A Nuclear Gene Encoding the Iron-Sulfur Subunit of Mitochondrial Complex II Is Regulated by B3 Domain Transcription Factors during Seed Development in Arabidopsis1[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 DEHYDRGENASE2-3 (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 (At5g27380), SDH2-2 (At5g40650), and SDH2-3 (At5g05165), 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 Tielen, 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 (Bisk 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 (Bisk and Pages, 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 (Lemaître 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 (Leon 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).
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 containing 50 μM 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
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 bZIP3 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
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
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 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).

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 2) 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.
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 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.

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 µg) from wild-type (wt) or homozygous sdh2-3 mutant plants (dSpm and DsLox). DNA was digested with EcoRV (E), HindIII (H), SpeI (S), XbaI (X), or PstI (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 HindIII 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.
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...
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 abi3 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 may 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; Welch et al., 2003; 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 O2 consumption and citric acid cycle activity increased rapidly during imbibition of maize (Zea mays) embryos. We have determined that 2-thienyltrifluoroacetone, 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 [Ler], 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.

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 BanH1 and NcoI 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-β-D-glucuronide as a substrate (Sigma-Aldrich). All absorbance measurements 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 (Eloeza 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 32P-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 Vicent and Delseny (1999) and further purified using the RNeasy Plant Mini Kit (Qagen), according to the manufacturer’s protocol. Northern-blot analyses were performed as described (Eloeza et al., 2006). Primers were obtained by PCR amplification using the following primer pairs: for SDH2-3, sdh2-58 and sdh2-63; for At2S3m, At2S3mF and At2S3mR; and for 18S rRNA, 18SF and 18SR.

cDNA synthesis was performed on 2 μg of RNA with random hexamers as primers, according to the instructions of the ImProm-II RT kit (Promega). PCR amplifications were performed in 50 μl, 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 AthZIP10, AthZIP25, AthZIP53, and ABRE1 proteins were cloned into the expression vector pET2a (Novagen), and FUS3 cDNA was cloned into pGEX-2T vector (Amer sham 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 [32P]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 probe, ABRE2m-3F and ABRE2m-3R.

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 ABRE2-3ABRE3 probe, ABRE2-ABRE3 and ABRE2-ABRE3R; and for the ABRE1-1RY probe, ABRE1-1RYF and ABRE1-1RYR. 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 30 μl of a solution containing 0.05% 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 μl of 2 N HCl, and the A492 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://tdbib.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 (DsLox0530G30) and the other in the Sainsbury Laboratory Arabidopsis Transposants dbp 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 DsLox line, primers sdh2-57 and sdh2-51 were used for the wild-type allele and primers dsm50 and sdh2-51 were used for the mutant one. For the DLox 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 50 mM sodium phosphate (pH 7.6) and 150 mM NaCl, and finally, staining was performed by incubation excised embryos were incubated for 1 h at 37°C. The following materials are available in the online version of this article.

Supplemental Data

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

Supplemental Figure S1. Binding of bZIP25 and bZIP33 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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