specific band of the interaction. B, Binding of FUS3 and ABI3 to the ABRE1-RY DNA. Increasing quantities (1:10–1) of extracts from bacteria expressing FUS3-GST or T7 tag-ABI3 were assayed for binding to DNA containing either ABRE1 and RY elements or ABRE2 and ABRE3 elements. Binding was measured by ELISA, with an antibody against GST or T7 tag, conjugated with HRP. Control binding reactions were performed with an extract from bacterial cells transformed with the empty vectors.

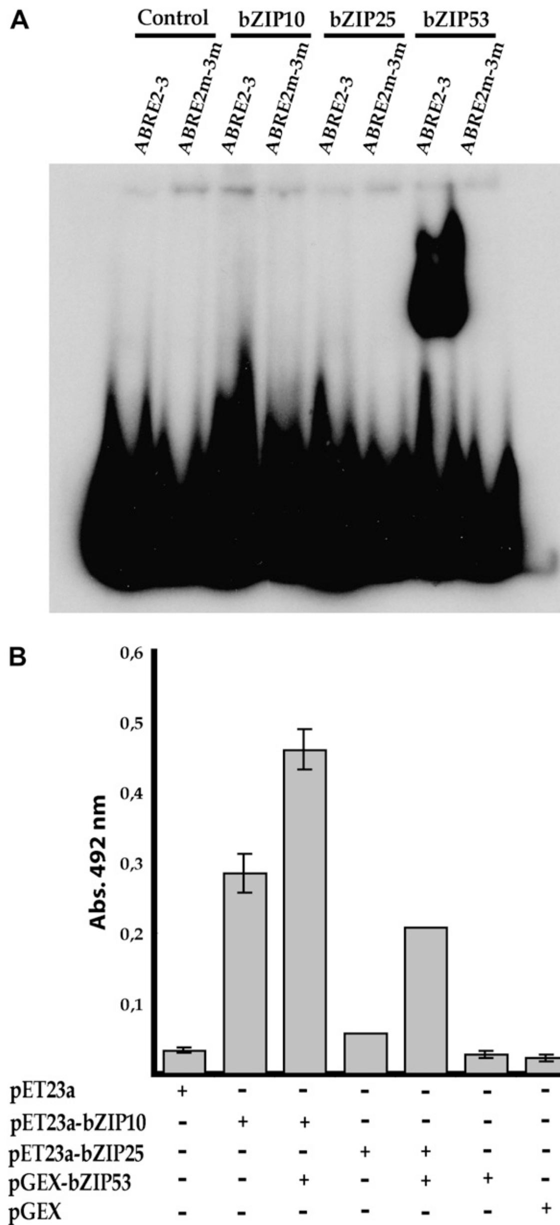


**Figure 7.** bZIP53 transcription factor binds the ABRE elements present in the *SDH2-3* promoter. A, EMSA of the native (ABRE2-3) and mutated (ABRE2m-3m) probes with protein extracts containing bZIP10, bZIP25, or bZIP53. Controls were performed with extracts from bacterial cells transformed with the empty pET23a vector. Equal concentrations of bZIP proteins in extracts were determined by western blot with appropriate antibodies. B, Binding of bZIP10 and bZIP25 to the ABRE2-ABRE3 DNA. Extracts from bacteria expressing T7 tag-bZIP10 or T7 tag-bZIP25 were assayed for binding to DNA containing ABRE2 and ABRE3 elements in the presence or absence of bZIP53-GST. Binding was measured by ELISA, with an antibody against the T7 tag (bZIP53-GST was not detected). Control binding reactions were performed with extracts from bacterial cells transformed with the empty vectors. Abs., Absorbance.

tion. Moreover, analysis of *sdh2-3* loss-of-function plants revealed its participation in early stages of seed germination.

**ABRE and RY Motifs Are Major cis-Elements Regulating *SDH2-3* Expression**

ABREs (G-boxes) have been implicated in SSP and LEA protein gene regulation and shown to function effectively when two copies are located in tandem or when it is associated with a coupling or enhancer element like the RY motif (Busk and Pagès, 1998; Leung and Giraudat, 1998; Nambara and Marion-Poll, 2003). In the *SDH2-3* promoter, ABRE2 and ABRE3 are separated by a short 6-bp sequence and ABRE1 is located eight nucleotides upstream of the RY enhancer element (Fig. 1A). When ABRE and RY motifs were mutated individually, promoter activities were significantly reduced, indicating that they all act synergistically to give high expression in mature seeds (Fig. 1B). Since ABRE2 mutation almost abolishes GUS expression, this element appears to be the most important by itself. Considering that the mutations introduced in ABRE and RY boxes have been shown to abolish the binding of bZIP and B3 domain transcription factors, respectively (Reidt et al., 2000; Bensmihen et al., 2002), these results suggest that transcription factors from these families may be directly involved in *SDH2-3* regulation.

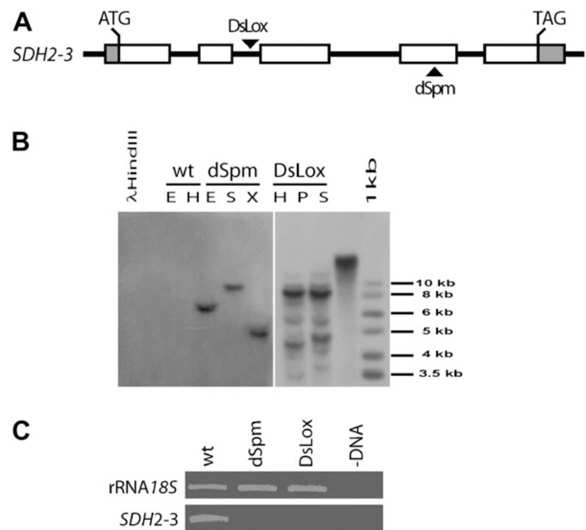


**Figure 8.** Identification of knockout mutant *sdh2-3* plants. A, Genomic organization of the *SDH2-3* gene. Exons are presented as boxes, and insertion sites are indicated by arrowheads. B, Southern-blot analysis was performed with total DNA (6 $\mu$g) from wild-type (wt) or homozygous *sdh2-3* mutant plants (dSpm and DsLox). DNA was digested with *Eco*RV (E), *Hin*dIII (H), *Spe*I (S), *Xba*I (X), or *Pst*I (P) and hybridized with a probe directed to the Basta resistance gene present in the T-DNA (DsLox) and transposon (dSpm). The probe identified one DNA fragment in dSpm mutant plants and at least two DNA fragments in DsLox mutant plants. The DNA fragment in the dSpm mutant and one of the two main DNA fragments in the DsLox mutant have the expected sizes. Phage λ DNA digested with *Hin*dIII and a 1-kb ladder were used as size markers. C, RT-PCR analysis of *SDH2-3* transcripts in wild-type and mutant plants. Thirty-five amplification cycles were used for *SDH2-3* and 15 cycles were used for the 18S rRNA load control. Lane –DNA corresponds to a PCR control without template.

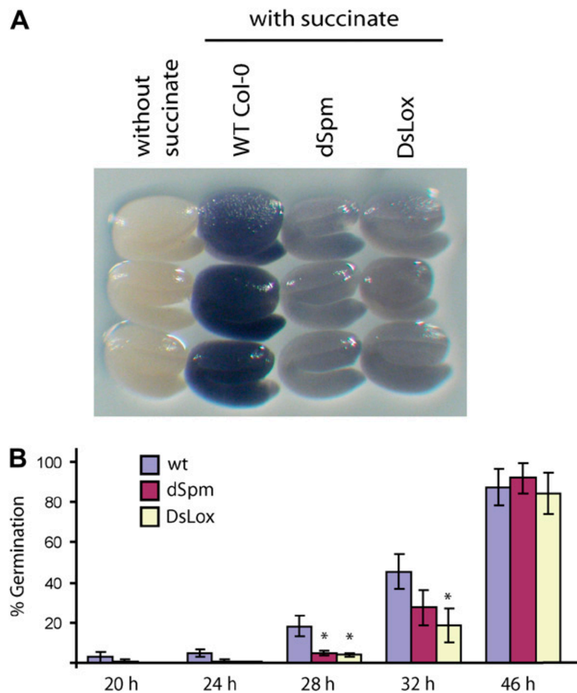


**Figure 9.** Effects of the *sdh2-3* mutations on seed SDH activity and germination. A, SDH activity in mature embryos of wild-type (WT) and *sdh2-3* dSpm and DsLox mutant plants. B, Germination of wild-type and *sdh2-3* mutant seeds. Values are means ± SE of three biological replicates carried out on batches of approximately 100 seeds. Asterisks indicate significant differences according to Student's *t* test ($P < 0.02$).

### B3 Domain Transcription Factors Regulate *SDH2-3* Expression in Vivo and Interact with the *SDH2-3* Promoter in Vitro

ABI3, FUS3, and LEC2 master regulators exhibit partially overlapping expression patterns and participate in an intricate and not fully understood network of cross-regulations involved in most seed maturation aspects, including storage compound synthesis (To et al., 2006; Santos-Mendoza et al., 2008, and refs. therein). We have used the severe *abi3-5*, *fus3-3*, and *lec2-1* mutants to address their role in *SDH2-3* expression. Here, we have demonstrated that *SDH2-3* transcript level is drastically reduced in *abi3-5*, *fus3-3*, and maturing *lec2-1* seeds (Figs. 2B, 4, and 5). These results obtained with individual mutants show that these three plant-specific transcription factors containing the conserved B3 DNA-binding domain regulate *SDH2-3* expression, likely at the level of transcription from the *SDH2-3* promoter, as shown for ABI3 (Figs. 2, C and D, and 3).

*LEC2*, *FUS3*, and *ABI3* expression begins early (from the heart stage) in maturation (Parcy et al., 1994; Kroj et al., 2003). However, *LEC2* expression decreases during desiccation, and only *ABI3* and *FUS3*, whose expression is maintained, are required to establish tolerance to desiccation. Interestingly, dependence of *SDH2-3* expression on LEC2 correlates with the known *LEC2* expression pattern, since once seeds entered desiccation, *SDH2-3* expression was not or was only slightly affected by the *lec2-1* mutation. Therefore, our results are consistent with a model in which *SDH2-3* expression is regulated by the three transcription factors before desiccation, and then *LEC2* becomes dispensable.

The described in vivo analysis of mutants in *ABI3*, *FUS3*, and *LEC2* does not elucidate if these transcription factors directly or indirectly trigger *SDH2-3* expression. Recent studies have demonstrated that they directly controlled the induction of SSP gene expression, recognizing the RY motifs present in the promoters of these target genes (Ezcurra et al., 2000; Reidt et al., 2000; Kroj et al., 2003; Mönke et al., 2004; Braybrook et al., 2006). Accordingly, we found that FUS3 is able to specifically bind the RY element of the *SDH2-3* promoter in EMSA assays (Fig. 6A), in agreement with observations made on the *Vicia faba* legumin and the Arabidopsis *At2S3* albumin promoters (Reidt et al., 2000; Kroj et al., 2003). Only using a more sensitive ELISA method have we also shown that ABI3 interacts directly with a probe containing the RY element (Fig. 6B). Altogether, our results suggest that

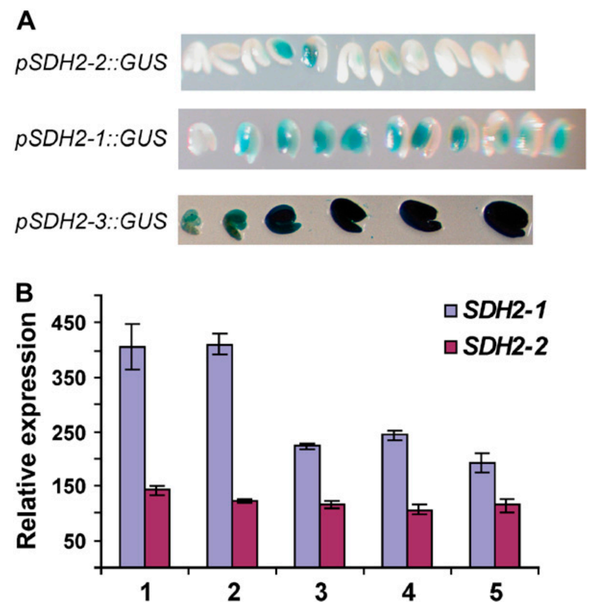


**Figure 10.** Expression of *SDH2* genes during embryo maturation. A, Embryos manually dissected from seeds were stained for GUS activity. Plants carrying the promoters of *SDH2-1*, *SDH2-2*, and *SDH2-3* fused to the GUS gene have been described (Elorza et al., 2004). Walking-stick to dry seed embryos were stained for 60 h (*pSDH2-2*::*GUS*) or 14 h (*pSDH2-1*::*GUS* and *pSDH2-3*::*GUS*). B, Expression of *SDH2-1* and *SDH2-2* during seed development. Data are from Schmid et al. (2005) and were obtained at www.bar.utoronto.ca. Mean signal intensities were averaged from three replicates. Expression was analyzed in whole seeds with mid to late torpedo embryos (lane 1), late torpedo to early walking-stick embryos (lane 2), walking-stick to early curled cotyledon embryos (lane 3), curled cotyledons to early green cotyledons (lane 4), and green cotyledons (lane 5).

ABI3 and FUS3 interact, probably with different affinities or by different mechanisms, with the *SDH2-3* promoter and directly regulate its expression. It is worth noting that, to the best of our knowledge, there are no previous reports showing that these B3 domain master regulators may directly regulate a gene involved in primary metabolism. Nevertheless, it has to be pointed out that indirect regulatory effects for B3-type transcription factors have also been reported (Gazzarrini et al., 2004; To et al., 2006) and cannot be ruled out in the case of *SDH2-3*, especially concerning its regulation by LEC2.

### bZIP Transcription Factors Bind the *SDH2-3* Promoter

Emerging models of SSP and LEA gene regulation by ABI3 suggest that, in addition to a likely weak direct interaction with RY promoter elements, ABI3 is recruited at the promoter level by bZIP proteins interacting with ABRE elements. For instance, ABI3 interacts with the bZIP transcription factor ABI5, which in turn is able to bind to LEA promoters and regulate their expression through a domain unique to ABI3 and not present in FUS3 or LEC2 (Nakamura et al., 2001). We found that *SDH2-3* expression was not reduced in *abi5* mutant plants (data not shown), suggesting that this bZIP transcription factor is not implicated in regulating the *SDH2-3* promoter. Similarly, ABI3 is also able to interact with bZIP10 and bZIP25 in regulating SSP genes (Lara et al., 2003).

We have found that ABRE elements in the *SDH2-3* promoter are strongly bound by bZIP53 (Fig. 7A) but less prominently by bZIP10 and bZIP25 binding (Fig. 7B). Furthermore, band-shift assays performed in the presence of bZIP53 and either bZIP25 or bZIP10 revealed interactions between the bZIP factors and the increased DNA-binding capacity of the heterodimers (Supplemental Fig. S1; data not shown). These data support the idea that the ABRE elements present in the *SDH2-3* promoter are recognized by group C bZIP/group S1 bZIP heterodimers.

Based on our results and on previous studies (Ezcurra et al., 2000; Kroj et al., 2003; Lara et al., 2003), we propose a model that predicts that the activation of *SDH2-3* would require proteins of the B3 (ABI3, FUS3, and LEC2) and bZIP (bZIP53 and either bZIP10 or bZIP25) classes. According to this model, FUS3 (and probably LEC2) and bZIP53/group C bZIP would recognize RY and ABRE sequences, respectively, whereas ABI3 may interact weakly with the *SDH2-3* promoter and also be tethered to the promoter through interactions with the bZIP heterodimers. Accordingly, mutations in RY would prevent FUS3 binding, and consequently, *SDH2-3* expression would be reduced. Similarly, mutations in ABRE boxes preclude bZIP heterodimer binding to the promoter and recruitment of ABI3, resulting in a drastic reduction of *SDH2-3* promoter transactivation, as observed when ABI3 itself is inactivated.

At present, only a few promoters of mitochondrial protein genes have been analyzed (Zabaleta et al., 1998; Satoh et al., 2002; Welchen et al., 2004; Dojcinovic et al., 2005; Weltmeier et al., 2006; González et al., 2007; Ho et al., 2007). However, to the best of our knowledge, the *SDH2-3* promoter is the first embryo-specific promoter characterized for a mitochondrial respiratory protein and the first gene promoter from TCA cycle enzymes to be characterized in plants.

### Respiratory Complex II in Mature Seeds and during Germination Mainly Contains SDH2-3

Several processes essential for seed viability and germination occur during the maturation phase in seed development. Although we may speculate that *SDH2-3* induction is part of the metabolic adaptations occurring during maturation, and that SDH2-3 is important for SDH activity under the conditions prevailing during maturation and desiccation stages, the lack of any visible phenotype in developing seeds from homozygous *sdh2-3* knockout plants indicates that *SDH2-3* is not essential for seed set and viability. The fact that *SDH2-1* is residually expressed in maturing embryos (Fig. 10) may explain this observation.

The steady-state abundance of *SDH2-3* transcripts is high in mature embryos, whereas *SDH2-1*and *SDH2-2* are expressed at very low levels (Elorza et al., 2006; Fig. 10). These observations correlate well with data obtained for SDH activity (Fig. 9A), which is greatly reduced in the two *sdh2-3* homozygous mutants. Residual SDH activity detected in the knockout lines is likely due to the presence of SDH2-1 and/or SDH2-2 and is sufficient to sustain germination. We have previously found that mitochondrial complex II is essential for gametophyte development in Arabidopsis using heterozygous mutant plants for the flavoprotein gene (*SDH1*; León et al., 2007). However, the lack of *sdh1/sdh1* homozygous seeds precluded the analysis of the effect of SDH absence on seed development and germination.

The imbibing seed resumes metabolic activity within minutes of water entering its cells, and rapid increases in respiration rate accompany the earliest stages of germination (Bewley and Black, 1994). Logan et al. (2001) showed that succinate-dependent $O_2$ consumption and citric acid cycle activity increased rapidly during imbibition of maize (*Zea mays*) embryos. We have determined that 2-thenoyltrifluoroacetone, a complex II inhibitor, completely blocks germination (data not shown), strongly supporting an essential role of complex II in this process. An appealing hypothesis is that high *SDH2-3* expression in mature embryos allows for a rapid increase in SDH activity upon imbibition, before expression of *SDH2-1* and *SDH2-2*. This assumption is supported by the observation that germination is retarded in the *sdh2-3* mutants (Fig. 9B). During germination and postgerminative growth, *SDH2-3* transcripts decline, and *SDH2-1* and *SDH2-2* transcript levels increase (Elorza et al., 2006), suggesting

that the embryo-specific SDH2-3-containing complex II is replaced by a complex II containing SDH2-1 or SDH2-2. This turnover may explain why the lack of *SDH2-3* has only an effect on germination kinetics. To our knowledge, this is the first study concerning the expression of TCA cycle genes during seed development and the characterization of phenotypic alterations during germination in mutant lines.

## MATERIALS AND METHODS

### Plant Growth and Transformation

Arabidopsis (*Arabidopsis thaliana* ecotype Columbia [Col-0], Landsberg *erecta* [L*er*], or C24) seeds were cold treated for 48 h at 4°C in darkness and then germinated and grown hydroponically at 20°C to 24°C under a 16-h-light/8-h-dark cycle (Gibeaut et al., 1997). *Agrobacterium tumefaciens*-mediated transformation of Arabidopsis plants was accomplished using the floral dip protocol (Clough and Bent, 1998). Seeds of the T1 generation were selected for resistance to hygromycin. At least 12 independent transgenic lines were obtained for each construct, and transgene presence was verified by PCR.

For germination assays, surface-sterilized seeds were stratified at 4°C for 48 h in the dark, sown on half-concentrated Murashige and Skoog medium solidified with 0.8% (w/v) agar, and incubated at 20°C to 24°C under a 16-h-light/8-h-dark cycle. Germination was scored at different times based on radicle emergence.

Heterozygous *abi3-5*, *fus3-3*, and *lec2-1* seeds were obtained from the Arabidopsis Biological Resource Center (ABRC; http://www.arabidopsis.org/abrc). Homozygous mature *abi3-5* and *lec2-1* mutant seeds were identified in siliques of heterozygous plants by their characteristic phenotype and sown to get homozygous mutant plants. Homozygous *fus3-3* seeds were selected before desiccation, because of the ability of immature embryos to germinate on 0.5× Murashige and Skoog medium solidified with 0.8% (w/v) agar supplemented with 1% (w/v) Suc. Growing plantlets were transferred and grown hydroponically. Seeds from a homozygous transgenic Arabidopsis line (A19, C24 background) carrying a transcriptional fusion between the double enhanced cauliflower mosaic virus 35S promoter and the *ABI3* cDNA (*35S*::*ABI3*) were kindly provided by François Parcy (Commissariat à l'Energie Atomique-INRA-Université Joseph Fourier, Grenoble, France).

Homozygous transgenic plants carrying a *SDH2-3* promoter fragment (1,603 or 391 bp) and the *SDH2-3* 5′ untranslated region (UTR) fused to GUS (Elorza et al., 2004) were crossed with homozygous *abi3-5* and *35S*::*ABI3* plants and their respective controls (L*er* and C24). F1 seeds from the *abi3-5* cross were germinated on hygromycin to select for the p*SDH2-3*::*GUS* construct, and homozygous *abi3-5* F2 seeds were identified by their green phenotype. F1 seeds from the *35S*::*ABI3* cross were germinated on hygromycin (p*SDH2-3*::*GUS* construct) and kanamycin (*35S*::*ABI3* construct).

### Promoter Mutagenesis and GUS Activity Assays

Mutagenesis was performed by PCR, using a construct containing the *SDH2-3* promoter and 5′ UTR fused to GUS as a template (construct P5; Elorza et al., 2004). Two PCRs were carried out for each mutant with the same template. For the promoter mutated in the RY element (RYm), one amplification was done with primers SDH2-3F and RYmR and the other was done with primers SDH2-3R and RYmF (Supplemental Table S1). Both amplification products were purified by electrophoresis through agarose gels, and their mixture was used as a template for a third PCR with primers SDH2-3F and SDH2-3R. This PCR product was cloned into pGEM-T plasmid (Promega), and the DNA fragment obtained by digestion with *Bam*HI and *Nco*I was ligated into pCAMBIA 1381 (http://www.cambia.org).

The same procedure was employed for constructs mutated in ABRE1 (ABRE1m), ABRE2 (ABRE2m), or ABRE3 (ABRE3m), with SDH2-3F, SDH2-3R, and the following primers: for ABRE1m, ABRE1mF and ABRE1mR; for ABRE2m, ABRE2mF and ABRE2mR; and for ABRE3m, ABRE3mF and ABRE3mR (for primer sequences, see Supplemental Table S1). To obtain the construct mutated in the three ABRE elements (3xABREm), the ABRE1 mutant was used as a template in the first step and mutation in ABRE2 was introduced as described. In the second step, mutation in ABRE3 was introduced with primers 3XABREmF and 3XABREmR.

The structures of constructs were verified by DNA sequencing. All constructs contain a promoter fragment (391 bp) and the 5′ UTR up to the first *SDH2-3* codon in frame with the GUS reporter gene and were introduced into *A. tumefaciens* GV3101 by electroporation. Arabidopsis transgenic plants carrying the mutations in the *SDH2-3* promoter fused to the GUS reporter gene were obtained, and soluble extracts of plant tissues were assayed for GUS activity by fluorometric measurements using 4-methylumbelliferyl-$\beta$-D-glucuronide (Sigma-Aldrich) as a substrate (Jefferson, 1987). Protein concentrations were determined by the Bradford method (Bradford, 1976), and GUS activities were calculated as nanomoles of 4-methylumbelliferone per hour per milligram of protein. Histochemical GUS staining was performed as described (Elorza et al., 2004).

### Nucleic Acid Preparation and Analysis

Total DNA was prepared from green leaves according to Ausubel et al. (1994). Southern-blot analysis was performed by standard procedures with a $^{32}$P-labeled probe derived from the Basta resistance gene by PCR amplification between primers BastaF and BastaR. Total RNA was extracted from seeds (50 mg) by the method of Vicient and Delseny (1999) and further purified using the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's protocol. Northern-blot analyses were performed as described (Elorza et al., 2006). Probes were obtained by PCR amplification using the following primer pairs: for *SDH2-3*, sdh2-58 and sdh2-63; for *At2S3*, At2S3F and At2S3R; and for 18S rRNA, 18SF and 18SR.

cDNA synthesis was performed on 2 $\mu$g of RNA with random hexamers as primers, according to the instructions of the ImProm-II RT system (Promega). PCR amplifications were performed in 50 $\mu$L, with one-twentieth of the cDNA and 0.5 units of AmpliTaq Gold (Applied Biosystems). The following primer pairs were used: for *SDH2-3*, sdh2-57 or sdh2-50 and sdh2-51; for the LEA *AtEm1* transcript, Em1F and Em1R; for actin mRNA, actinF and actinR; and for 18S rRNA, 18SF and 18SR.

### EMSA

The cDNAs encoding AtbZIP10, AtbZIP25, AtbZIP53, and ABI3 proteins were cloned into the expression vector pET23a (Novagen), and FUS3 cDNA was cloned into pGEX-2T vector (Amersham Biosciences) as a translational fusion to GST. Expression in *Escherichia coli*, preparation of protein extracts, and EMSAs were as described previously (Lara et al., 2003). To obtain the DNA probes, overlapping oligonucleotides were annealed and end labeled with [$^{32}$P]dATP by the fill-in reaction (Klenow exo-free DNA polymerase; USB Corporation [http://www.usbweb.com]) and purified by 8% PAGE (39:1 cross-linking). The following oligonucleotides were used: for the wild-type RY probe, RY1 and RY2; for the mutated RYm probe, RYm1 and RYm2; for the wild-type ABRE2-3 probe, ABRE2-3a and ABRE2-3b; and for the mutated ABRE2m-3 m probe, ABRE2m-3mF and ABRE2m-3mR.

### DNA-Protein Interaction Analysis by ELISA

The ELISA technique was used to study DNA-protein interaction as described by Mönke et al. (2004). Streptavidin-coated microwell strips (Nunc) were used, and the following double-stranded biotinylated DNA fragments were prepared according to Mönke et al. (2004): for the ABRE2-ABRE3 probe, ABRE2-ABRE3F and ABRE2-ABRE3R; and for the ABRE1-RY probe, ABRE1-RYF and ABRE1-RYR. Antibodies against T7 tag (Novagen) and GST (Amersham Biosciences) conjugated with horseradish peroxidase (HRP) were used to detect bound transcription factors. HRP activity was determined by adding 60 $\mu$L of a solution containing 0.015% hydrogen peroxide (Sigma-Aldrich) and *o*-phenylenediamine dihydrochloride (one tablet from Dako [http://www.dako.com] dissolved in 3 mL). After incubation at 37°C, the reaction was stopped with 60 $\mu$L of 2 N HCl, and the $A_{492}$ was measured with a plate reader (Tecan).

### Isolation of Insertional *sdh2-3* Mutants

Insertional mutant lines were searched at two Web sites: the Arabidopsis Insertion Database (http://atidb.org/cgi-perl/index) and the T-DNAExpress (http://signal.salk.edu/cgi-bin/tdnaexpress). Two mutant alleles were identified, one in the Wisconsin DsLox T-DNA population (DsLox503G03) and the other in the Sainsbury Laboratory Arabidopsis Transposants dSpm population (line SM_3.623). Arabidopsis seeds from these lines (Col-0 background)

were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham) and the ABRC, respectively. To isolate homozygous mutant plants, seedlings were genotyped by a PCR-based approach using total DNA extracted from one cotyledon or one small leaf and primers flanking the insertion point for the wild-type allele and a gene-specific and left border-specific primer pair for the mutant alleles. For the dSpm line, primers sdh2-57 and sdh2-51 were used for the wild-type allele and primers dspm3′ and sdh2-51 were used for the mutant one. For the DsLox line, the wild-type allele was amplified with sdh2-50 and sdh2-51 and the mutant allele was amplified with LBW and sdh2-51.

## Histochemical SDH Assay

A simplified protocol, based on the procedure described by Baud and Graham (2006), was used. Seeds were imbibed for 12 h at 4°C in the dark, excised embryos were incubated for 1 h at 37°C in 50 mM sodium phosphate (pH 7.6) and 150 mM NaCl, and finally, staining was performed by incubation for 3 h at 30°C in 50 mM sodium phosphate (pH 7.6), 7.5 mM succinate (fresh), and 2.4 mM nitroblue tetrazolium (Calbiochem). As controls, embryos were incubated in parallel in the same buffer without succinate. After staining, embryos were washed with distilled water and viewed using a stereoscopic microscope (Nikon SMZ800), and photographs were taken with a Nikon Coolpix 4500 CCD digital camera.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AB013395 and AJ278912.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Binding of bZIP25 and bZIP53 proteins to the ABRE2-3 sequence of the *SDH2-3* promoter.

**Supplemental Figure S2.** *SDH2-3* is not essential for Arabidopsis growth and development.

**Supplemental Table S1.** List of oligonucleotides.

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