PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE
Facultad de Ciencias Biológicas
Programa de Doctorado en Ciencias Biológicas
Mención Biología Celular y Molecular

"CARACTERIZACION DE LA REGULACION TRANSCRIPCIONAL Y FUNCION DEL GEN SDH2-3 DE *Arabidopsis thaliana* "

FRANKO RESTOVIC CARVAJAL

Abril 2013

PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE
Facultad de Ciencias Biológicas
Programa de Doctorado en Ciencias Biológicas
Mención Biología Celular y Molecular

"CARACTERIZACION DE LA REGULACION TRANSCRIPCIONAL Y FUNCION DEL GEN SDH2-3 DE *Arabidopsis thaliana* "

Tesis presentada a la Pontificia Universidad Católica de Chile como parte de los requisitos para optar al grado de Doctor en Ciencias Biológicas con mención en Biología Celular y Molecular

Por

FRANKO RESTOVIC CARVAJAL

Director de Tesis : Xavier Jordana de Buen

Comisión de Tesis: Dr. Jose Casaretto Vargas
Dra. Maria del Pilar Carvallo
Dr. Rodrigo Gutiérrez Ilabaca

Abril 2013

Acknowledgements

I would like to thank all the people who helped me through the years. It has been a long way, with ups and downs, that at the end took me to a better place than the one I used to be.

First of all, I would to express my infinite gratitude to my advisor, Dr. Xavier Jordana. He took me as a student in a difficult personal time and he trusted me all the way. He taught me how to be a rigorous, enthusiastic and responsible scientist. I could not ask for better guidance.

Also, I would like to specially thank María Isabel Gómez. Without her invaluable help this thesis would have been a lot harder. But more important, was her great advice in life matters that I will miss the most.

I would also like to thank my friends throughout these years. To Dani: thank you for all your support and for been there always with the right words. To Elena: for your incredible sincerity. To Cristián: for all the pipet tips I stole from you (I won´t pay you back). To Sara: for the incredible moments together. Believe me, this would not have been the same without you guys.

I would also like to specially thank Anita, Paula, Seba, Roberto, Esteban, Hannetz, Marcos, Peter, Claudia, Marco, Alejandro and all XJ former lab members.

Also, I would like to thank Dr. Loreto Holuigue, and all her lab members, especially Grace (for all the food and friendship), Luis, Ariel, Aldo, Daniel, Fran, Gigi, Ema, Eva, Marce, Trini and all actual and former lab members.

I would also like to thank my lifetime brothers Joaco, Tuna, Dani, Jimmy, Chino, Toño, Mame and Zorro for more than 20 years of friendship. Moreover, I would like to thank Mónica, Vero Codelia, Carmen, Gabo, Pao, Amparo, Vero Burzio, Sole, Claudio, Rodrigo and all my "science-related" friends.

I would like to express my deep thankfulness to Dr. Jesús Vicente-Carbajosa and Dr. Joaquín Medina, who kindly received me in their lab in Madrid (CBGP). Also, I would like to specially thank Eva, Maik, Iván, Jan, Paloma, Inés, Pilar, Estrella, Mar, Marcela, Manuel, Alicia, David, Vivi, Patri and all the beautiful people that I knew during these wonderful seven months abroad.

I would like to thank my family, which are my main support in life. My Mother and Father, which have always believed in me, I love you. I could not ask for better parents. A mi abuelita y abuelito, que los adoro con toda mi alma. My Aunt Claudia, Uncle JC, Maxi, Milán, Vic and Alexis. Also I would like to thank my mother-in-law Patricia and especially Uncle Gonzalo for all the support.

And last but not least, I would like to thank my wife Daniela. You are the only one that can really understand the importance of this big step. Thank you for being always in the good, and specially, the bad times. I love you deeply.

SUBJECT INDEX

FIGURE INDEX

GLOSSARY

5-MeC	5-methylcytosine
ABA	Abscisic acid
ABRC	Arabidopsis Biological Resource Center
ABRE	Abscisic acid Response Element
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AuxRE	Auxin Response Element
bp	Base pairs
bZIP	Basic Leucine Zipper Domain
CaMV	Cauliflower mosaic virus
Col-0	Columbia
Ct	Threshold cycle
CTAB	Hexadecyltrimethylammonium bromide
DOB	Drop out base
DOF	DNA binding with One zinc Finger
DTT	Dithiothreitol
DEPC	Diethylpyrocarbonate
DNase	Deoxyribonuclease
DsLox	Mutant lines carrying a Ds transposon and LoxP sites
dSpm	Defective suppressor-mutator
ETC	Electron-transport chain
EDTA	Ethylen diamino tetraacetic acid
FAD	Flavin adenine dinucleotide

GST	Glutathione S-transferase
GUS	β-glucuronidase
JGI	Joint Genome Institute
LB	Luria-Bertrani medium
LEA	Late Embryogenesis Abundant
MES	2-(N-morpholino)-ethanesulfonic acid
MMLV	Moloney Murine Leukemia Virus
MS	Murashige and Skoog médium
MU	4-methylumberiferone
MUG	4-Methylumbelliferyl-β-D-glucuronide hydrate
MUN	2'-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate
NADH	Nicotinamide adenine dinucleotide
NAN	*small* cytoplasmic sialidase gene of *Clostridium perfringens*
O2	Opaque2 transcription factor from maize
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PDC	Pyruvate dehydrogenase complex
PEG	Polyethylene glycol
PEP	Phosphoenolpyruvate
PSV	Storage Protein Vesicles
PVP	Polyvinylpyrrolidone
qPCR	Quantitative PCR
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate

SDH	Succinate dehydrogenase
SD	Standard deviation
SE	Standard error
SSP	Seed Storage Proteins
T7	11 amino acids from the leader sequence of the T7 bacteriophage gene10
TAE	Tris-acetate-EDTA buffer
TAG	Triacylglycerides
TCA	Tricarboxylic acid
TEMED	N,N,N´,N´-tetramethylethylenediamine
TTFA	4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione
UTR	Untranslated región
UV	Ultraviolet
V	Volts
X-gal	Bromo-chloro-indolyl-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide

ABSTRACT

Our group has undertaken the study of mitochondrial function in plants focusing on complex II (succinate dehydrogenase, SDH) of *Arabidopsis thaliana*. This complex plays a pivotal role in the tricarboxylic acid (TCA) cycle and the electron transport chain, two fundamental processes in the energetic plant metabolism. We have described the existence of three nuclear genes coding for iron-sulfur proteins (SDH2), named *SDH2-1*, *SDH2-2* and *SDH2-3*. Two of them, *SDH2-1* and *SDH2-2*, are almost identical (96%) and share the same exon-intron structure. *SDH2-3* on the other hand, has a different exon-intron structure and has approximately 70% identity to the other two. Additionally, unlike *SDH2-1* and *SDH2-2* that are expressed in adult plants, *SDH2-3* expression is confined to seed maturation and it decreases during germination, while *SDH2-1* and *SDH2-2* transcripts are low in seeds and they begin to accumulate after germination and during vegetative growth. We have described seed-specific *cis*-elements necessary for promoter activity (three ABA-responsive elements or ABRE and one RY element) and determined *in vitro* binding of seed-specific transcription factors to them (bZIP10, bZIP25 and bZIP53 binding to ABREs; ABI3 and FUS3 binding to RY). Moreover, we have determined that ABI3 and FUS3 are necessary for promoter activity *in planta*, as mutant lines of these factors showed decreased *SDH2-3* levels in seeds. On the other hand, we have determined that SDH2-3 is important for seed germination, indicating a specific role during this developmental stage.

The main focus of this thesis work is to study the seed-specific expression and the function of *SDH2-3*. It is worth noting that no reports of mitochondrial proteins bearing

this singular expression pattern have been published. Therefore, we will determine essential regions of the *SDH2-3* promoter and additional putative *cis*-elements controlling transcriptional regulation, transcription factors involved, and the function of this protein during seed maturation and postgerminative growth.

Regarding the *SDH2-3* promoter, during this thesis we were able to determine a minimal region necessary and sufficient for promoter activity, between -114 and +49 from the transcription start site. Moreover, the 5´UTR region (+1 to +49) is essential for *SDH2-3* promoter activity, as determined by loss-of-function experiments.

In addition, transient expression assays showed that ABI3 is able to activate *SDH2-3* transcription *in vivo* alone or in combination with bZIP factors bZIP10, bZIP25 or bZIP53. However, single bZIP factors were unable to activate the promoter, and only transfection with bZIP10 and bZIP53 was able to induce expression. Moreover, *SDH2-3* transcript levels are significantly reduced in *bzip53* mutant dry seeds. These results indicate the *SDH2-3* promoter is activated by bZIP transcription factors and corroborate the importance of ABI3.

On the other hand, we determined that SDH2-3 influences seed development and maturation, as lack of this protein resulted in decreased seed weight. Interestingly, protein content also showed a reduction in *sdh2-3* mutants while lipid content did not show any biologically significant variation. This is an interesting feature since seed metabolism is directed during maturation towards the formation of seed proteins and lipids. Thus, the decrease in protein content would explain the lower total weight.

Although it has been suggested that mitochondria plays a minor role during seed maturation, these results suggest that its role should be reconsidered, as it may carry out important metabolic tasks during this stage.

Mitochondrial role during postgerminative growth is well characterized. Here we show that *sdh2-3* mutants have impaired hypocotyl growth in the dark. Moreover, TTFA treatment (complex II inhibitor) abolishes hypocotyl growth and seedling establishment. All these results suggest an essential role for complex II during postgerminative growth and establishment. Wild seeds in nature generally germinate underground, in conditions where they lack direct sunlight. A seed with a non-functional SDH2-3 would be in disadvantage over wild-type seeds, which would elongate their hypocotyls further until they reach light in order to promote photoautotrophic growth. SDH2-3 gives an important advantage to the plant in energy-consuming processes such as germination and early stages towards seedling establishment. This work shows the importance that this non-essential gene can have in critical stages of plant development.

The existence of a *SDH2-3*-like gene in the moss *Physcomitrella patens* has drawn our attention because *SDH2-3* has been described as a seed-specific expressed gene in angiosperms, and mosses do not have seeds. We confirmed the *SDH2-3*-like gene is expressed and increases under osmotic stress, in contrast to saline stress, desiccation and high ABA content. Moreover, we determined that Physcomitrella *SDH2-3* promoter lacks significant activity in Arabidopsis, either in seeds or vegetative tissue. These results indicate that the transcriptional regulation of this gene in Physcomitrella evolved in an independent way as compared to Arabidopsis.

1. INTRODUCTION

Mitochondria are considered essential eukaryotic organelles, which have evolved from an α-proteobacterial endosymbiont ancestor. They play an essential role in modern eukaryotic cells, housing both the tricarboxylic acid (TCA) cycle and the electron-transport chain (ETC). This organelle is an important source of ATP and carbon skeletons, which in turn are associated to other metabolic pathways. Even though several mitochondrial processes are well conserved through different organisms, plant mitochondria possess some unique characteristics. The presence of alternative NADH dehydrogenases different from the complex I of the ETC, as well as the existence of an alternative oxidase different from the cytochrome c oxidase are examples of plant mitochondrial proteins which are not present for instance, in mammals. In addition, mitochondrial genome structure and size are far more variable within plants as compared to other eukaryotes (Wolstenholme & Fauron, 1995).

1.1. Arabidopsis succinate dehydrogenase complex

Our laboratory has focused on the study of plant mitochondria and its association with different plant processes and developmental stages (Elorza et al., 2004, Elorza et al., 2006, Figueroa et al., 2002, Figueroa et al., 2001, Leon et al., 2007, Roschzttardtz et al., 2009). In order to accomplish this, we have taken complex II (succinate dehydrogenase) as a model to study expression of genes encoding mitochondrial proteins and also mitochondrial function, using mutants to analyze the effects of mitochondrial dysfunction on plant development and physiology. Succinate:ubiquinone oxidoreductase (succinate

dehydrogenase [SDH]; EC 1.3.5.1), commonly referred to as mitochondrial complex II, is a central component of the cellular respiration process. This mitochondrial membrane-bound complex is a functional part of both the TCA cycle and the aerobic respiratory chain (ETC), catalyzing the oxidation of succinate to fumarate and the reduction of ubiquinone to ubiquinol. Complex II has been well characterized in bacteria and heterotrophic eukaryotes (Lemire & Oyedotun, 2002, Yankovskaya et al., 2003) where it is composed by four subunits. SDH1 is a peripheral membrane-bound flavoprotein that forms the succinate-binding site and is linked to a FAD molecule which acts as an acceptor of a hydride ion during succinate oxidation. This flavoprotein is bound to the iron-sulfur protein or SDH2 subunit, which conducts the electrons from SDH1 through its three non-heme iron-sulfur centers to the membrane. The SDH1-SDH2 subcomplex is anchored to the matrix side of the inner membrane by the SDH3 and SDH4 subunits, which also contain a b-type heme and the ubiquinone binding site (figure 1) (Yankovskaya et al., 2003). Furthermore, it has been reported that plant complex II may contain additional subunits of unknown function, along with the four previously mentioned (Millar et al., 2004).

The four main subunits of complex II are encoded in the nuclear genome of *Arabidopsis thaliana* (Figueroa et al., 2002, Figueroa et al., 2001, Millar et al., 2004). All of them but SDH4, are encoded by more than one gene in this organism (Figueroa et al., 2002). For instance, we have described three nuclear genes, designated as *SDH2-1* (At3g27380), *SDH2-2* (At5g40650) and *SDH2-3* (At5g65165), encoding the iron-sulfur protein (Figueroa et al., 2001). *SDH2-1* and *SDH2-2* have an identity of 96% and are

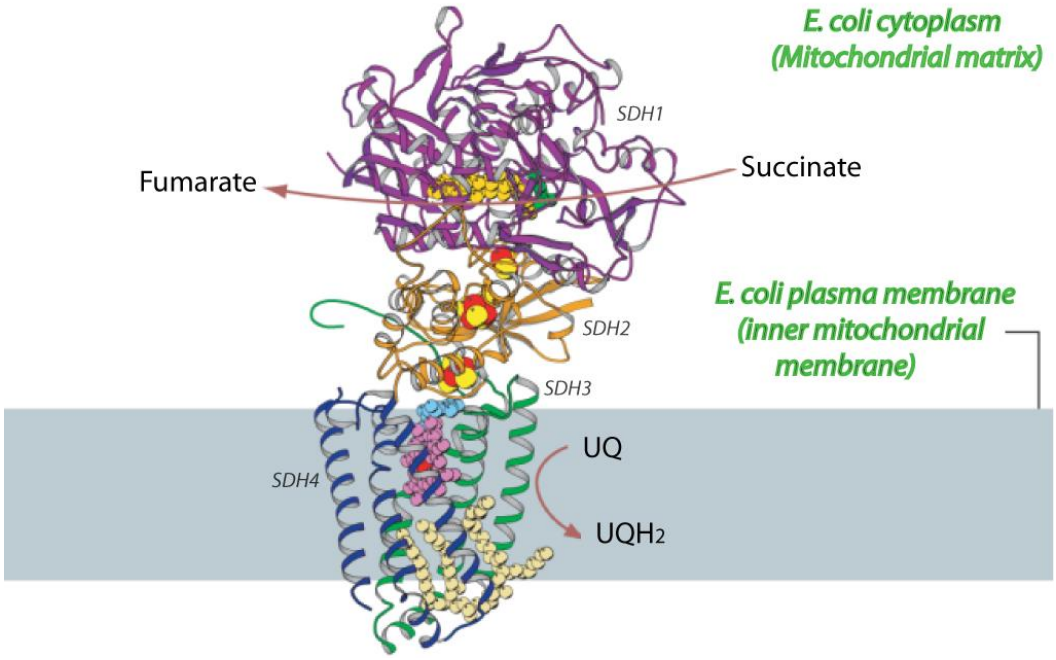


Figure 1. *E. coli* succinate dehydrogenase structure.

E. coli succinate dehydrogenase structure obtained by x-ray diffraction. Peripheral subunits SDH1 and SDH2 are shown in purple and orange, respectively. Membrane-bound subunits SDH3 and SDH4 are shown in green and blue, respectively. FAD molecule is shown in yellow and oxaloacetate (competitive inhibitor) in green within SDH1. The three iron-sulfur centers of SDH2 are shown in yellow and red. Ubiquinone in pink is located in the interface between subunits 2, 3 and 4. Modified image from Yankovskaya et al., 2003.

expressed in all organs from adult plants, indicating a possible redundant function (figure 2a and 2b) (Elorza et al., 2004, Figueroa et al., 2001). On the other hand, *SDH2-3* has approximately 67% identity to *SDH2-1* and *SDH2-2* and its expression is confined to seed development and desiccation, suggesting a specific role during these stages (figure 2c) (Elorza et al., 2006, Roschzttardtz et al., 2009). It has to be pointed out that there is only one additional report describing more than one SDH2 gene in a eukaryotic organism, a sheep nematode (Roos & Tielens, 1994). The three of them are probably functional since they are highly conserved when compared with homologs from other organisms and contain the Cys motifs which are essential for electron transport (Figueroa et al., 2001). Moreover, we have shown that *sdh2-3* mutants have impaired SDH activity in dry seeds and show a delay in germination (Roschzttardtz et al., 2009), raising interesting questions about its function during seed development and germination, and potential functional differences with SDH2-1 and SDH2-2.

1.2. Embryo-specific *SDH2-3* expression: promoter characterization & transcription factors

Detailed analysis of the *SDH2-3* promoter has shown that this gene is transcriptionally regulated during seed development and has a functional promoter ranging from 223 bp upstream of the transcription start site to 49 bp downstream of it (figure 3a; Elorza et al., 2006). *In silico* predictions revealed the putative existence of three abscisic acid (ABA) *cis*-responsive elements (ABREs) containing the characteristic ACGT core consensus (Busk & Pages, 1998, Leung & Giraudat, 1998), and a RY-like *cis* element (Nambara & Marion-Poll, 2003). Both ABRE and RY elements are typically found in seed

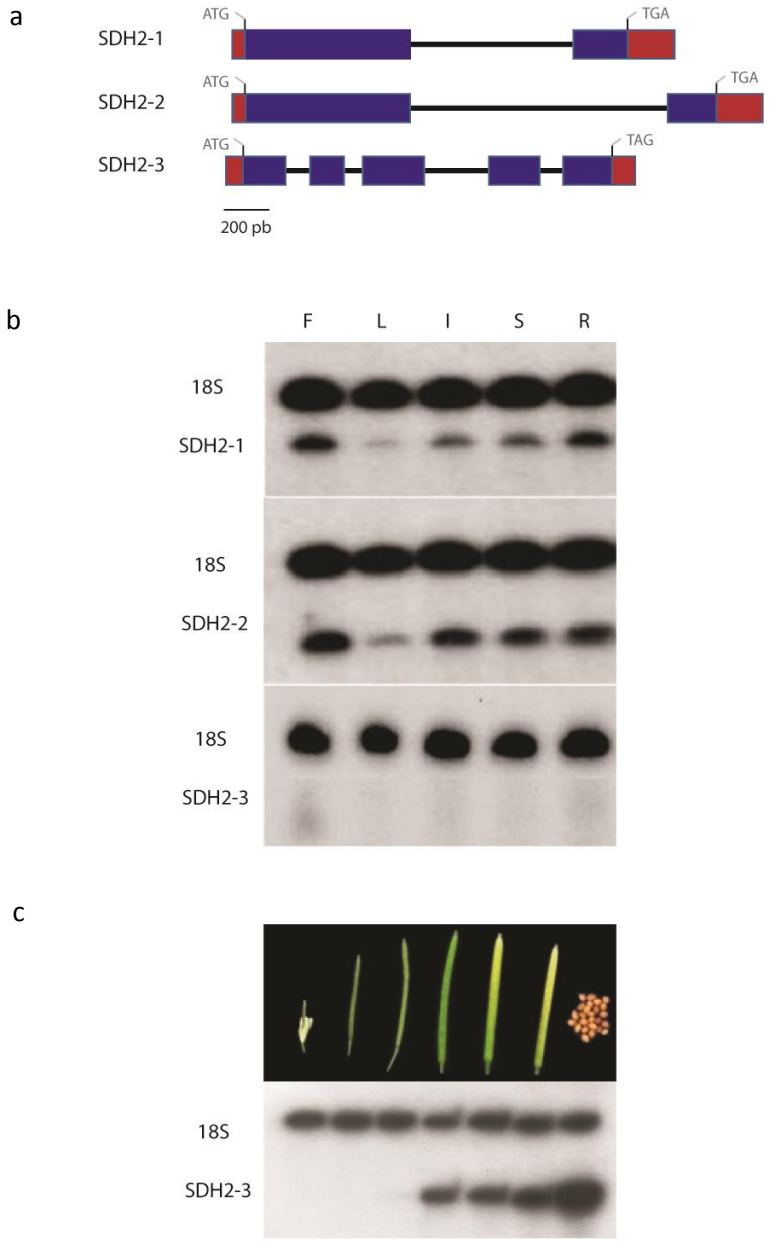


Figure 2. *SDH2-3* struture and expression.

a) Exon/intron structure of the three Arabidopsis SDH2 genes. b) RT-PCR multiplex analysis of expression of the three SDH2 genes. Total RNA was obtained from flowers (F), leaves (L), inflorescences (I), stems (S), and roots (R). Amplification of 18S rRNA was employed as internal control of RNA concentration. Figure from Elorza et al., 2004. c) RT-PCR multiplex analysis of *SDH2-3* transcripts during silique development. Total RNA was obtained from opened flowers (lane 1), short siliques (<5 mm long, lane 2), thin green siliques (5-10 mm long, lane 3), long and thick green siliques (10-15 mm long, lane 4), siliques with yellow tips (lane 5), yellow siliques (lane 6) and dry mature seeds (lane 7). Figure from Elorza et al., 2006.

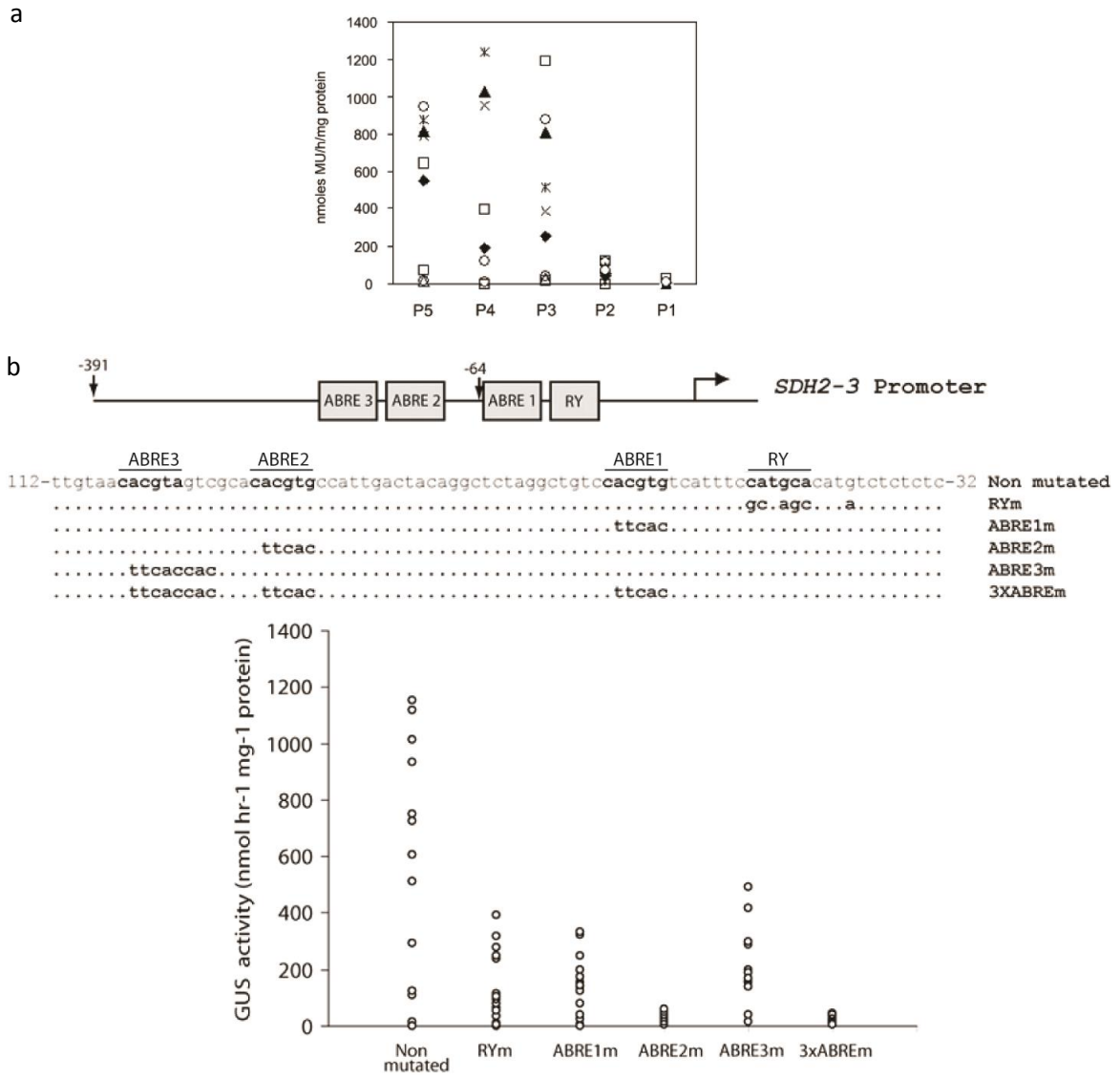


Figure 3. *SDH2-3* promoter in Arabidopsis.

a) Minimal functional promoter for seed expression. GUS activity was measured in dry seed extracts from transformed plants carrying progressive deletions of the *SDH2-3* promoter fused to GUS. Promoter lengths are 1603 bp (P5), 391 bp (P4), 223 bp (P3) and 64 bp (P2). In the P1 construct, the whole promoter and 16 bp from the 5´UTR were deleted. Figure from Elorza et al., 2006. Each symbol represents one independent transgenic line. b) Mutation analysis of ABRE and RY elements present in the *SDH2-3* promoter. GUS activity was measured in dry seed extracts from transformed plants containing the indicated constructs (Roschzttardtz et al., 2009). RYm, ABRE1m, ABRE2m and ABRE3m are mutated RY, ABRE1, ABRE2 and ABRE3 elements, respectively. 3xABREm is a promoter construct mutated in the three ABRE elements. The sequence of the wild-type promter is shown, and the mutated nucleotides in each construct are indicated below. Each point represents one transgenic line. All constructs contain a promoter fragment of 391 bp plus the 5´UTR (P4).

specific promoters, especially those encoding abundant seed storage proteins (SSP) (Hughes & Galau, 1989) and late embryogenesis abundant proteins (LEAs) (Parcy et al., 1994). As expected, mutation analysis showed that these four elements are required for normal *SDH2-3* promoter activity (figure 3b; Roschzttardtz et al., 2009). However, Roschzttardtz (2007) showed that those elements, although necessary, were not sufficient for *SDH2-3* expression in seeds. Indeed, fusing a DNA fragment comprising the three ABRE and RY elements (-114 to -32) to a minimal CaMV35S promoter was not able to confer promoter activity in seeds. Thus, one aim of this thesis work is to define more precisely the minimal necessary promoter region able to direct embryo *SDH2-3* transcription, and identify if possible additional known *cis* elements are necessary for this expression.

ABSCISIC ACID INSENSITIVE3 (ABI3), FUSCA3 (FUS3), and LEAFY COTYLEDON2 (LEC2) are transcription factors that belong to the B3 superfamily, and are considered master regulators of seed development (Giraudat et al., 1992, Luerssen et al., 1998, Santos-Mendoza et al., 2008, Stone et al., 2001). Binding to promoter regions, such as the RY element, occurs through their B3 domain (Ezcurra et al., 2000, Suzuki et al., 1997). These B3 domain transcription factors have different spatial and temporal expression patterns from early embryogenesis onwards, they are also interlocked in a cross-regulated network, share some redundant functions and possess novel functions due to differences in protein domains (Agarwal et al., 2011, Suzuki & McCarty, 2008). Roschzttardtz et al. (2009) determined that ABI3 and FUS3 are required for *SDH2-3* transcription in dry seeds, while LEC2 is necessary before desiccation (figure 4a). Furthermore, they demonstrated that ABI3 and FUS3 bind *in vitro* to a promoter region

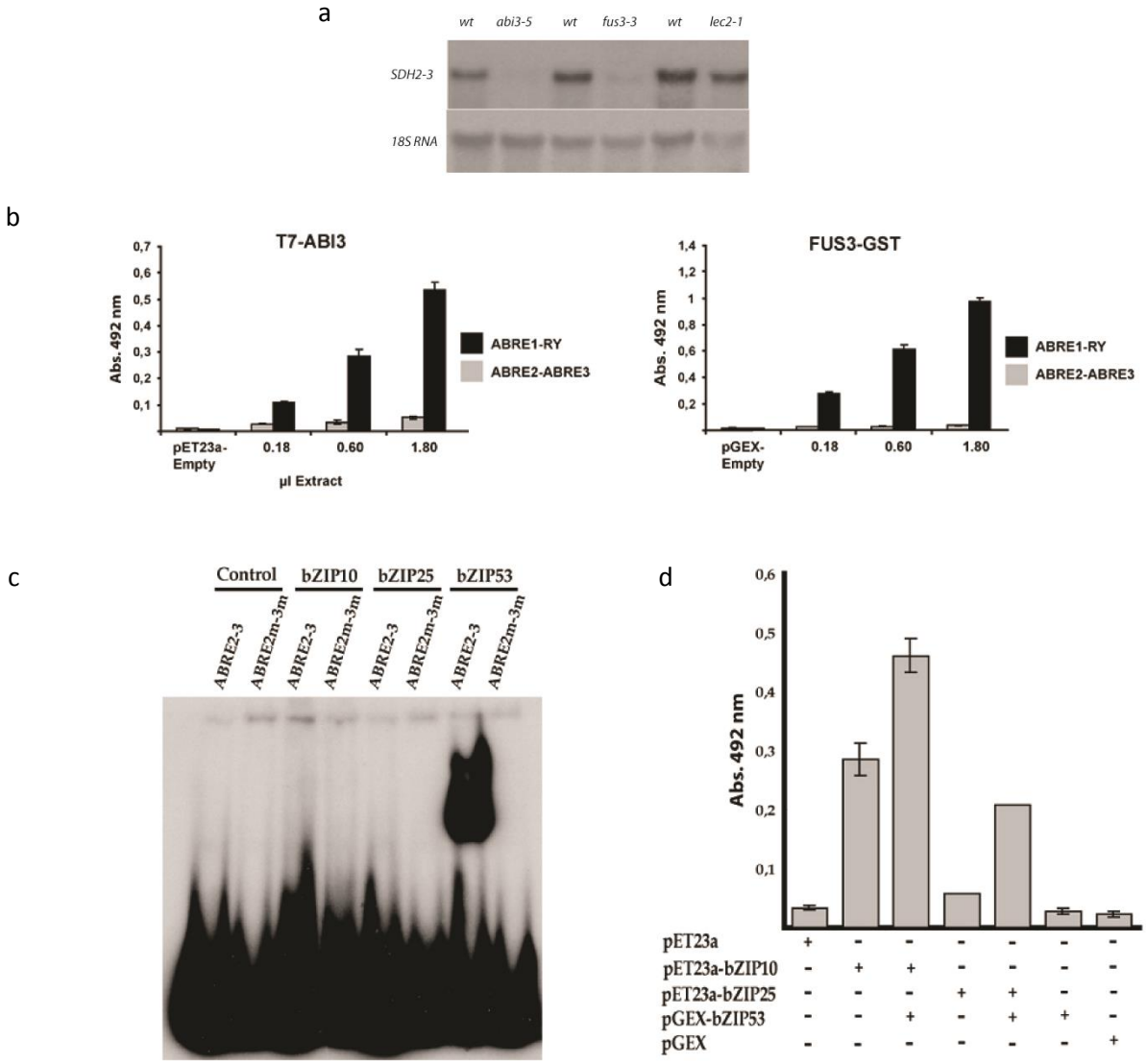


Figure 4. Transcription factors involved in *SDH2-3* promoter.

a) *SDH2-3* expression in dry seeds of *abi3-5*, *fus3-3*, *lec2-1* mutants and their respective wild-type ecotypes. Transcripts were analyzed by Northern blot. b) ABI3 and FUS3 binding to an ABRE1-RY probe. Increasing quantities of bacterial extracts expressing T7tag-ABI3 or FUS3-GST were assayed. A probe containing the ABRE3-ABRE2 sequence was used a negative control. Binding was measured by ELISA, with an antibody against GST or T7 tag. c) EMSA using probes containing a wild-type (ABRE2-ABRE3) or mutated (ABRE2m-ABRE3m) sequence with protein extracts containing bZIP10, bZIP25 or bZIP53. Controls were performed with extracts from bacterial cells transformed with the empty PET23a vector. d) bZIP10 and bZIP25 binding to a probe containing the ABRE2-ABRE3 sequence in the presence or absence of bZIP53. Extracts from bacteria expressing T7tag-bZIP10 or T7tag-bZIP25 were assayed in the presence or absence of bZIP53-GST. Binding was measured by ELISA, with an antibody against the T7 tag (bZIP53 is not detected). Adapted from Roschzttardtz et al. (2009).

containing the RY element (figure 4b). Additionally to the B3 superfamily proteins, bZIP transcription factors have also been involved in seed development regulation. OPAQUE2 (O2) is a bZIP transcription factor from maize, and probably one of the most studied in plants. Mutations in either the O2 gene or its *cis*-binding site, greatly affects the transcription of a SSP gene during embryo development (Schmidt et al., 1992). Two Arabidopsis bZIPs factors, closely related to O2, have been characterized (bZIP10 and bZIP25). Both are part of the C group of bZIP transcription factors, are expressed during embryo development and regulate SSP genes *At2S1* and *At2S3* (Lara et al., 2003). On the other hand, bZIP53 is a bZIP transcription factor of the S1 group, is highly expressed during seed development and it has also been involved in transcriptional activation of SSP genes *At2S1* and *AtCRU* (Alonso et al., 2009). These transcription factors bind to ABRE elements through their basic leucine zipper domain, which also function as a dimerization domain. bZIP53 has been described to heterodimerize with group C bZIPs such as bZIP10 and bZIP25 (Ehlert et al., 2006, Weltmeier et al., 2006).

We have been able to show that bZIP53 can bind *in vitro* to a probe containing the ABRE2-ABRE3 elements of the *SDH2-3* promoter either as an homodimer (figure 4c) or an heterodimer along with bZIP10 and bZIP25 (figure 4d; Roschzttardtz et al., 2009). However, we have until now no evidence for an *in vivo* role of these bZIP transcription factors in the regulation of *SDH2-3* expression. Thus, one of the aims of this work is to evaluate the *in vivo* role of bZIP transcription factors in *SDH2-3* expression, using bzip mutant and overexpressing lines and transient expression assays in protoplasts.

The sesquiterpenoid ABA is a plant phytohormone that is synthesized from xanthophylls and has been described to regulate various physiological responses to abiotic stress (water, salt, hypoxic and cold stresses) or biotic conditions such as wound or pathogen attacks (Leung & Giraudat, 1998, Shinozaki & Yamaguchi-Shinozaki, 2000). ABA regulated processes include the synthesis of seed storage proteins and lipids, the acquisition of desiccation tolerance and dormancy of the seed and the inhibition of germination (Leung & Giraudat, 1998, Rohde et al., 2000). At the transcriptional level, ABA signal is transduced through ABA responsive elements (ABRE). This element contains an ACGT core motif, which is recognized by bZIP transcription factors and is often found in tandem with other ABREs or coupled to an RY element (Shen et al., 1996). ABRE elements are typically found in a variety of seed-specific promoters, such as SSP and LEA genes and also the *SDH2-3* promoter (Elorza et al., 2006, Kroj et al., 2003). We have shown that exogenous ABA is able to promote *SDH2-3* transcript accumulation (figure 5a) and promoter activation (figure 5b) in vegetative tissue ectopically expressing ABI3 (Roschzttardtz et al., 2009). But until now, we have no direct evidence supporting a role for ABA in *SDH2-3* expression during seed development. In order to evaluate the requirement for ABA in seed *SDH2-3* expression, we will evaluate *SDH2-3* transcript levels in four mutant lines (*aba2-1*, *aba2-3*, *aba3-1* and *aba3-2*) deficient in embryo ABA biosynthesis (Barrero et al., 2006, Schwartz et al., 1997).

Epigenetic processes such as DNA methylation have been tightly related with embryogenic development (Xiao et al., 2006). Parent-of-origin effects on seed development have been shown to require DNA methylation and affect embryogenesis,

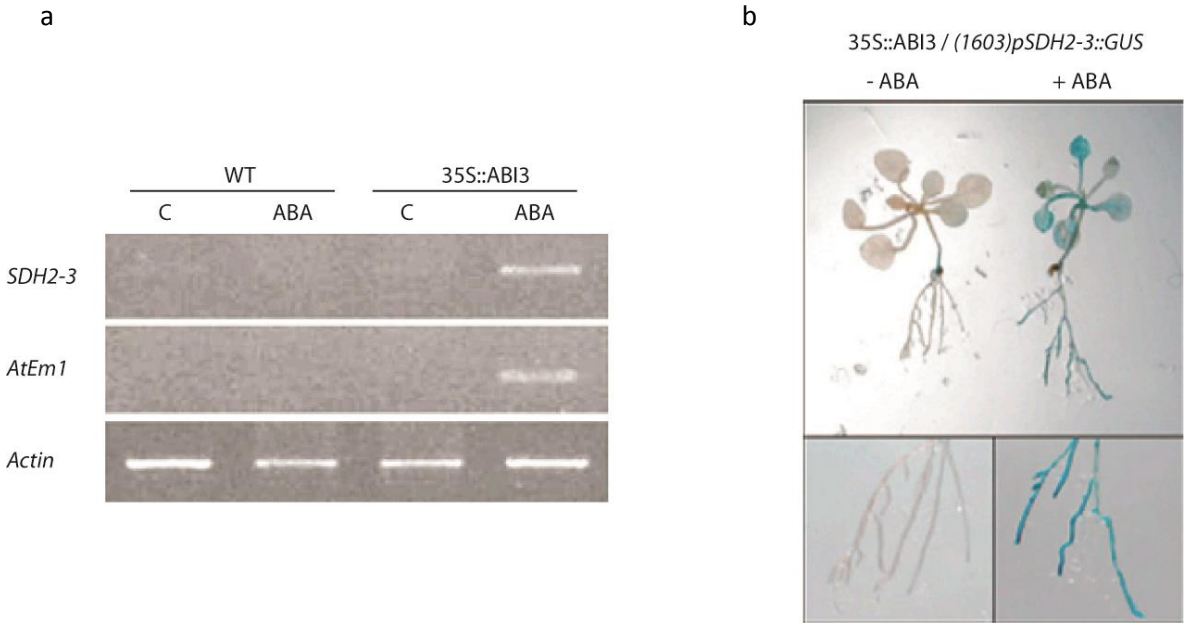


Figure 5. *SDH2-3* induction by ABA and ABI3 in vegetative tissue.

SDH2-3 induction by exogenous ABA and ABI3 in vegetative tissue. a) ABA induction of *SDH2-3* in 35S::ABI3 seedlings. RT-PCR analyses of *SDH2-3* and *AtEm1* transcripts were performed on RNA prepared from 2-week-old wild-type and transgenic 35S::ABI3 seedlings incubated for 48 h with or without 50 µM ABA. b) ABA induction of *SDH2-3promoter::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 µM ABA and then stained for GUS activity. Adapted from Roschzttardtz et al. (2009).

viability and seed size (Adams et al., 2000). *SDH2-3* has a very tight spatial and temporal expression and is not expressed at all in vegetative tissues, and DNA methylation may be involved in *SDH2-3* promoter repression after germination. Here we address this issue by analyzing the effect of a DNA methylation inhibitor and DNA methyltransferase mutants on *SDH2-3* expression.

1.3. Seed development and germination, seedling establishment: putative mitochondrial role

One aspect in the success of gymnosperms and specially angiosperms in the modern world is seed set. Seeds replaced spores of seed-less plants as a mean of propagation, dispersal and/or storage units. The dry seed, after development and acquisition of dormancy, is a structure that is fitted to survive long periods of time under unfavorable conditions. Seed development can be somehow divided into three major stages: an early morphogenesis phase consisting of a series of cell divisions that give the basic architecture of the plant (Mayer et al., 1991); a second step in which the embryo goes into a period of cellular expansion and differentiation occupying the sac, which is simultaneous with the accumulation of storage products (Raz et al., 2001, Goldberg et al., 1994); and a final maturation stage, when the seed becomes metabolically quiescent and tolerant to desiccation (Vicente-Carbajosa & Carbonero, 2005). Metabolism in developing seeds focuses on two main pathways: formation of triacylglycerides (TAG) and storage seed proteins (SSP) (figure 6). Both pathways are fueled at early stages mostly by

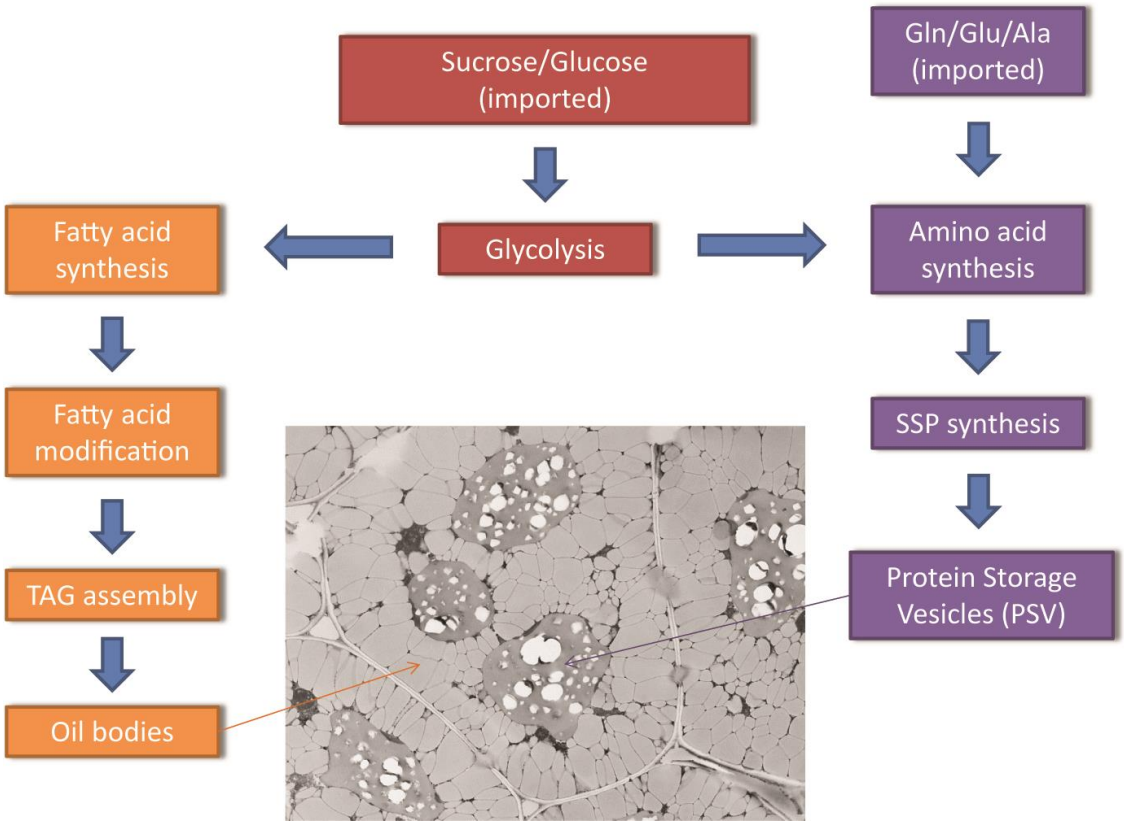


Figure 6. Seed development primary metabolism in Arabidopsis.

Simplified model for seed development primary metabolism in Arabidopsis. Imported sucrose/glucose fuel both TAG and SSP production. Fatty acids synthesized in the plastid are exported to the cytosol as acyl-CoAs, acylated on a glycerol backbone forming TAG and then deposited in oil bodies. Seed storage protein synthesis is also fueled by imported amino acids (Glu, Gln and Ala) and occurs within the embryo. SSPs are finally stored in protein storage vesicles (PSVs). Modified figure from Baud et al. (2008).

imported organic compounds such as sucrose, glucose, Gln, Glu and Ala (Schwender & Ohlrogge, 2002, Schwender et al., 2006). Cleavage of sucrose by invertase, and the subsequent formation of hexose-phosphate molecules, fuels a transient starch formation in plastids, the oxidative pentose phosphate pathway in order to gain reducing power for fatty acids synthesis, and most important, the cytosolic and plastidial glycolytic pathways. Moreover, the main purpose of these glycolytic pathways is thought to be the formation of TAG. Cytosolic phosphoenolpyruvate (PEP) formed during glycolysis in the cytoplasm (Ruuska et al., 2002, White et al., 2000), is rather imported to the plastid than converted to pyruvate (Kubis et al., 2004). It is within the plastid that PEP is transphosphorylated, resulting in ATP and pyruvate, which is later decarboxylated into acetyl-CoA, CO_2 and NADH by the pyruvate dehydrogenase complex (PDC). This plastidial acetyl-CoA is a direct precursor in the formation of fatty acids. Regarding protein accumulation, it has been described that the majority of the cytosolic oxaloacetate is used for Asp synthesis and amino acids derived from it. Moreover, most of the nitrogen present in the developing seed is imported mainly as Gln and Ala, and used for the production of other amino acids and SSPs (Schwender et al., 2006). It has been suggested that mitochondrial metabolism is not important during embryo maturation, being involved only in the provision of carbon skeletons (as citrate) for cytosolic fatty acid elongation, with little importance in ATP production (Schwender et al., 2006). Moreover, reports indicate that the photosynthetic pathway is the main ATP and NADPH source, at least during the first stages of seed maturation (Goffman et al., 2005). Even though mitochondrial seed metabolism is largely

unknown and further work is needed to define its role, the current view is that this organelle does not play a prominent role during seed maturation.

In contrast to seed maturation, an important role for mitochondria has been proposed during germination and postgerminative growth. Albeit little is known about mitochondria in dry seeds, it has been recognized that oxygen consumption is one of the early events after imbibition (Bewley, 1997, Benamar et al., 2003). Along with hydration, this organelle needs to become rapidly competent for oxidative phosphorylation since it has been reported that oxygen is absolutely needed during germination (Al-Ani et al., 1985) and fermentative processes are not able to supply the energetic needs of the embryo. ATP production during the first hours post imbibition relies on oxidation of NADH and succinate by the respiratory chain (Benamar et al., 2008, Logan et al., 2001).

After germination sensu-stricto (radicle protrusion), postgerminative growth is tightly associated with degradation and mobilization of accumulated reserves (lipids, proteins, starch) in order to resume plant life cycle. Until transition to autotrophy, this phase is heterotrophic, being fueled by the oxidation of carbon resources produced during seed maturation. For instance, hypocotyl elongation and seedling establishment are dependent on early heterotrophic metabolism, reflecting the importance of reserve accumulation during previous stages (Eastmond et al., 2000). During this phase, glyoxisomal β-oxidation of fatty acids derived from TAGs gives acetyl-CoA, which enters the glyoxilate cycle to produce succinate (Eastmond et al., 2000, Hayashi et al., 1998). This newly formed succinate enters mitochondria where it is converted to malate through an incomplete TCA cycle. This malate is later exported to the cytosol where it enters the

gluconeogenic pathway, fueling the synthesis of soluble carbohydrates (figure 7)(Beevers, 1980).

Thus, current knowledge suggests that mitochondria and especially complex II, could play an important role during germination and early postgerminative growth. However, experimental data demonstrating this role is very scarce and we have taken advantage of the availability of *sdh2-3* mutants and complex II inhibitors to address this issue.

Regarding SDH2-3 function, we have previously determined that SDH activity is greatly reduced but not absent in mutant embryos of two *sdh2-3* homozygous mutants (figure 8a)(Roschzttardtz et al., 2009). This suggests that SDH2-3 is the main iron-sulfur subunit of embryo complex II during seed maturation, and that residual activity in *sdh2-3* mutants is likely due to a low level expression of *SDH2-1* and/or *SDH2-2* (Roschzttardtz et al., 2009). Interestingly, the mutants lacking a functional SDH2-3 protein show a delay in germination, however all seeds germinated at 46 h (figure 8b)(Roschzttardtz et al., 2009). At the transcript level, we have previously found that *SDH2-3* transcripts are abundant in dry seeds and decrease rapidly during germination, whereas *SDH2-1*/*SDH2-2* expression is induced, reaching a maximum at 1-2 days postimbibition (Elorza et al., 2006). These results are consistent with a role of SDH2-3 containing complex II during germination (first 24 h) and with replacement of SDH2-3 by SDH2-1/SDH2-2 during postgerminative growth.

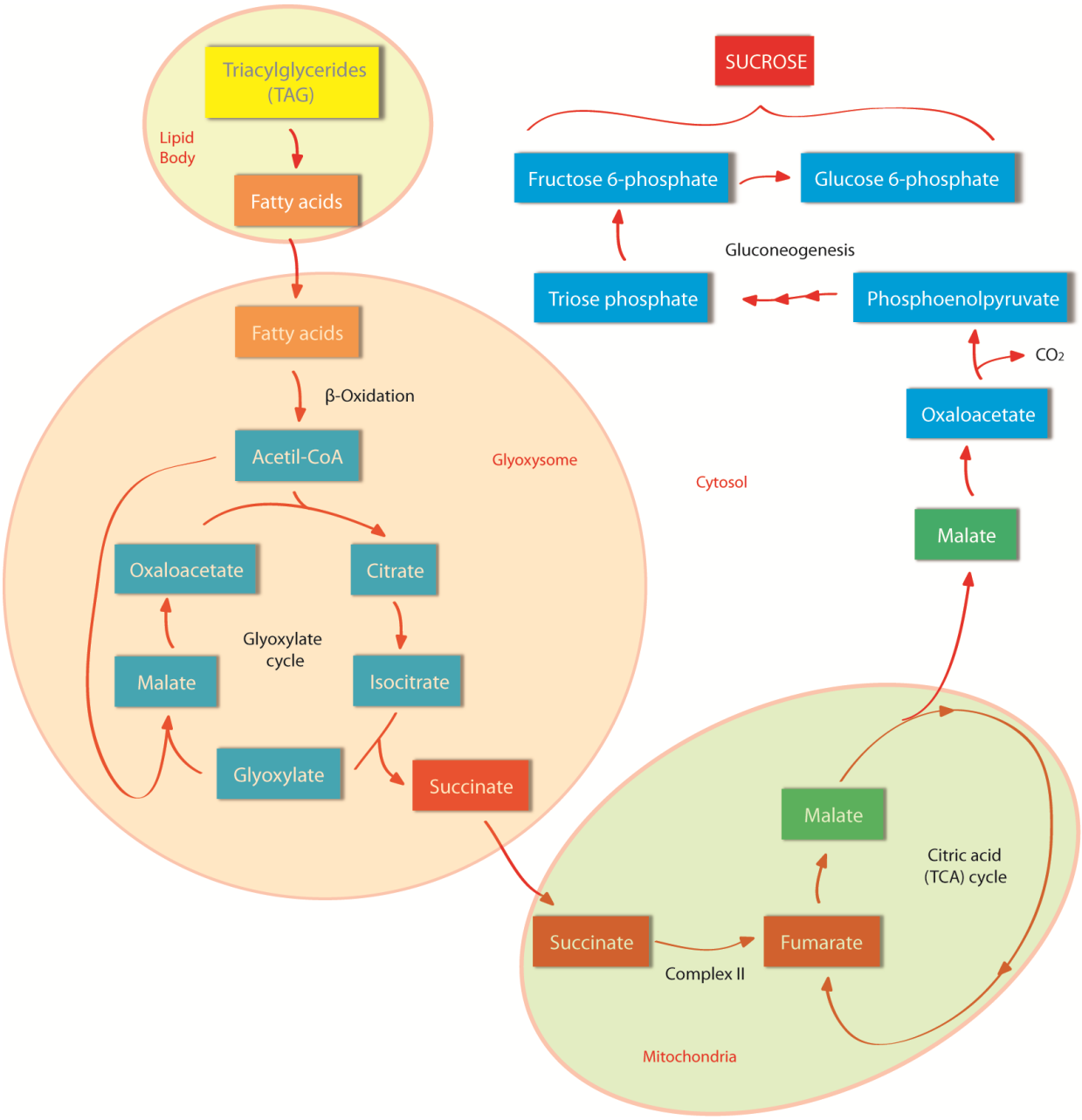


Figure 7. Heterotrophic metabolism of germinating seeds.

Lipids from oleaginous seeds (such as Arabidopsis) are mobilized from oil bodies to the glyoxysome as acyl-CoA, where they are oxidized to acetyl-CoA. The latter is substrate for the glyoxylate cycle, in which one mole of succinate is made per two of acetyl CoA, without the loss of carbon atoms as CO_2. The generated succinate is then transported to the mitochondrion where by action of the SDH complex it is converted to fumarate. Further reactions produce malate, which is exported to the cytosol, where it enters the gluconeogenic pathway to produce sucrose. Modified figure from Penfield et al. (2005).

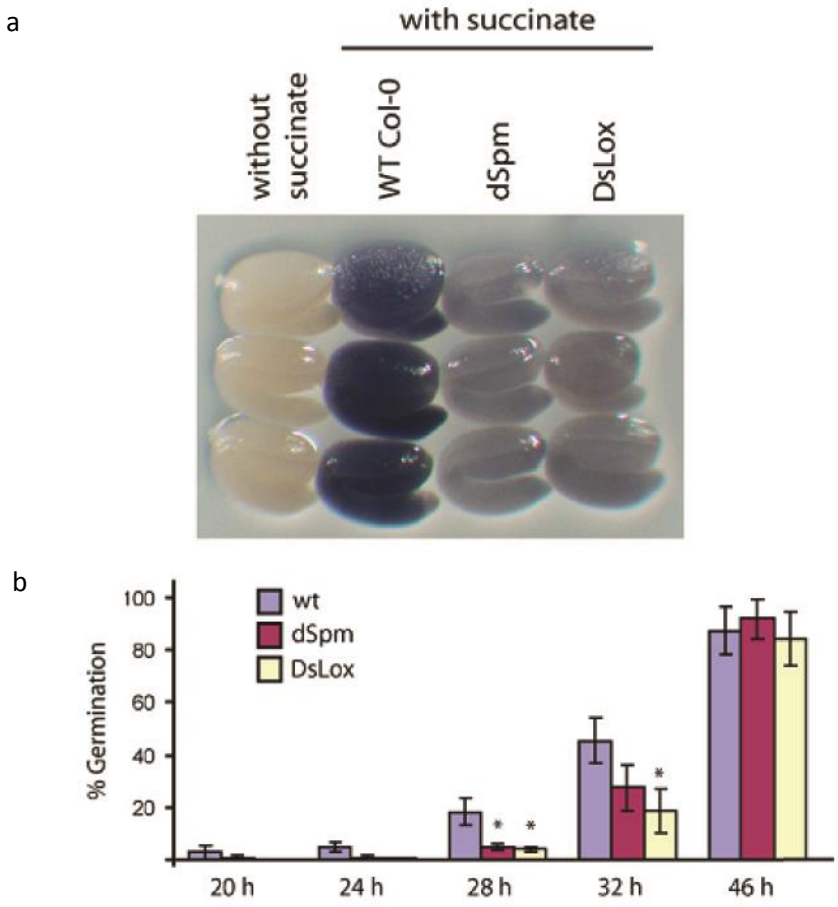


Figure 8. Effects of the *sdh2-3* mutation on seed SDH activity and germination-

a) Effects of the *sdh2-3* mutation on SDH activity in mature embryos. SDH activity was qualitatively assayed in mature embryos of wild-type and *sdh2-3* dSpm and DsLox mutants. b) Effects of the *sdh2-3* mutation on germination. Values are means ± SE of three replicates and asterisks indicate significant differences according to student´s t test ($p < 0.02$). Figure from Roschzttardtz et al. (2009).

1.4. Evolutionary considerations on the presence of SDH2-3

As already mentioned, the presence of an embryo-specific iron-sulfur subunit poses intriguing questions about its function and its evolutionary origin. Concerning this last point, we have been able to identify *SDH2-3*-like genes in several seed plants, both monocotyledonous and dicotyledonous (unpublished results, figure 9). Thus, the presence of an *SDH2-3* type gene is conserved among angiosperms. Interestingly, during this thesis work, an *in silico* analysis allowed us to identify a *SDH2-3*-like gene in the moss *Physcomitrella patens* (figure 9). This moss, as well as others, does not have structures homologous to seeds. However, it shares different fundamental physiological and genetic processes with vascular plants, even though both lineages diverged early in plant evolution. Moreover, mosses are considered the first land plants that evolved approximately over 450 million years ago and Physcomitrella has emerged as an excellent model organism to address evolutionary issues (Cove, 2005, Schaefer & Zryd, 2001). Mosses, as ferns and seed plants, alternate generations (Prigge & Bezanilla, 2010). However, the haploid gametophyte generation is predominant in the life cycle of Physcomitrella (figure 10). In this thesis, we decided to analyze if the *SDH2-3* like gene in Physcomitrella is functional, i.e. if it is expressed and how this expression is regulated. This may give us some clues about the evolutionary origin of this gene which is specifically expressed in seeds in extant seed plants. In the long term and in a broader view, this type of analysis may help to understand how such complex structures as seeds, essential for earth colonization, evolved.

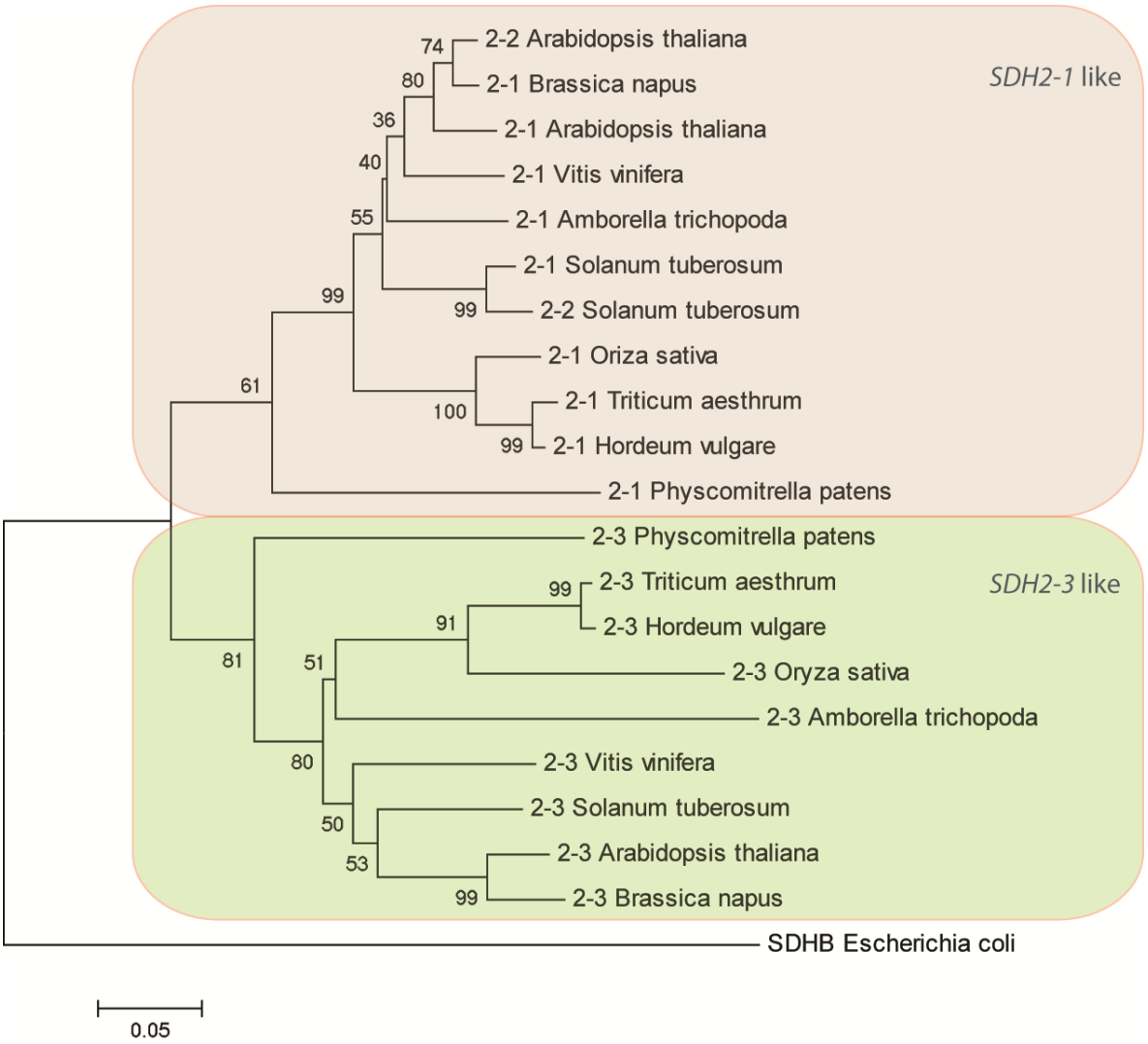


Figure 9. Evolutionary relationships of *SDH2* genes from nine plant species.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 2000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl & Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 171 positions in the final dataset. *SDHB* gene from *Escherichia coli* was used as an outgroup control. Phylogenetic analyses were conducted in MEGA5 (Tamura et al., 2011).

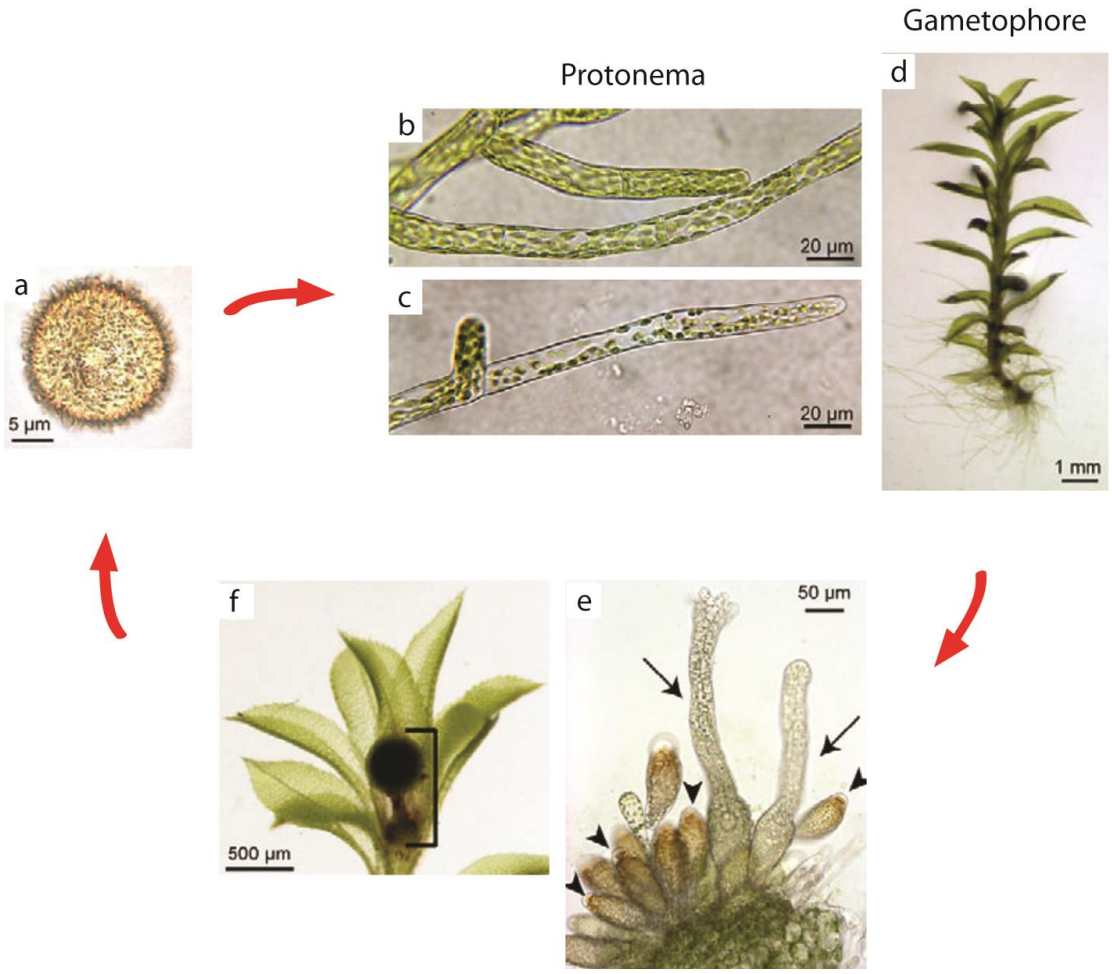


Figure 10. Physcomitrella life cycle.

a) An haploid spore germinates giving way to a b) chloronemal cell. This cell will continue to grow until it differentiates into a c) caulonemal cell. Both of these cells are the components of the protonema tissue, which is dependent on an aqueous environment. Afterwards, a d) gametophore rises from the protonema. This gamete-production shoot is ultimately anchored by rhizoids that grow by tip growth from the base. e) Formation of both male (antheridia; arrows) and female (archegonia; arrowheads) organs is promoted at the apex of the gametophore. Then, a motile flagellate sperm fertilizes the egg and the f) sporophyte is developed (marked with the bracket). After, the cycle is resumed as the sporophyte produces the haploid spore. Adapted from Prigge & Bezanilla (2010).

All the aforementioned allow us to suggest that *SDH2-3* expression in seeds is under a strict combinatorial control carried away by specific transcription factors responsible for the recruitment of the basal transcriptional machinery, providing specificity and strength to this promoter activity. Additionally, it has a unique expression pattern for a mitochondrial protein and we have proved its role during germination. Therefore, according to the above, the working hypothesis of our work is:

HYPOTHESIS

The *SDH2-3* gene is transcriptionally regulated *in vivo* by seed-specific transcription factors (ABI3, bZIP10, bZIP25 and bZIP53) and ABA, and it is important for seed maturation and early postgerminative growth.

GENERAL AIM

Determine the *in vivo* regulation of the *SDH2-3* gene and its function during seed development and postgerminative growth.

SPECIFIC AIMS

1) To define precisely the minimal promoter able to direct embryo *SDH2-3* transcription and identify new regulatory regions (different from the ABRE and RY *cis*-elements).

2) To evaluate the *in vivo* regulation of *SDH2-3* expression by seed-specific transcription factors (ABI3, bZIP10, bZIP25 and bZIP53) and ABA. To determine if DNA methylation may be involved in *SDH2-3* promoter repression after germination.

3) To determine the role of SDH2-3 during seed maturation, germination and postgerminative growth.

4) To determine if the *SDH2-3*-like gene is expressed in *Physcomitrella patens* and how this expression is regulated.

2. MATERIALS

2.1. Plant Material

Arabidopsis thaliana wild-type seeds ecotype Columbia (Col-0) were obtained from the Arabidopsis Biological Resource Center (ABRC). Plants overexpressing bZIP10, bZIP25 and bZIP53 and the respective homozygous mutants *bzip10*, *bzip25* and *bzip53* were kindly donated by Dr. Jesús Vicente-Carbajosa, from the CBGP, Madrid, Spain (Alonso et al., 2009, Lara et al., 2003). *drm1/drm2/cmt3* triple-null mutant and the *met1-6* null mutant were kindly donated by Dr. Rodrigo Gutiérrez, from the Pontificia Universidad Católica de Chile, Santiago, Chile. *SDH2-3* mutant lines DsLox and dSpm were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham) and the ABRC, respectively. Homozygous mutant plants were previously obtained (Roschzttardtz et al., 2009). Transgenic plants carrying a *SDH2-3* promoter fragment (391 or 223 bp) and the *SDH2-3* 5´ untranslated region (5´UTR, 49bp) fused to GUS have been previously obtained (Elorza et al., 2004). Transgenic plants carrying a mutated promoter, either in individual ABRE or RY elements (mABRE3, mABRE2, mABRE1 and mRY) or in the three ABRE elements (mABREx3) were obtained by Roschzttardtz et al. (2009). These mutated constructs contain 391 bp of promoter and the 5´UTR (49 bp), up to the first *SDH2-3* codon in frame with the GUS reporter gene. Transgenic plants carrying either the -114 to -32 fragment of the *SDH2-3* promoter plus the minimal cauliflower mosaic virus (CaMV) 35S promoter (-64 to +41) or only the minimal CaMV35S promoter fused to GUS were also previously obtained (constructs -114/-32/-64 p35S and -64 p35S; Roschzttardtz, 2007). *aba2-1*, *aba2-3*, *aba3-1* and *aba3-2* mutant lines were obtained from the Nottingham

Arabidopsis Stock Centre (University of Nottingham). Arabidopsis Col-0 plants transformed with a construction containing the reporter GUS gene under the control of the Physcomitrella *PpSDH2-3* promoter was kindly donated by Dr. Vicente-Carbajosa.

2.2. Bacterial strains

E. coli XL1-Blue: *endA*1, *gyrA*96, *hsdR*17, *lac-*, *recA*1, *relA*1, *supE*44, *thi*-1, F'[*proAB*$^{+}$, *lacI*^{q}, *lacZ*ΔM15, Tn*10* (tet^{r})].

E. coli DH5α: *endA*1, *gyrA*96, *hsdR17, recA1, relA1, supE44, thi-1, lambda*$^{-}$, *ΔlacU169Ø80(lacZΔM15)*.

A. tumefaciens GV3101: *pMP90 (pTiC58ΔT-DNA) RifR GmR.*

2.3. Yeast strains

The yeast strains BY4741 (MATa; his3D1; leu2D0; met15D0; ura3D0) and BY4742 (MATα; his3D1; leu2D0; met15D0; ura3D0), both harboring a mutated YLL041c ORF (accession numbers Y01529 and Y11529, respectively) were ordered from Euroscarf. The open reading frame YLL041c, encoding SDHB, has been replaced by kanMX4 in both strains, a kanamycin resistance gene confering G418 resistance in *Saccharomyces cerevisiae*.

2.4. Solid growth medium for propagation and selection of plants

For Arabidopsis propagation, we used agar plates containing 0.5 X MS medium (825 mg/L NH_4NO_3, 950 mg/L KNO_3, 3.1 mg/L H_3BO_3, 166.1 mg/L $CaCl_2$, 0.0125 mg/L $CoCl_2x6H_2O$, 0.0125 mg/L $CuSO_4$ $x5H_2O$, 18.63 mg/L $Na_2EDTAx2H_2O$, 13.9 mg/L $FeSO_4x7H_2O$, 90.35 mg/L $MgSO_4$, 8.45 mg/L $MnSO_4xH_2O$, 0.125 mg/L $Na_2MoO_4x2H_2O$, 0.415 mg/L KI, 85 mg/L KH_2PO_4, 4.3 mg/L $ZnSO_4x7H_2O$) supplemented with 29 mM sucrose, pH 5.7. When required, 50 µg / mL of kanamycin were added to the plates to select for resistant plants.

For Physcomitrella propagation, we used BCD medium containing 1 mM $MgSO_4$, 10 mM KNO_3, 45 µM $FeSO_4$ and 1.8 mM KH_2PO_4 (pH 6.5 adjusted with KOH). One ml of trace elements solution (0.22 µM $CuSO_4$, 0.19 µM $ZnSO_4$, 10 µM H_3BO_3, 0.1 µM Na_2MoO_4, 2 µM $MnCl_2$, 0.23 µM $CoCl_2$, and 0.17 µM KI) was added per 1 L of BCD medium. BCDATG medium is BCD medium (plus trace elements) supplemented with 1 mM $CaCl_2$, 5 mM diammonium (+)-tartrate, 0.5% (w/v) glucose and 0.8% (w/v) agar.

2.5. Bacterial growth medium

Bacteria were grown in LB medium: 10 g / L peptone, 5 g / L yeast extract, 5 g / L NaCl, pH 7 with or without 0.6% (w/v) agar. Medium was autoclaved and adequate antibiotics for transformants selection were added. Additionally, 0.5 mM IPTG and 50 µg / mL X-gal were added to distinguish recombinant colonies from non-recombinant ones.

2.6. Yeast growth medium

Transformed yeast strains were plated in Yeast Synthetic Drop-out Medium lacking uracil (Sigma-Aldrich).

For complementation assays, transformed yeasts were plated in fermentative YPD (1% (w/v) Bacto-peptone (Difco), 2% (w/v) yeast extract, 2% (w/v) glucose and 1.6% (w/v) bactoagar) or non-fermentative YPG (1% (w/v) Bacto-peptone (Difco), 2% (w/v) yeast extract, 3% (v/v) glycerol and 1.6% (w/v) bactoagar) or YPE (1% (w/v) Bacto-peptone (Difco), 2% (w/v) yeast extract, 2% (v/v) ethanol and 1.6% (w/v) bactoagar).

2.7. Enzymes and commercial kits

All the restriction enzymes used were from New England Biolabs. Taq DNA polymerase, RNase-free DNase I and RNase A were obtained from Invitrogen. T4 DNA ligase, Wizard DNA Clean-up System, pGEM^{tm}-T Easy Vector System and MMLV were from Promega. Miniprep and Maxiprep systems were obtained from Axygen. EZNA Purification Band System was obtained from Omega Bio-Tek, Inc.

2.8. Reagents and materials

dNTP mix, ammonium persulfate, sucrose, EDTA, acrylamide, bisacrylamide, TEMED, Trizol, yeast tRNA and agarose were obtained from Invitrogen.

Lithium chloride, glycerol, acetic acid, ammonium acetate, magnesium sulfate, iron(II) sulfate, copper(II) sulfate, monopotassium phosphate, boric acid, potassium

nitrate, zinc sulfate, calcium chloride, sucrose, mannitol, sorbitol, magnesium acetate, sodium molybdate, manganese(II) chloride, cobalt(II) chloride, diammonium (+)-tartrate, sodium hydroxide, potassium hydroxide, sodium acetate, chloroform, glacial acetic acid, formamide, formaldehyde, chloridric acid, Hydrochloric acid, triethanolamine, copper (II) nitrate trihydrate, isopropanol, isoamyl alcohol, ethanol and methanol were obtained from Merck.

Abscisic acid was obtained from Phytotechnologie laboratories.

4-Methylumbelliferyl-β-D-glucuronide hydrate (MUG), 2'-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate (MUN), N-Lauroylsarcosine sodium salt (sarkosyl), Zebularine, Hexadecyltrimethylammonium bromide (CTAB), succinic acid and fumaric acid were obtained from Sigma-Aldrich.

Cellulase R10 and Macerozyme R10 were obtained from Yakult Pharmaceutical Ind. Co., Japan.

Sodium metabisulphite and quinol were obtained from BDH Chemicals.

Silwet L-77, used in plant transformation, was obtained from OSi Specialities.

2.9. Primers

AtpSDH2-3F HindIII	5´-AAGCTTCATTGTAACACGTAGTC-3´ (*Hind*III site underlined)
Atp2S1F HindIII	5´-AAGCTTATGAGTTACACATGGTT-3´ (HindIII site underlined)
Atp2S1R XhoI	5´-CTCGAGTTTTTGCTATGTGTGTATGTTTTG-3´ (XhoI site underlined)
bzip10-F	5´-CTCGAGATGAACAGTATCTTCTCC-3´ (XhoI site underlined)
bzip10-R	5´-GAATTCTCAGTCCACGCATTTTTTCG-3´ (EcoRI site underlined)
bzip25-F	5´-GAGCTCATGCACATCGTCTTCTCTG-3´ (SacI site underlined)
bzip25-R	5´-GGATCCTTAATGCTTGTGATTCC-3´ (BamHI site underlined)
bzip53-F	5´-GAGCTCATGGGGTCGTTGCAAAT-3´ (SacI site underlined)
bzip53-R	5´-CCCGGGTCAGCAATCAAACATATC-3´ (SmaI site underlined)
abi3-F	5´-GAGCTCATGAAAAGCTTGCATGTGG-3´ (SacI site underlined)
abi3-R	5´-GGATCCTCATTTAACAGTTTGAGAAGTTG-3´ (BamHI site underlined)
fus3-F	5´-CTCGAGATGATGGTTGATGAAAATGTGG-3´ (XhoI site underlined)
fus3-R	5´-GGTACCCTAGTAGAAGTCATCGAGA-3´ (KpnI site underlined)
sdh2-3-F	5’-TTGGATTCCATTGTAACACGTAG-3’ (BamHI site underlined)

sdh2-3-R	5’-ATTCCATGGTCTGTTCGCTTGATC-3’ (NcoI site encompassing the ATG start codon underlined)
mAuxRE-R	5’-GCAGGAGATCTAGATGTGCATG-3’ (mutated nucleotides underlined)
mAuxRE-F	5’-CATGCACATCTAGATCTCCTGC-3’ (mutated nucleotides underlined)
mDOF-F	5’-CCTGCAGATATCTACTCACCAAG-3’ (mutated nucleotides underlined)
mDOF-R	5’-CTTGGTGAGTAGATATCTGCAGG-3’ (mutated nucleotides underlined)
mAuxRE/mDOF-F	5’-CACATCTAGATCTCCTGCAGATATCTACTC-3’ (mutated nucleotides underlined)
mAuxRE/mDOF-R	5’-GAGTAGATATCTGCAGGAGATCTAGATGTG-3’ (mutated nucleotides underlined)
RYR	5’-GGAGAGAGACATGTGCATGG-3’
sdh2-3/35S	5’-CACATGTCTCTCTCC*ACAATCCCACTATC*-3’ (sequence from the *SDH2-3* promoter underlined, sequence from the CaMV 35S minimal promoter in italics)

GUS35S-R	5’-ACGTACC**ATG***GGGGACTGACC*-3’ (NcoI site underlined, GUS start codon in bold, CaMV35S 5’UTR in italics)
sdh2-3´´	5’ CACGTTTGAATTCGACCCGATAC 3’ (EcoRI site underlined)
sdh2-3-F´	5’-TTGAATTCATTGTAACACGTAG-3’ (EcoRI site underlined)
35S-5UTR1-R	5’ *TAGAGTCCCCCGTGTT*GTCAAAATCCTTGG 3’ (sequences complementary to CaMV35S 5’UTR and *SDH2-3* promoter in italics and underlined, respectively)
35S-5UTR2-R	5’ GGGGATCCTC*TAGAGTCCCCCG* 3’ (overlap with previous primer in italics)
35S-5UTR3-R	5’-GACTGACCACCC*GGGGATCCTCT* 3’ (overlap with previous primer in italics, overlap with GUS35-R underlined).
RT-sdh2-3-F	5´-TGCATATTGTGCGCGTGTTG-3´
RT-sdh2-3-R	5´-TATTCATCGCGGCTATCGCT-3´
RT-ABI3F	5´-GGCAGGGATGGAAACCAGAAAAGA-3´
RT-ABI3R	5´-GGCAAAACGATCCTTCCGAGGTTA-3´
RT-cln-F	5´-AATACGCGCTGAGTTCCCTT-3´
RT-cln-R	5´-AGCACCGGGTTCTAACTCAA-3´
RT-ubq10-F	5´-GGCCTTGTATAATCCCTGATGAATAAG-3´

RT-ubq10-R	5´-AAAGAGATAACAGGAACGGAAACATAGT-3´
RT-express-F	5´-ATCGAGCTAAGTTTGGAGGATGTAA-3´
RT-express-R	5´-TCTCGATCACAAACCCAAAATG-3´
RT-ppsdh2-3-F	5´-GTGTTTGTGTTCCGTCAATATGGAC-3´
RT-ppsdh2-3-R	5´-GACATCAAATACTCTTTGTTCTCAGGA-3´
RT-ppβtubulin1-F	5´-GAGTTCACGGAAGCGGAGAG-3´
RT-ppβtubulin1-R	5´-ATATCTTTCAGGCTCCACCG-3´
RT-actin2-F	5´-GCGAAGAGCGAGTATGACGAG-3´
RT-actin2-R	5´-AGCCACGAATCTAACTTGTGATG-3´
RT-histone3.2-F	5´-CGTCCAGGAACAGTCGCTCTT-3´
RT-histone3.2-R	5´-TTCACAGCCTACGCCCTCTCT-3´
RT-ppshp1-F	5´-GCTTACACGTTCGTTGATGT-3´
RT-ppshp1-R	5´-CTAGCCAACGATGACGTAGA-3´
Met1	5’-ATTAYGTTTGGATTYGATTYGATATAGGATTYGGTTT-3´
Met2	5’-TACTRCTCAAACRCACTTTCTCCRCCRCTAA-3´
AtSDH2-1S EcoRI	5’-GAATTC*ATG*GCGTCTGGTTTGATCGG-3´ (EcoRI site underlined, start codon in italics)

AtSDH2-2S EcoRI	5´-GAATTC*ATG*GCGTTTGGTTTGATCGG-3´ (EcoRI site underlined, start codon in italics)
AtSDH2-3S EcoRI	5’-GAATTC*ATG*TCGTCTGTCTTGCGGTT-3´ (EcoRI site underlined, start codon in italics)
PpSDH2-1S BamHI	5´-GGATCC*ATG*GCAGCGATAACGAGGGC-3´ (BamHI site underlined, start codon in italics)
PpSDH2-3S EcoRI	5’-GAATTC*ATG*GCGCACCGAAAGACATT-3´ (EcoRI site underlined, start codon in italics)
AtSDH2-1AS XhoI	5’-CTCGAG*TCA*ACGCTGAAGTTGCTTGATG-3´ (XhoI site underlined, stop codon in italics)
AtSDH2-2AS XhoI	5´-CTCGAG*TCA*ACCAGATTTCTGAAGCT-3´ (XhoI site underlined, stop codon in italics)
AtSDH2-3AS XhoI	5’-CTCGAG*CTA*GACACTCTCTGTTCTCACT-3´ (XhoI site underlined, stop codon in italics)
PpSDH2-1AS XhoI	5´-CTCGAG*CTA*TTAGGCTTATCTTGTAATC -3´ (XhoI site underlined, stop codon in italics)
PpSDH2-3AS XhoI	5’-CTCGAG*TTA*TATCATGTCAGGGCGATC-3´ (XhoI site underlined, stop codon in italics)

YSDHBS EcoRI	5’-GAATTC*ATG*TTGAACGTGCTATTGAGAA-3´ (EcoRI site underlined, start codon in italics)
YSDHBAS XhoI	5´-CTCGAG*CTA*GGCAAATGCCAAAGATT-3´ (XhoI site underlined, stop codon in italics)
YSDHBAS	5’-TCTGGATTCCATCTGTAAAC-3´
YSDHBS1	5’-GTTTACAGATGGAATCCAGA-3´
YSDHBS2	5’-GTTTACAGATGGAATCCTGA-3´
promppsdh2-3-F	5´-GAGAGTAAAATGTGAAAGGA-3´
promppsdh2-3-R	5´-GAGGTCCTGCGTCGCATTAG-3´

3. METHODS

3.1. Arabidopsis growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) seeds were surface sterilized with a 10% (v/v) solution of commercial bleach for 10 min, rinsed thoroughly with sterile water and plated on half strength MS agar plates (2.2 g / L) supplemented with 1% (w/v) sucrose and 0.8% (w/v) agar. Seeds were stratified at 4°C for 48h in the dark, and then plates were incubated at 22°C, under a 16h light/8h dark cycle. For transformed plant selection, 25 µg / ml hygromycin was added to the plates. For hypocotyl length analysis, seeds were sterilized and stratified as above, but grown at 22°C in the dark in vertically placed plates. Sometimes sucrose (1% w/v for routine growth), succinate, fumarate, zebularine, TTFA and ABA were added to the plates at the stated concentrations. In order to obtain seeds or adult plants for protoplast preparation, seeds were either sown on plates and transferred to soil after two weeks at 22°C under a 16 h light/ 8 h dark cycle, or planted directly into soil. Then plants were grown at 22°C under long day conditions (16 h light/ 8 h dark) to promote flowering for transformation and collecting seeds, or under short day conditions (8 h light/ 16 h dark) to promote vegetative growth and obtain big leaves suitable for protoplast preparation.

3.2. Nucleic acids preparation

3.2.1. RNA extraction from vegetative tissues

Total RNA from Arabidopsis vegetative tissues was extracted with Trizol reagent using the recommended manufacturer protocol. Approximately 50-100 mg of tissue were grounded in liquid nitrogen and 1 ml of Trizol was added. After processing all the samples and incubating for 5 min at room temperature, 0.2 volume of chloroform was added and the tubes agitated in a vortex for 15 seconds. Later, the tubes were centrifuged for 15 min at 4°C and the resulting aqueous phase was transferred to a new tube and 500 µl of isopropanol were added. The RNA was precipitated for 10 min at room temperature and then centrifuged at 12000*g for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed with 70% ethanol. The pellet was air dried and dissolved in DEPC-treated water.

Total RNA from Physcomitrella gametophore or protonema tissue was performed following the same protocol.

3.2.2. RNA extraction from seeds

RNA extraction was performed according to Onate-Sanchez and Vicente-Carbajosa (2008) with minor modifications. 20-25mg of seeds were grounded in a mortar with liquid nitrogen and then transferred to a 1.5 ml eppendorf tube. Then, 550 µl of extraction buffer (0.4M LiCl; 0.2M Tris-HCl pH 8; 25mM EDTA pH 8; 1% w/v SDS; DEPC-treated water) and 550 µl of chloroform were added. After agitation in a vortex and centrifuging at

12000*g for 3 min at room temperature, the aqueous phase was carefully transferred to a new 1.5 ml tube. Afterwards, 1 volume of water-saturated phenol was added, and the tube was agitated in a vortex and centrifuged under the same conditions. The aqueous phase was transferred to a new 1.5 ml tube and extracted with 1 volume of chloroform. After centrifugation at 12000*g, the upper phase was transferred to a new 1.5 ml tube and RNA was precipitated by the addition of ⅓ of the initial volume of 8M LiCl and incubation at -20°C for 1 hour or overnight at 4°C. Then, a centrifugation step at 12000*g and 4°C was performed for 20-30 min. The pellet was dried for 15 min at room temperature, resuspended in 30 µl of DNase buffer containing 10 units of RNase-free DNaseI (Promega) and incubated for 30 min at 37°C. Then, 470 µl of water, 7 µl of 3M NaAc pH 5.2 and 250 µl of absolute ethanol were added. The tube was then centrifuged at 12000*g and 4°C for 10 min. Supernatant was carefully transferred to a new 1.5 ml tube, and 43 µl of 3M NaAc pH 5.2 and 750 µl of ethanol were added and incubated for at least 1 hour at -20°C. We then centrifuged at 12000*g for 20 min at 4°C, washed the pellet with 70% ethanol, air-dried and resuspended in 20 µl of DEPC-treated water. RNA concentration was determined by A_{260} nm, in a Nanodrop spectrophotometer.

3.2.3. DNA extraction from vegetative tissues

Genomic DNA was prepared from seedlings or leaves of 4-6 week old plants. We grounded approximately 200 mg of plant tissue in a mortar with liquid nitrogen and mixed with 500 µl of CTAB buffer (2% (w/v) Hexadecyl trimethyl-ammonium bromide (CTAB); 100 mM Tris pH 8; 20 mM EDTA pH 8; 1.4 M NaCl; 1% (w/v) PVP 40. The CTAB/plant

extract mixture was agitated in a vortex and transferred to a microfuge tube and incubated at 55°C for 15 min. After incubation, the sample was centrifuged at 12000*g for 5 min at room temperature in order to spin down debris. Supernatant was carefully transferred to a new microfuge tube, and 250 µl of chloroform:Isoamyl-alcohol (24:1) were added. After agitation in a vortex, a centrifugation step was performed at 12000*g for 1 minute at room temperature. The aqueous phase was transferred to a new tube and 50 µl of 7.5 M ammonium acetate followed by 500 µl of ice cold absolute ethanol were added. After mixing by slow inversion and incubation for 1 hour at -20°C, precipitated DNA was recovered by centrifugation at 12000*g for 15 min at 4°C. The resulting pellet was washed with 70% ethanol and resuspended in 20 µl of DEPC-treated water

3.2.4. DNA extraction from seeds

Approximately 25-50 mg of dry seeds were grounded in a mortar with liquid nitrogen and then resuspended in 500 µl of the CTAB buffer described above. The CTAB/plant extract mixture was incubated at 55°C and extracted with chloroform:isoamyl-alcohol (24:1) exactly as described for DNA from vegetative tissues (methods 3.2.3.). Then the aqueous phase was extracted 3 times with two volumes of phenol:chloroform:isoamyl-alcohol (25:24:1). After the last centrifugation at 12000*g, the aqueous phase was transferred to a new tube, sodium acetate was added to a final concentration of 0.3 M and DNA was precipitated by addition of 0.5 volumes of ethanol and incubation overnight at -20°C. After centrifugation at 12000*g for 15 min at 4°C, the pellet was resuspended in 100 µl of a Ribonuclease A solution (10 µg / ml; Promega) and

incubated for 30 min at 37°C. After this, samples were diluted with 400 µl of water, sodium acetate was added to a final concentration of 0.041 M and carbohydrates were precipitated by adding 250 µl of cold (-20°C) ethanol. After 2-3 min at room temperature, centrifugation was performed at 12000*g for 10 min at 4°C, and the supernatant was transferred to a new tube. Afterwards, 750 µl of ethanol and sodium acetate to a final concentration of 100 mM were added. After centrifugation at 12000*g for 20 min at 4°C, the pellet was washed twice with 70% ethanol and resuspended in 20 µl of DEPC-treated water. DNA concentration was determined by measuring the absorbance at 260 nm using the Nanodrop spectrophotometer.

3.2.5. RNA extraction from Physcomitrella

Approximately 50-100 mg of gametophore tissue or 20-50 mg of dry protonema tissue were grounded in a mortar with liquid nitrogen. Further RNA extraction was performed as described in section 3.2.1. (methods).

3.2.6. DNA extraction from Physcomitrella

Approximately 50-100 mg of gametophore tissue were grounded in a mortar with liquid nitrogen and DNA extraction was performed exactly as described in section 3.2.3. (methods).

3.2.7. Yeast RNA extraction

Yeasts were grown in liquid YPD medium with permanent agitation at 30°C. Cells were pelleted by centrifugation at 12000*g for 5 min and total RNA extraction was performed using the same protocol described in section 3.2.1. (methods) with 1 mL of the reagent per 5-10 x 10^6 yeast cells.

3.3. Recombinant DNA techniques

3.3.1. cDNA synthesis

cDNAs were obtained using the MMLV^{tm} Reverse Transcription System from Promega. cDNA synthesis was performed according to the supplier protocol. Between 0.5 and 2 µg of DNaseI-treated RNA were mixed with 50 ng of random hexamers in a final volume of 12 µl. Samples were incubated at 80°C for 10 min and then transferred to ice. After a 5 min incubation on ice, 4 µl of 5X reaction buffer (50 mM Tris-HCl pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 10 mM DTT), 1 µl of a 10 mM dNTP mix, 1 µl of RNAsin reagent from Promega and 1 µl (50 U) of the MMLV reverse transcriptase were added. Then, samples were incubated at 25°C for 10 min and at 37°C for 1 hour. The reaction was stopped by incubating the samples at 70°C for 15 min.

3.3.2. PCR

Routine PCR reactions were made in a final volume of 20 µl and Taq DNA polymerase from Invitrogen was used. Buffers and primer concentrations (200 pmoles each primer) were as recommended by the supplier. After a denaturation step at 94°C for

3 min, 35 cycles of the following steps were performed: 30 seconds at 94°C (denaturation), 30 seconds at 55-57°C (hybridization) and 30 seconds per 1000 nucleotides at 72°C (extension). Then, a final elongation step (72°C) for 10 min was performed and the samples were cooled down to 4°C. Samples were analyzed by agarose gel electrophoresis (section 3.3.6. in Methods).

For transcript relative quantification, RT-qPCR assays were performed using the Mx3000P QPCR System (Stratagene) according to the manufacturer´s instructions and the SensiMix Plus SYBR kit provided by Quantace (www.bioline.com). Clathrin and PpActin2 were selected as expression normalizers by the geNorm program for Arabidopsis and Physcomitrella, respectively (Vandesompele et al., 2002). This program selects the most stable expressed reference (housekeeping) gene from a set of tested candidate reference genes which include clathrin, ubiquitine 10 and an unknown Arabidopsis expressed protein for Arabidopsis, and tubulin1, actin2 and histone3.2 for Physcomitrella.

In order to analyze the quality and efficiency of the used primers, template DNAs of known concentration have to be prepared. To this end, three PCR reactions were made for each primer pair, using as template 1 µl of cDNA from seeds or leaves. PCR products were mixed and purified by electrophoresis on 1% agarose gels and extraction with commercial kit “EZNA Purification Band System”. DNA concentration was then determined in a Nanodrop spectrophotometer, and dilution series ranging from 5 pg / µl to 0.00005 pg / µl were prepared. These dilutions were used as templates for qPCR reactions. The qPCR mix used was the following: 12.5 µl of 1X Sensimix^{tm} Plus SYBR from Quantace (buffer reaction; heat-activated DNA polymerase; dNTPs; 6mM $MgCl_2$; SYBR Green I and ROX

internal standard); 0.5 µl of each 10 µM primer; 2 µl of each template dilution and 9.5 µl of MiliQ water. A technical duplicate was performed for each amplification. The qPCR program was the following: 95°C for 10 min (enzyme activation); 40 cycles of 95°C for 30 seconds, 55-57°C for 30 seconds, and 72°C for 30 seconds; and a final cycle of 72°C for 10 min. Melting curves were performed to verify the amplification of a single product and results were analyzed with the MxPro software. Standard curves were generated for each primer pair by plotting the Ct obtained for each dilution (Ct is defined as the cycle in which fluorescence is significantly different from the background) against the log of the template DNA (in pg). To validate a primer pair, the correlation coefficient (R^2) for the linear regression must be greater than 0.985 and efficiencies must range between 90 and 110%. Efficiencies (E) are calculated from the slope of the curve [$E = 10^{(-1/\text{standard curve slope})}$].

For transcript level determinations in different RNA preparations, we used the same procedure. In general, three biological replicates (RNAs and cDNAs) were prepared for each condition (genotype or treatment) and each cDNA was amplified twice (technical replicates). RNA levels were determined in relation to the housekeeping gene, taking into account the efficiencies of amplification of the gene of interest and of the housekeeping gene.

To evaluate gene expression in Arabidopsis, the following primer pairs were used: RT-sdh2-3-F and RT-sdh2-3-R for *SDH2-3*; RT-ABI3F and RT-ABI3R for *ABI3*; RT-cln-F and RT-cln-R for c*lathrin*. For Physcomitrella gene expression, the following primers were used: RT-ppsdh2-3-F and RT-ppsdh2-3-R for *PpSDH2-3*; RT-actin2-F and RT-actin2-R for *actin2*; RT-ppshp1-F and RT-ppshp1-R for *PpSHP1*.

3.3.3. Cloning of DNA fragments in plasmids

DNA fragments obtained by PCR, RT-PCR or plasmid digestion with restriction enzymes were purified by electrophoresis on 1% agarose gels and extracted with commercial kit "EZNA Purification Band System". Then, they were ligated into the corresponding plasmids with T4 DNA ligase (as recommended by the suppliers).

Competent cells were prepared by inoculating 5 ml of LB medium with 150 µl of a bacterial culture in stationary phase. After incubation with constant agitation (200 r.p.m.) at 37°C overnight in the presence of the adequate antibiotic, this preculture was inoculated into 250 ml of LB medium. Bacterial growth was carried out at 37°C and constant agitation (200 r.p.m.) until an $O.D._{600nm}$ of 0.5 (2-3 hours approximately). Cells were then centrifuged at 2000*g for 10 min at room temperature in 50 ml polypropylene tubes. The resulting pellet was resuspended in 25 ml of a sterile and ice-cold solution of 75 mM $CaCl_2$, maintained in ice for 1 hour and centrifuged under the same conditions. Afterwards, the bacterial pellet was resuspended in 5 ml of ice-cold 75 mM $CaCl_2$, and glycerol (14% (v/v) final concentration) was added. Competent cells were stored in 100 µl aliquots in 1.5 ml eppendorf tubes at -80°C.

In order to proceed to transformation, 100 µl of competent bacterial cells were mixed with 10-100 ng of plasmid and incubated in ice for 30 min. After a 1 min heat-shock at 42°C, the tube was transferred to ice for 2 min and then, 800 µl of LB medium were added. After incubation at 37°C with constant agitation for 60 min, 200 µl were spread on

1.5% (w/v) LB agar plates supplemented with the appropriate antibiotic (100 μg / ml ampicillin or 50 μg / ml kanamycin).

To determine if the resulting colonies contained the plasmid, two methodologies were used. The first method was to analyze the transformed colonies by PCR of the cloned fragment. For this, a single colony was touched with a sterile tip and then introduced in a tube containing a PCR mix with the appropriate set of primers. PCR was performed as described before. The resulting fragments were analyzed by agarose gel electrophoresis. The second method consisted in small scale plasmid purification and its analysis by restriction enzyme digestion and agarose gel electrophoresis (Sections 3.3.4., 3.3.5. and 3.3.6. in Methods).

3.3.4. Plasmid DNA extraction from bacterial cultures

E. coli strains (XL-1 Blue or DH5-α) containing the plasmids of interest were grown in liquid LB medium (10 g / L bactotryptone, 5 g / L yeast extract and 5 g / L NaCl) with permanent agitation at 37°C and with the appropriate antibiotic (50 μg / ml kanamycin or 50 μg / ml ampicillin). Plasmids were purified from 5 ml of bacterial cultures for Miniprep extractions or 100 ml for Maxiprep extractions. Plasmids were purified following the recommended protocol from Axygen.

3.3.5. Digestion with restriction enzymes

For plasmid digestions, DNA (0.2-1 µg) was incubated with 1-10 units of the appropriate restriction enzyme in a final volume of 10 µl for 2 hours or o/n at 37°C in the presence of the recommended buffer. The DNA fragments were separated by electrophoresis on agarose gels.

3.3.6. Agarose gel electrophoresis for DNA separation

DNA fragments were separated by electrophoresis on 1.5 % (w/v) agarose gels prepared with 1X TAE solution (40 mM Tris-acetate; 1 mM EDTA; pH 8). One µl of a 10 mg / ml ethidium bromide solution was added for every 200 ml of gel. After solidification of the agarose gel, 10 µl of DNA samples were mixed with 2 µl of loading buffer (20 % (v/v) Glycerol; 0.1 % (w/v) sarkosyl; 0.1 % (w/v) blue bromophenol; 25 mM EDTA), and loaded into the gel. Electrophoresis was performed at 80 mV in the presence of 1X TAE buffer. DNA fragments were visualized in an UV transilluminator and photographed using a Canon PC 1130 camera.

In some cases PCR-obtained DNA fragments were eluted from the agarose gel using the “EZNA Purification Band System”, following the recommended protocol.

3.3.7. Sequence analysis

Sequence reads from plasmids were obtained from Macrogen Online Sequencing Order System (dna.macrogen.com). Alignments against sequences obtained from

Genbank (www.ncbi.nlm.nih.gov) were done by using the Clustal algorithm in the Megalign option from the DNastar program.

New *cis* elements in the *SDH2-3* promoter were determined using the PLACE online program (PLAnt Cis-regulatory Elements; Higo et al. (1999)). This program has a *cis* elements database of seed-specific gene promoters, both in monocotyledons and dicotyledons.

3.4. *SDH2-3* promoter analysis in transgenic plants

3.4.1. Constructs for promoter analysis

The construct -223/+49 (numbers in relation to the *SDH2-3* transcription initiation site), containing the *SDH2-3* promoter and 5' untranslated region (UTR) fused to the β-glucuronidase (GUS) coding sequence, has been previously described (P3 construct, Elorza et al. 2004; see also Results, figure 11). To shorten the promoter, the region between -114 and +49 was amplified by PCR using Arabidopsis genomic DNA as template, forward primer sdh2-3F and reverse primer sdh2-3R. Mutagenesis of putative AuxRE and DOF elements was performed by PCR, using the P3 construct as template. Two PCRs were carried for each mutant with the same template. For the promoter mutated in the AuxRE element (mAuxRE), one of the amplifications was done with primers sdh2-3F and mAuxRE-R and the other was performed with primers sdh2-3R and mAuxRE-F. A mixture of both amplification products was used as a template for a third PCR with primers sdh2-3F and sdh2-3R. The same procedure was employed to obtain constructs mutated in DOF (mDOF) or in both AuxRE and DOF elements (mAuxRE/mDOF), using sdh2-3F, sdh2-3R and the

following *mutated* primers: mDOF-F and mDOF-R for the mutated DOF element; mAuxRE/mDOF-F and mAuxRE/mDOF-R for the double mutant. PCR products (-114/+49 wild type and mutated constructs) were cloned into pGEM-T plasmid (Promega), and the DNA fragments obtained by digestion with *Bam*HI and *Nco*I were ligated into pCAMBIA1381 (http://www.cambia.org).

To fuse the -223 to -32 fragment of the *SDH2-3* promoter to the minimal cauliflower mosaic virus (CaMV) 35S promoter (construct -223/-32/-64p35S, see figure 11 in Results section), two PCRs were performed. The -223/-32 region was first amplified using the P5 construct (Elorza et al. 2004) as template, forward primer sdh2-3´´ and reverse primer RYR. The CaMV35S minimal promoter (-64 to +41) was amplified using pBI121 (Clontech) as template, forward primer sdh2-3/35S and reverse primer GUS35S-R. A mixture of both amplification products was employed as template for a third PCR with primers sdh2-3´´ and GUS 35S-R. PCR products were cloned into pGEM-T plasmid, and DNA fragments digested with *Eco*RI and *Nco*I were ligated into pCAMBIA1381.

To obtain the region upstream of the *SDH2-3* transcription initiation site (-114 to -1) fused to the CaMV35S 5'UTR (construct -114/-1/35S5´UTR in figure 11, Results section), four serial PCRs were performed using the same forward primer (sdh2-3F') and different partially overlapping reverse primers designed to reconstitute the 5'UTR. These reverse primers were 35S-5UTR1-R, 35S-5UTR2-R, 35S-5UTR3-R and finally primer GUS35S-R. Final PCR product was cloned into pCAMBIA1381 as described above.

The construct -114/-32/-64p35S, containing the -114 to -32 fragment of the *SDH2-3* promoter fused to the minimal CaMV35S promoter and 5´UTR (-64 to +41), and its control construct -64p35S, containing only the minimal CaMV35S promoter and 5´UTR fused to GUS, have been previously described (Roschzttardtz, 2007).

The *PpSDH2-3* promoter was obtained by PCR using Physcomitrella genomic DNA as template, forward primer promppsdh2-3-F and reverse primer promppsdh2-3-R. Final PCR product was cloned into pCAMBIA1381 as described above.

The structures of all the constructs made during this thesis were verified by DNA sequencing. PCR conditions were as described in section 3.3.2. of Methods.

3.4.2. *Agrobacterium tumefaciens* transformation with plasmids

For the preparation of competent cells, 50 ml of LB medium were inoculated with 1 ml of a saturated Agrobacterium culture (strain GV3101) and incubated at 28°C with agitation (120 rpm) until the culture reached an OD_{600nm} of 0.5 (12 hours approximately). Then, the cells were centrifuged for 10 min at 3000*g in 50 ml polypropylene tubes and the cell pellet was resuspended in a cold and sterile 20 mM $CaCl_2$ solution and maintained in ice for 15 min. From this point the cells were considered competent for transformation. Cells were mixed with glycerol (14% (v/v) final concentration), and aliquots of 100 µl were frozen in liquid nitrogen and stored at -80°C. For transformation, 5 µl (1 µg) of plasmid were added to a tube with 100 µl of frozen Agrobacterium cells and incubated at 37°C for 5 min. Then, 1 ml of LB medium was added and cells were incubated for 3 hours at 28°C

with agitation. Finally, 100 µl were spread on LB agar plates with 50 µg / mL of kanamycin and incubated 48-72 hours at 28°C, until colonies became visible.

Colonies were analyzed by PCR in order to verify plasmid presence, following the procedure described above (section 3.3.3.).

3.4.3. Arabidopsis transformation

The floral dip protocol described by Clough and Bent (1998) was used for Arabidopsis transformation. Arabidopsis plants were grown in soil and stems were cut when they reached approximately 10 cm, in order to induce growth of more lateral stems. The procedure was repeated once to obtain plants with more stems and flowers. On the other hand, 2 ml of LB medium with 50 µg / ml kanamycin, 50 µg / ml rifampicin and 50 µg / ml gentamicin were inoculated with a strain of Agrobacterium GV3101 transformed with the plasmid of interest, and incubated for 2 days at 28°C with constant agitation. Then, 200 ml of LB supplemented with the same antibiotic mix were inoculated with 1 ml of the pre-culture and incubated for 1 day at 28°C with constant agitation. Cells were centrifuged at 5000*g for 20 min at 20°C, and then the pellet was resuspended in transformation medium (5% (w/v) sucrose; 0.05% (v/v) Silwet L-77) to an approximately OD_{600nm} of 0.8 (normally 300 ml of transformation medium). For transformation, 300 ml of the Agrobacterium suspension was transferred to a wide recipient with a magnetic stirrer, in order to homogenize the bacterial medium. Aerial parts of the plants were completely submerged for 15 seconds in the bacterial suspension, and then plants were grown under normal conditions until seeds (T1 generation) were collected.

Transformed seeds should have the hygromycin resistance given by the T-DNA of the pCAMBIA1381 plasmid. Thus, seeds obtained from the transformed plants were sown on agar plates containing 25 µg / µl of hygromycin. Non-resistant plants did not develop true leaves and long roots as compared with resistant seedlings after 6 days. Ten days post germination, transformed seedlings were transferred to soil and plants were grown under standard conditions in order to obtain T2 seeds. Construct presence was also confirmed by PCR using genomic DNA from leaves.

3.4.4. Quantitative GUS analysis

Arabidopsis transgenic plants carrying the different constructs were selected and soluble extracts of different tissues were assayed fluorometrically for GUS activity. Tissue samples were collected, stored at -80°C, grounded in liquid nitrogen and transferred to a tube with 500 µl of extraction buffer (50 mM sodium phosphate pH 7; 10 mM β-mercaptoethanol; 10 mM EDTA; 0.1% (w/v) sarkosyl; 0.1% (v/v) Triton X-100) per 50 mg of tissue. After agitation in a vortex and centrifugation at 12000*g for 10 min at 4°C, GUS activity was measured by mixing 200 µl of the extraction buffer with 50 µl of the protein extract and 250 µl of GUS activity buffer (extraction buffer with 2 mM 4-methylumbelliferyl-β-D-glucuronide as substrate). Incubation was performed for 1 hour at 37°C. A 100 µl aliquot was mixed with 1.9 ml of Na_2CO_3 0.2 M in order to stop the reaction. The reaction product (4-methylumbelliferone, 4MU) was quantified by determining the emitted fluorescence in a Hoefer TKO fluorometer (λ_{ex}=365 nm, λ_{em}=460 nm) calibrated with known 4-MU concentrations (10-100 nM). In order to obtain GUS

specific activities, protein concentrations of the samples were determined by the Bradford method (Bradford, 1976), and GUS activities were calculated as nanomoles of 4-methylumbelliferone produced per hour and milligram of protein.

3.4.5. Histochemical GUS assay

Mature embryos were manually separated from the testa and endosperm with syringe needles using a stereoscopic microscope (Nikon SMZ800), and incubated in GUS staining buffer (50 mM sodium phosphate pH 7.2; 0.5 mM $K_3Fe(CN)_6$; 0.5 mM $K_4Fe(CN)_6$; 0.1% (v/v) Triton X-100) with 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide; 100mM stock solution in dimethylformamide). Vacuum was applied for 10 min and samples were incubated at 37°C for 12 hours (Jefferson et al., 1987). After incubation, samples were clarified by several washing steps with 70% ethanol at 37°C. Stained samples were observed with a stereoscopic microscope (Nikon SMZ800) and photographed with a Nikon Coolpix 4500 CCD digital camera.

3.5. *SDH2-3* promoter analysis by transient expression in protoplasts

3.5.1. Constructs for transient expression

Two reporter plasmids carrying different promoters fused to GUS were prepared. The construct -393/+49 (P4 construct; Elorza et al., 2004) was used as template to amplify the wild type *SDH2-3* promoter and 5´UTR (-114/+49) with forward primer AtpSDH2-3 F HindIII and reverse primer sdh2-3-R NcoI. A 234 bp DNA fragment containing the promoter and 5´UTR of the gene encoding *Arabidopsis thaliana* 2S1 albumin (At2S1) was

obtained by PCR with primers Atp2S1 F Hind III and Atp2S1 R XhoI, and DNA from Col-0 seedlings as template. PCR products were cloned into pGEM-T plasmid, and the DNA fragments obtained by digestion with HindIII and NcoI (XhoI for 2S1 promoter) were ligated into pBT10-GUS (Sprenger-Haussels and Weisshaar, 2000).

To obtain the effector plasmids driving the expression of bZIP and B3 transcription factors, total cDNA was prepared using DNase-treated RNA from seeds and the protocol described in Methods, section 3.3.1. Then, cDNAs for each transcription factor were generated by PCR amplification, using cDNA prepared from Arabidopsis seed RNA as template and the following primer pairs: forward primer bZIP10-F and reverse primer bZIP10-R for bZIP10; forward primer bZIP25-F and reverse primer bZIP25-R for bZIP25; forward primer bZIP53-F and reverse primer bZIP53-R for bZIP53; forward primer ABI3-F and reverse primer ABI3-R for ABI3. PCR products were cloned into pGEM-T plasmid and the DNA fragments obtained by digestion with the appropriate restriction enzymes (XhoI and EcoRI for bZIP10; SacI and BamHI for bZIP25 and ABI3; SacI and SmaI for bZIP53; XhoI and KpnI for FUS3) were ligated into PUC pSS.

In addition to a reporter plasmid and effector plasmids, a plasmid containing the NAN gene under the control of the CaMV35S promoter, kindly donated by Dr. Vicente-Carbajosa (CBGP, Madrid, Spain) was used as described in Kirby and Kavanagh (2002) to normalize for protoplast transfection efficiency. The NAN gene encodes for an optimized version of the “small” cytoplasmic sialidase of *Clostridium perfringens* (Kirby & Kavanagh, 2002).

3.5.2. Protoplast isolation and transfection

Protoplast isolation and transfection were performed as described by Yoo et al. (2007) with minor modifications. Leaves were obtained from 6-8 week-old healthy plants grown in a short day cycle (8 hours light / 16 hours dark). The middle part of the leaves was cut into 0.5-1 mm leaf strips that were transferred immediately to a freshly prepared enzyme solution (20 mM MES pH 5.7; 1.5% (w/v) cellulase R10; 0.4% (w/v) macerozyme R10; 0.4 M mannitol; 20 mM KCl; 10 mM $CaCl_2$) previously filtered through a 0.45 µm syringe filter. Approximately 20 leaves and 10-20 ml of enzyme solution were used per experiment. Leaf strips were vacuum infiltrated with the enzyme solution for 30 min in the dark, and enzymatic digestion of the cell wall was carried out at 22°C in the dark for 12-16 hours. After digestion, the solution containing the released protoplasts was filtered through a 75 µm nylon mesh humified with W5 solution (2 mM MES pH 5.7; 154 mM NaCl; 125 mM $CaCl_2$; 5 mM KCl) into a 50 ml polypropylene tube. The tube was then centrifuged at 100 x g for 2 min and the supernatant was discarded. Ten ml of the W5 solution were added and the protoplasts were gently mixed and centrifuged as described before. The supernatant was discarded and 10 ml of W5 solution were added and the tube gently swirled. A 10 µl aliquot was used for cell counting on a Neubauer Chamber and the rest of the protoplasts were kept on ice for 3-5 hours. Protoplasts settle at the bottom of the tube by gravity after 15 min, so the supernatant was carefully discarded and the cells were resuspended at 2 x 10^5 ml^{-1} in MMG solution (4 mM MES pH 5.7; 0.4 M mannitol; 15 mM $MgCl_2$).

For DNA-PEG-calcium transformation, 20 µl of the plasmid mix (20 µg in total) were added to a 2 ml microfuge tube. Three types of plasmids were used: a reporter plasmid (6 µg) with either the *SDH2-3* promoter or the *At2S1* promoter fused to GUS (pBT10-GUS vector), effector plasmids (4 µg each) expressing transcription factors (PUC pSS vector, which was added to complete 12 µg when less than 3 effectors plasmids were used) and a control plasmid for transfection (2 µg, 35S::NAN). Then, 200 µl of the protoplast solution were added and mixed gently with DNA, followed by the addition of 220 µl of PEG solution (40% (w/v) PEG 4000; 0.2 M mannitol; 100 mM $CaCl_2$). Tubes were mixed by inversion for 12-14 times and incubated at room temperature for 20 min. Afterwards, protoplasts were diluted with 800 µl of W5 solution and centrifuged at 100 x g for 2 min. Supernatant was discarded and protoplasts were resuspended in 250 µl of WI solution (4 mM MES pH 5.7; 0.5 M mannitol; 20 mM KCl) and incubated at 22°C for 12-16 hours without agitation. After incubation, supernatant was carefully removed and protoplasts were frozen in liquid nitrogen and kept at -80°C until further analysis. All transfections were performed in triplicate.

3.5.3. GUS and NAN activity assays

Frozen protoplast pellets were resuspended in 150 µl of resuspension buffer (50mM sodium phosphate pH 7.2; 10mM EDTA; 0.1% (v/v) Triton X-100; 0.03% (w/v) sarkosyl) and strongly agitated in a vortex for 3 min. For GUS assay, a 100 µl aliquot was transferred to a tube with 100 µl of GUS activity buffer (50mM sodium phosphate pH 7.5; 10mM EDTA; 0.1% (v/v) Triton X-100; 0.03% (w/v) sarkosyl; 1 mM MUG) and incubated for

1 hour at 37°C. The reaction was stopped by mixing 100 µl of the reaction mix with 100 µl of 0.2 M Na_2CO_3. For NAN assay, a 10 µl aliquot was added to 10 µl of NAN activity buffer (50mM sodium phosphate pH 7; 10mM EDTA; 0.1% (v/v) Triton X-100; 0.03% (w/v) sarkosyl; 1mM MUN). After incubation at 37°C for 30 min, a 10 µl aliquot was mixed with 190 µl of 0.3 M Na_2CO_3 to stop the reaction. The product of both GUS and NAN reactions is MU, of which fluorescence was measured using a The Synergy™ HT fluorometer from BioTek (λ_{ex}=365 nm, λ_{em}= 460 nm). Promoter activity was scored as the ratio of GUS activity to NAN activity for each individual transfection. Every column represents the mean of three independent transfections.

3.6. Epigenetic regulation of the SDH2-3 promoter

3.6.1. DNA bisulphite treatment

The bisulphite treatment is based on Clark et al. (2006) with minor modifications. This method exploits the different sensitivities of cytosine and 5-methylcytosine (5-MeC) to deamination under acidic conditions, in which cytosine undergoes conversion to uracil, whereas 5-MeC remains unreactive. The first step is a denaturation process, in which 500-2000 ng of seed or 15 day-old seedling DNA were incubated in 20 µl of 0.3 M NaOH for 15 min at 37°C, and 2 min at 90°C. The tubes were immediately placed on ice for 5 min and the following components were added: 208 µl of a freshly made saturated sodium metabisulphite solution (pH 5), 12 µl of 10mM quinol and 200 µl of mineral oil. After gently mixing, incubation was carried out at 55°C for 16 hours. During this incubation, sulphonation and hydrolytic deamination convert non methylated C residues to U

residues. Treated DNA was desalted using the Promega Wizard Clean-Up kit, following the manufacturer protocol. One ml of the Promega Wizard Clean-Up resin was added to each tube and mixed by inversion in order to bind the DNA to the silica gel slurry. Afterwards, the tube content was passed through a minicolumn and washed with 2 ml of 80% isopropanol. The minicolumn was transferred to a new 1.5 ml tube and the DNA was eluted with 50 µl of water. For the final step of alkali desulphonation, 5.5 µl of 3 M NaOH were added to the tube and incubated for 15 min at 37°C. Then, 1 µl of 10 mg / ml yeast tRNA was added and nucleic acids were precipitated by addition of 33.3 µl of 5 M ammonium acetate (pH 7) and 330 µl of ice cold ethanol. After incubation at -20°C for 1 hour, DNA was recovered by centrifugation at 4°C for 15 min at 12000*g. The pellet was air-dried and resuspended in 20 µl of water.

In order to analyze bisulphite-treated DNA, PCR reactions using *SDH2-3* promoter-specific primers were performed. After treatment, DNA should have different methylation profiles, so suitable primers are needed. These primers were designed as follows: for the forward primer (Met1), all the cytosines in CG context were replaced by Y (pyrimidine; C or T) and all other Cs were replaced by Ts; and for the reverse primer (Met2), all the guanines in CG context were exchanged for R (purine; A or G) and all the remaining Gs were replaced by As. Since in the Met1 forward primer the G residues were not changed to A (or R) and in the Met2 reverse primer the C residues were not changed to T (or Y), these primers would not amplify the deaminated antisense strand and would be specific for the sense strand. In other words, if the C residues in the antisense strand are not methylated, our PCR strategy would not allow us to amplify this DNA strand: obtained

results are consistent with specific amplification of the sense strand, and thus C methylation was analyzed only in this strand. Finally, PCR products were analyzed by agarose gel electrophoresis, cloned and sequenced.

3.7. Characterization of seeds and postgerminative growth

3.7.1. Determination of seed weight

In order to determine individual seed weight, seven replicates of approximately 200 seeds of wild-type and mutant *sdh2-3* seeds were weighed. Seeds were counted using a stereoscopic microscope (Nikon SMZ800).

3.7.2. Seed protein analysis

Total seed protein content was measured by grinding 30 seeds in liquid nitrogen in a mortar in the presence of 200 µl of extraction buffer (50mM sodium phosphate pH 7.2, 10mM β-mercaptoethanol, 10mM EDTA, 0.1% (w/v) sodium lauroyl sarcosine, 0.1% (v/v) Triton X-100), followed by Bradford quantification method (Bradford, 1976).

For total protein separation, 20 seeds were grounded in 20 µl of SDS sample buffer (0.25M Tris-HCl pH 6.6; 5% (v/v) glycerol; 5% (w/v) SDS; 0.1% (w/v) bromophenol blue) in an eppendorf tube using a pestle. Then, 15ul of the protein extract were separated by a 12% (w/v) SDS-PAGE and stained with Coomassie Brilliant Blue for visualization.

For storage protein staining in seeds, tissues were fixed a 2.5% (v/v) glutaraldehyde, 1% (w/v) Osmium tetraoxide, 0.48% (v/v) formaldehyde in 0.025 M Pipes

buffer pH 7.2, dehydrated and embedded in LR White for protein and carbohydrates. Cross sections (2 µm) were mounted on slides and stained for proteins with 1% (w/v) Aniline Blue Black, 7% (v/v) acetic acid at 50°C for 10 min (Bronner, 1975, O´Brien, 1981). Then, they were rinsed in 7% (v/v) acetic acid and dried off quickly with cool air from a hair drier. Finally, we mounted coverslips on the slides and the samples were viewed using a Nikon Eclipse 80i microscope and photographed with a Nikon Digital Sight DS-SMc camera.

3.7.3. Lipid determination

Lipid determination protocol was performed based on Chen and Vaidyanathan (2012). Fifty seeds were counted using a stereoscopic microscope (Nikon SMZ800) and grounded with liquid nitrogen in a mortar in the presence of 100 µl of R1 solution (25% methanol in 1 N NaOH). The resulting mix was transferred to a new 2 ml microfuge tube and 900 µl of the R1 solution were added. Then, the mixture was saponified at 100°C for 30 min and tubes were agitated in a vortex every 5 min in order to release fatty acids. After saponification, the tube was cooled down to room temperature, and a 600 µl aliquot of the mixture was pipetted to a new tube containing 900 µl of R2 solution (chloroform / methanol, 2:1 v / v) and agitated in a vortex for 2 min. A centrifugation step was performed at 12000*g for 2 min and two 200 µl aliquots of the organic phase were transferred to two different tubes, one with 200 µl of R3 solution (1 M triethanolamine / 1 N acetic acid, 9:1 v / v) and the other with 200 µl of R4 buffer (1 M triethanolamine / 1 N acetic acid / 6.45% (w / v) $Cu(NO_3)_2$ x $3H_2O$, 9:1:10 v / v). Both tubes were mixed by

agitation in a vortex for 2 min and centrifuged at 12000*g for 2 min. Then, the absorbance at 260 nm of 100 µl of the organic phase of each tube was measured. Background absorbance for each tube was made following the same protocol with a sample-less mixture. The final absorbance for each sample due to fatty acids was obtained by subtracting absorbance of the R3 tube from that of the R4 one. A calibration curve was obtained by using linoleic acid as standard (12.5 to 100 µg).

3.7.4. Electron microscopy

For electron microscopy analysis, we fixed, dehydrated and embedded the dry seeds in LR white resin (London Resin, Basingstoke, U.K.). We used a fixative solution containing 10% (v/v) dimethyl sulfoxide, 4% (v/v) paraformaldehyde, 1% (v/v) glutaraldehyde and 0.06 M sucrose in 0.05 M of cacodylate buffer pH 7.4 for 1 hour. The fixed samples were cut into slices of < 1 mm thick and treated for another 2 hours with a freshly prepared fixative solution. For ultrastructural studies, the fixed samples were then postfixed with 1.5% (w/v) osmium tetroxide in the same cacodylate buffer for 3 hr. After washing with the same buffer, the slices were stained in 1% (v/v) uranyl acetate for 2 hours, with subsequent dehydration in a graded ethanol series at room temperature. The samples were treated with propylene oxide and infiltrated with propylene oxide–Epon (Epon 812 resin; TAAB Laboratories, Aldermaston, UK) solution (propylene oxide–Epon resin, 1:1 [v/v]) overnight. The samples were then embedded in Epon resin that was allowed to polymerize at 60°C for 48 hours. Ultrathin sections were cut on an ultramicrotome (Leica, Reichert Division, Vienna, Austria) and mounted on copper grids.

The sections were then stained with 4% (v/v) uranyl acetate and lead citrate. Observation was made in a Philips Tecnai 12 Biotwin (Eindhoven, The Netherlands) transmission electron microscope at 80 kV.

3.7.5. Germination and postgerminative phenotype scoring

To evaluate germination (radicle emergence), cotyledon expansion and seedling establishment (presence of true leaves), seeds were sown on 0.5X MS plates without sucrose and with or without TTFA, succinate or fumarate, at different concentrations. After stratification for 2-3 days at 4°C in the dark, incubation was carried out at 22°C under a 16 h light / 8 h dark cycle. Seeds and seedlings were viewed with a stereoscopic microscope (Nikon SMZ800) and photographs were taken with a Nikon Coolpix 4500 CCD digital camera. To measure hypocotyl elongation, seeds were sown on 0.5X MS with or without sucrose or TTFA at different concentrations, stratified as described, and then plates were placed vertically and incubated at 22°C in the dark. Plates were scanned in an Epson Perfection v700 Photo Scanner and images were later processed with the IMAGEJ software (http://rsb.info.nih.gov/ij/).

3.8. Yeast complementation with Arabidopsis SDH2 isoforms

3.8.1. Constructs for complementation analysis

The three Arabidopsis iron-sulfur isoforms (*AtSDH2-1*, *AtSDH2-2* and *AtSDH2-3*), the two Physcomitrella isoforms (*PpSDH2-1* and *PpSDH2-3*) and the Yeast SDHB CDS were amplified by PCR using as templates cDNAs synthetized with Arabidopsis RNA,

Physcomitrella RNA and Yeast RNA, respectively. Primers pairs used for amplification were the following: AtSDH2-1S EcoRI and AtSDH2-1AS XhoI for *AtSDH2-1*; AtSDH2-2S EcoRI and AtSDH2-2AS XhoI for *AtSDH2-2*; AtSDH2-3S EcoRI and AtSDH2-3AS XhoI for *AtSDH2-3*; PpSDH2-1S BamHI and PpSDH2-1AS XhoI for *PpSDH2-1*; PpSDH2-3S EcoRI and PpSDH2-3AS XhoI for *PpSDH2-3*; YSDHBS EcoRI and YSDHBAS XhoI for *YSDHB*. PCR products were later cloned into pGEM-T and then digested with the following enzymes: EcoRI and XhoI for *AtSDH2-1*, *AtSDH2-2*, *AtSDH2-3*, *PpSDH2-3* and *YSDHB*; BamHI and XhoI for *PpSDH2-1*. DNA fragments were then subcloned into a modified pYES2 yeast expression vector, containing the ADH1 strong promoter controlling the expression of the mentioned genes (kindly donated by Dr. Vicente-Carbajosa from CBGP, Spain).

The chimeric constructs encoding the N-Terminal region of YSDHB fused to AtSDH2-1, AtSDH2-3 or PpSDH2-3 were obtained as follows. Amplification of the region encoding the YSDHB N-terminal region was performed using the pGEM-T vector containing the *YSDHB* gene as template along with forward primer YSDHBS EcoRI and reverse primer YSDHBAS. To obtain the regions from *AtSDH2-1*, *AtSDH2-3* and *PpSDH2-3* encoding the conserved SDH2 protein sequence (see figure 36), PCR amplifications were made using as template the pGEM-T vectors containing the CDS for *AtSDH2-1*, *AtSDH2-3* and *PpSDH2-3*, respectively, and the following primers: YSDHBS1 and AtSDH2-1AS XhoI for *AtSDH2-1*; YSDHBS2 and AtSDH2-3AS XhoI for *AtSDH2-3*; YSDHBS2 and PpSDH2-3AS XhoI for *PpSDH2-3*. PCR products were analyzed by agarose gel electrophoresis and then a second amplification round was performed using a mix of the first PCR amplification product and each one of the products of the second PCR amplification as template, with

the following primers: forward primer YSDHBS EcoRI and reverse primer AtSDH2-1AS XhoI for the *YSDHB*/*AtSDH2-1* chimeric construct; forward primer YSDHBS EcoRI and reverse primer AtSDH2-3AS XhoI for the *YSDHB*/*AtSDH2-3* chimeric construct; forward primer YSDHBS EcoRI and reverse primer PpSDH2-3AS XhoI for the *YSDHB*/*PpSDH2-3* chimeric construct. The three PCR products were cloned into pGEM-T and then digested with EcoRI and XhoI. Digestion products were subcloned into the modified pYES2 yeast expression vector containing the ADH1 promoter.

PCR conditions were as described in section 3.3.2. of Methods.

3.8.2. Yeast transformation

For transformation, wild type and SDHB-mutated yeast strains were grown on YPD (1% (w/v) bacto-peptone, 2% (w/v) yeast extract, 2% (w/v) glucose and 1.6% (w/v) bactoagar) medium plates. After 3 days of growing at 28°C, colonies were scraped off the plates separately and cultured in 15ml of YPD medium. Next day, 50 µl of the 15 ml culture were added to 1 ml of water and then cells were centrifuged at top speed for 5 seconds. The resulting pellet was resuspended in 1 ml of 100 mM LiAc and incubated for 5 min at 30°C. After that, cells were centrifuged at 12000*g for 30 seconds at room temperature and the supernatant was discarded. Then, the following components were added in the following order: 240 µl of 50% PEG 3500, 36 µl of 1M LiAc, 25 µl of 2 mg / ml single-stranded carrier DNA, 1 µg of plasmid DNA, and water to a total volume of 400 µl. Cells were agitated in a vortex and incubated for 20 min at 42°C (or overnight at room temperature). Later, after a 12000*g centrifugation for 30 seconds, cells were

resuspended in water and plated in DOB medium plates lacking uracil in order to identify transformed colonies. Resulting colonies were photographed between 4-7 days later after incubation at 28°C.

3.8.3. Yeast complementation assay

For the complementation assay, colonies of the transformed yeasts were scraped off the plates and inoculated separately in 3-5 ml of DOB liquid medium lacking uracil followed by incubation at constant agitation for 16-18 hours at 28°C. Yeast cells were counted in a Neubauer Chamber and 1/10 dilutions in water were made starting from 10^6 to 10^2 cells. Then, 2 µl of the different dilutions were placed in fermentative (glucose) or non-fermentative (glycerol or ethanol) plates (see Materials), and photographed 2-4 days later after incubation at 28°C.

3.9. *Physcomitrella patens SDH2-3*-like gene analysis

3.9.1. Physcomitrella growth conditions

Physcomitrella patens (Hedw.) Bruch & Schimp subsp. *Patens* (Tan, 1979) collected in Grandsen Wood, Huntingdonshire, UK was our wild type strain. Protonema and gametophore of this moss were grown in 9-cm plates on BCDATG medium (see materials for details). For protonema culture, the solidified medium was covered with a layer of cellophane to facilitate collection from the medium. The plates were incubated at 25°C under continuous light (40 µmol photons m^{-2} s^{-1}). For vegetative propagation, gametophore was subcultured into new plates every 2-3 weeks, whereas protonema was

collected every 1-2 weeks and grounded with a polytron PT2100 homogenizer (Kinematica AG, Lucerne, Switzerland). The ground protonema was soaked on the BCDATG medium using the same conditions described above (Nishiyama et al., 2000). Protonema and gametophores of Physcomitrella were subjected to different types of abiotic stresses. For osmotic stress treatment, both protonema and gametophore were transferred to BCDATG plates supplemented with 600 mM of mannitol or 600 mM sorbitol. High salinity treatment was performed by adding 250 mM NaCl to BCDATG plates. ABA treatment was carried out by adding 100 µM *(+)-cis-trans* ABA to the BCDATG plates. For the dehydration experiments, both protonema and gametophore were transferred to filter paper for 2 and 6 hours, and allowed to become desiccated at room temperature (Kroemer et al., 2004).

3.9.2. qPCR primers and conditions

Used primers and conditions are described in section 3.3.2. of Methods.

4. RESULTS

4.1. *SDH2-3* promoter characterization

We have previously shown that the region between -223 and +49 (numbers relative to the transcription start site) is sufficient to confer high level expression of the GUS reporter gene in mature seeds (Elorza et al., 2006; figure 11). This region contains three ABRE elements (ABRE1, ABRE2 and ABRE3) and one RY-like enhancer element which were shown by mutagenesis to be required for high embryo *SDH2-3* promoter activity (Roschzttardtz et al., 2009). Moreover, we showed that the fragment comprising the three ABRE and the RY elements (-114 to -32) fused to a minimal cauliflower mosaic virus (CaMV) 35S promoter containing TATA and CAAT boxes (construct -114/-32/-64p35S), does not have any noticeable promoter activity (figure 11; Roschzttardtz, 2007). This raises the question whether the region upstream of the ABRE elements (-223 to -114) or the region downstream of the RY element (-32 to +49) is required for seed transcriptional control. Two constructs were made (methods 3.4.1), one in which the region between -223 and -114 was removed (construct -114/+49; figure 11a), and the other in which the -223 to -32 region was fused to the CaMV 35S minimal promoter (construct -223/-32/-64p35S; figure 11a) and GUS activity was determined in mature T2 seeds (methods 3.4.4). Interestingly, deletion of the promoter to -114 did not significantly affect GUS activity (construct -114/+49; figure 11b), indicating that the -114 to +49 region contains all the elements required for high seed *SDH2-3* expression. In contrast, loss of the region downstream the RY element abolished GUS activity almost completely (construct -223/-32/-64p35S; figure 11b). Furthermore, a construct in which the -114 to -1 region of the

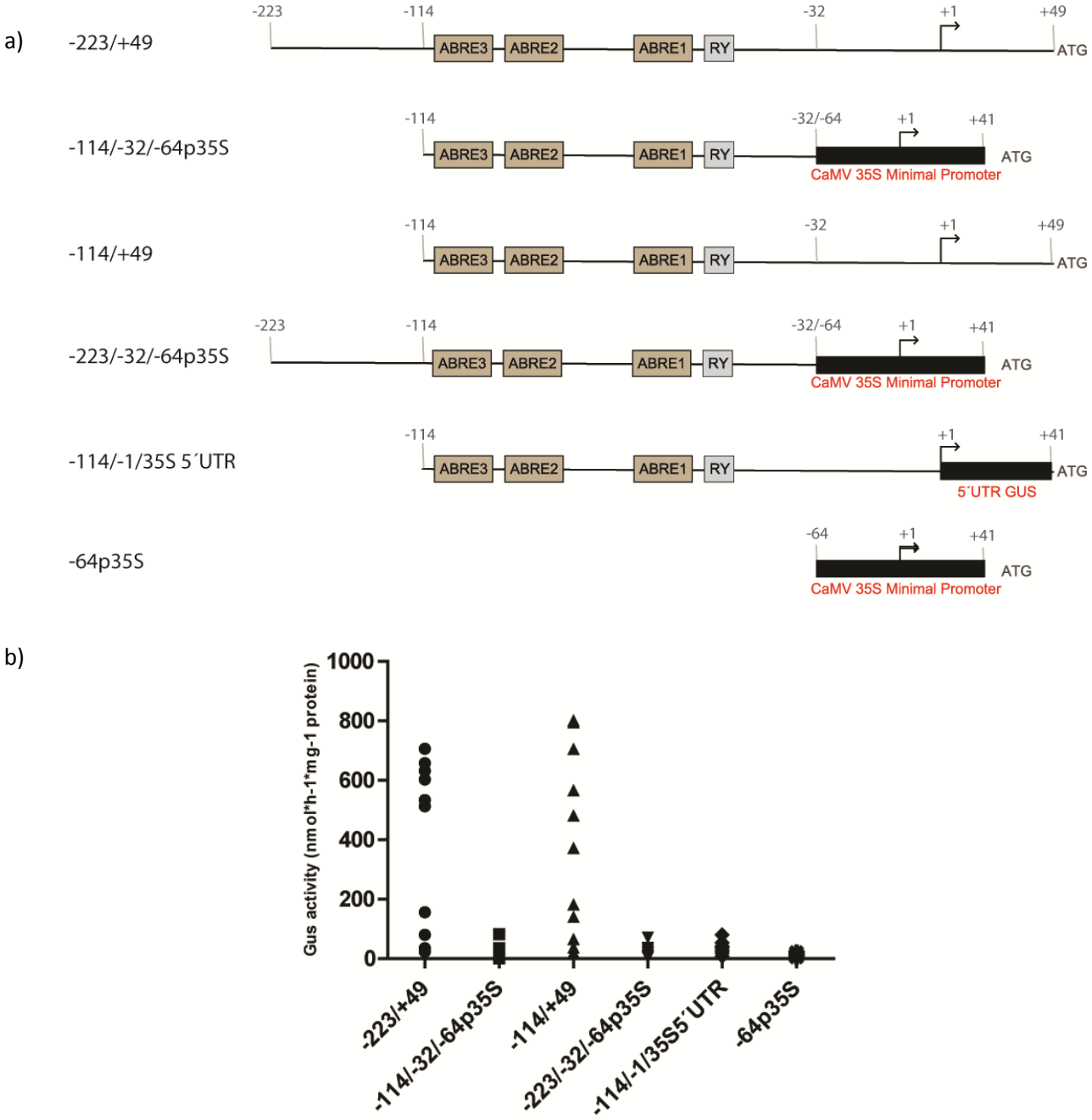


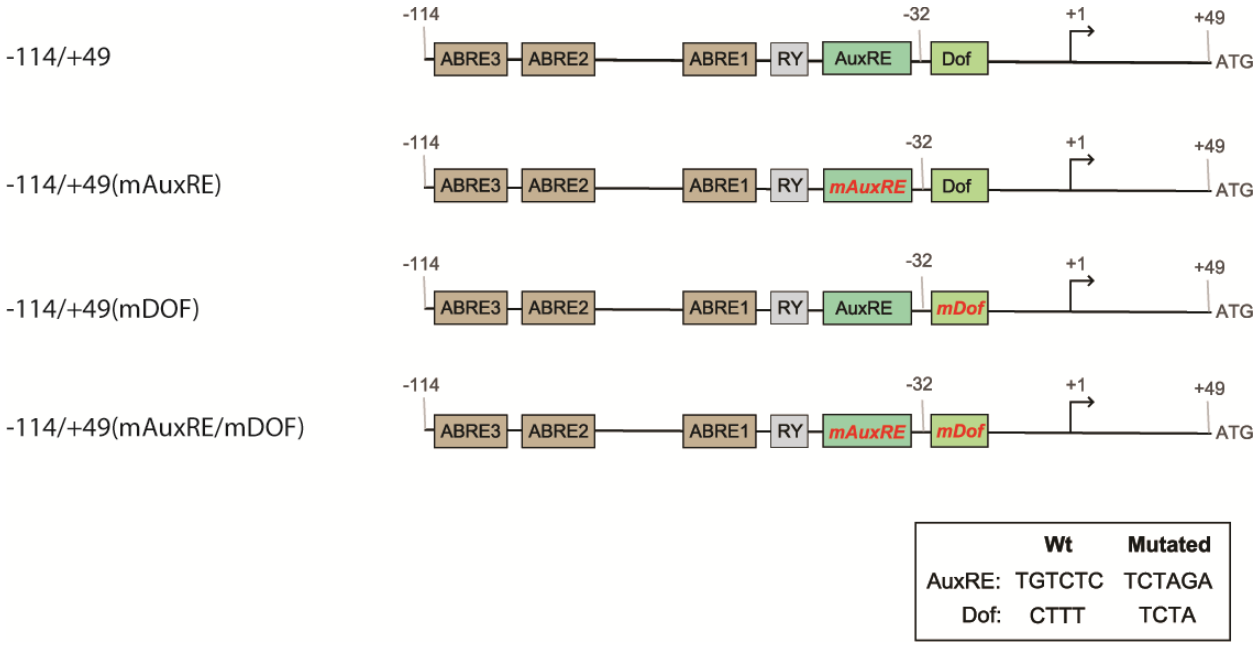
Figure 11. Identification of the *SDH2-3* functional promoter.

a**)** Structure of the constructs fused to GUS (not shown). Thin lines with motifs shown in boxes represent the *SDH2-3* promoter. Numbers are in relation to the transcription initiation site, indicated by a curved arrow. Black boxes represent the CaMV 35S minimal promoter and the region encoding its 5'UTR. b**)** GUS activity was measured in seed extracts from 10, 17, 11, 11, 11 and 6 independent transgenic lines carrying the -223/+49, -114/-32/-64p35S, -114/+49, -223/-32/-64p35S, -114/-1/35S5'UTR and -64p35S constructs fused to GUS, respectively. Each symbol represents one transgenic line.

SDH2-3 promoter was fused to the 41 bp encoding the 5' untranslated region (UTR) of transcripts driven by the CaMV 35S promoter in pBI121 showed very weak or no GUS activity (construct -114/-1/35S 5'UTR; figure 11). These results indicate that, in addition to the already described ABRE and RY elements located between -114 and -32, further *cis* elements between -32 and +49 are necessary for a functional *SDH2-3* promoter. Moreover, they suggest the region encoding the 5' UTR is crucial for promoter activity.

In silico analysis of the sequence downstream of the RY element showed the presence of two putative *cis* elements, an auxin-responsive element (AuxRE) and a DOF transcription factor-binding site (figure 12a). AuxRE elements are known targets for Auxin Response Factors (ARF), which are part of the B3 superfamily of transcription factors along with ABI3, FUS3 and LEC2 and are known to be involved in seed development (Friml, 2003, Schruff et al., 2006, Suzuki & McCarty, 2008). On the other hand, DNA binding with One zinc Finger (DOF) transcription factors have been described as transcriptional regulators of seed storage proteins (SSP) in cereal endosperm, binding the prolamin box which contains the AAAG consensus sequence (Diaz et al., 2002, Marzabal et al., 2008). Constructs containing substitution mutations in putative AuxRE, DOF site or both were made using the -114/+49 construct as template (methods 3.4.1). GUS activity was determined in mature T2 seeds from transgenic plants carrying the wild type (-114 to +49) or mutated promoters (figure 12b). Mutating any of the two elements or both appear to cause a mild reduction of GUS activity (means of 379, 178, 256 and 245 nmoles h^{-1} mg^{-1} protein and medians of 373, 207, 255 and 291 nmoles h^{-1} mg^{-1} protein for wild type, mutated AuxRE, mutated DOF and mutated AuxRE and DOF, respectively). However, data analysis using

a)



b)

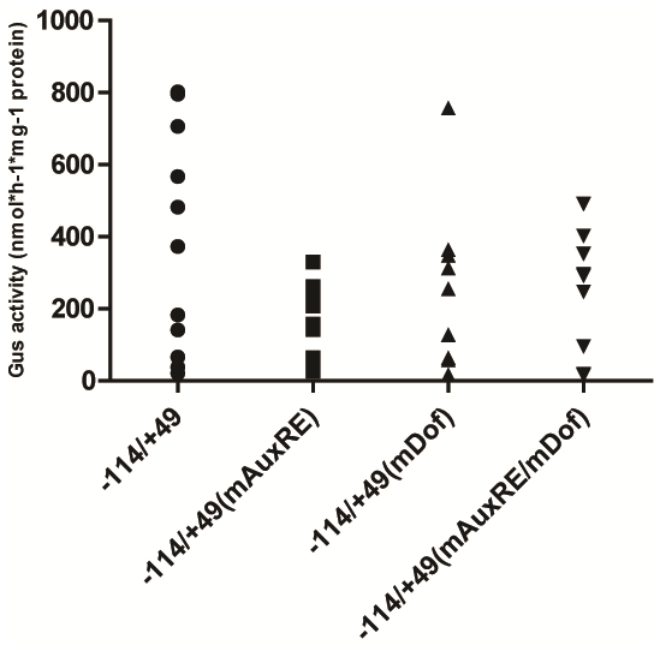


Figure 12. Mutation analysis of putative AuxRE and DOF elements.

a) Structure of the mutant constructs fused to GUS, all made on the -114/+49 promoter. Mutated elements are indicated as mAuxRE and mDOF, and altered nucleotides are shown below. b) GUS activity was determined in seed extracts from 11 (-114/+49 control construct), 11 (mAuxRE), 9 (mDOF) and 9 (mAuxRE/mDOF) independent transgenic lines. Each symbol represents one transgenic line.

non parametric statistical tests like the Kruskall-Wallis test followed by Dunn's multiple comparison test or the Mann-Whitney test for pairwise comparisons showed that these differences were not statistically significant. Thus, AuxRE and DOF sites play a minor role, if any, in modulating *SDH2-3* transcription, and further work will be required to identify the elements located between -32 and +49 that are necessary for *SDH2-3* expression during seed maturation.

We have thus defined the minimal promoter able to direct embryo SDH2-3 transcription (-114 to +49) and showed that, in addition to the ABRE and RY elements, *cis* elements located between -32 and +49 are required for promoter activity

4.2. Module-specific regulation of the *SDH2-3* promoter in mature seeds

The *SDH2-3* promoter is highly expressed during seed maturation and is completely silent during vegetative growth in Arabidopsis (Elorza et al., 2006). As in many other seed-specific promoters, ABRE and RY elements are necessary, but not sufficient for normal transcriptional activity (Roschzttardtz, 2007; figure 11). Here we show that mutations in the ABRE and RY elements uncover different histochemical GUS staining patterns in Arabidopsis mature embryos (methods 3.4.5; figure 13). Interestingly, mutations in each of the ABRE elements showed more pronounced decreases in hypocotyl and radicle staining than in cotyledon staining. Additionally, in the triple ABRE mutant promoter (mABREx3), hypocotyl and radicle staining are almost completely abolished. On the other hand, mutation in the RY element results in increased radicle staining, whereas

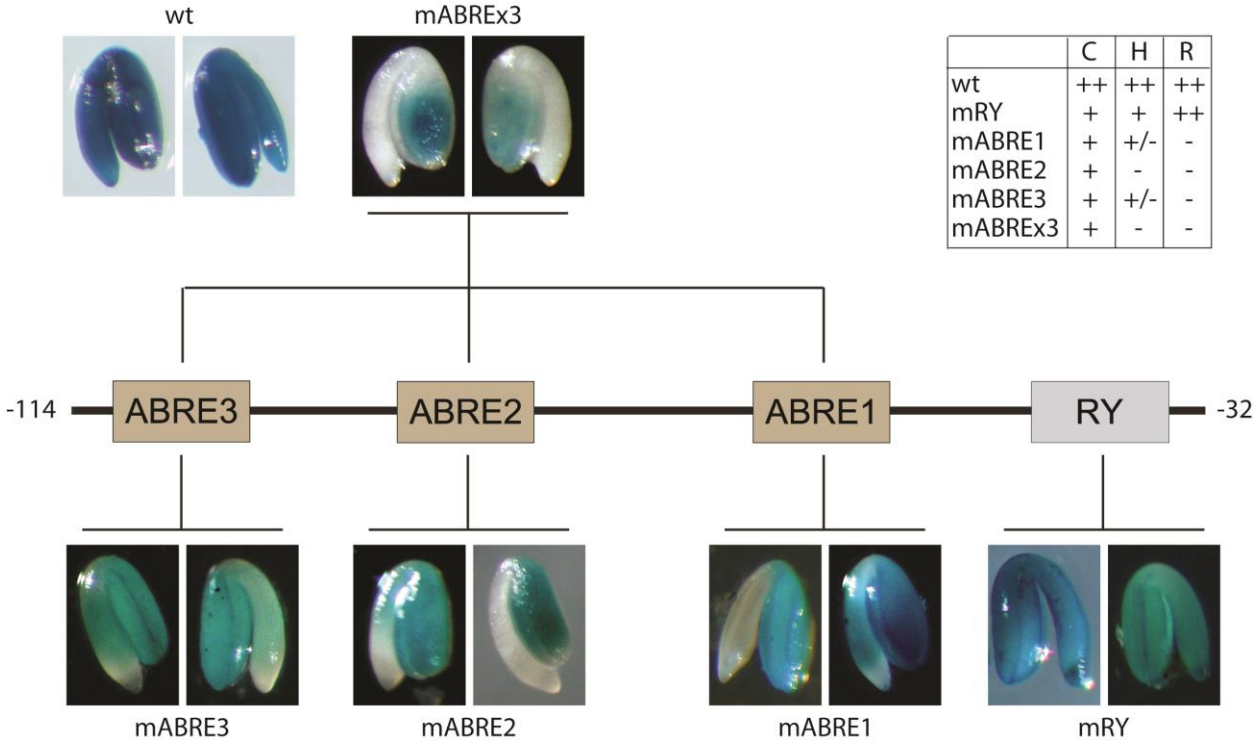


Figure 13. *Cis*-elements involved in spatial regulation of the *SDH2-3* promoter.

Histochemical staining for GUS pattern expression in *Arabidopsis* embryos carrying GUS fusions, was done by incubation with staining buffer for 12 hours. Representative mature embryos, all of them obtained from dry seeds are shown for lines mutated in the indicated *cis*-element(s). The table on the right indicates cualitative expression (+) or lack of it (-) in three regions (modules) of the embryo: C, cotyledon; H, hipocotyl; and R, radicle.

other regions showed decreased GUS activity. These results suggest a module-specific effect of these *cis*-elements in the promoter activity, with partially overlapping roles.

4.3. Different combinations of bZIP and B3 transcription factors activate transcription in leaf protoplasts

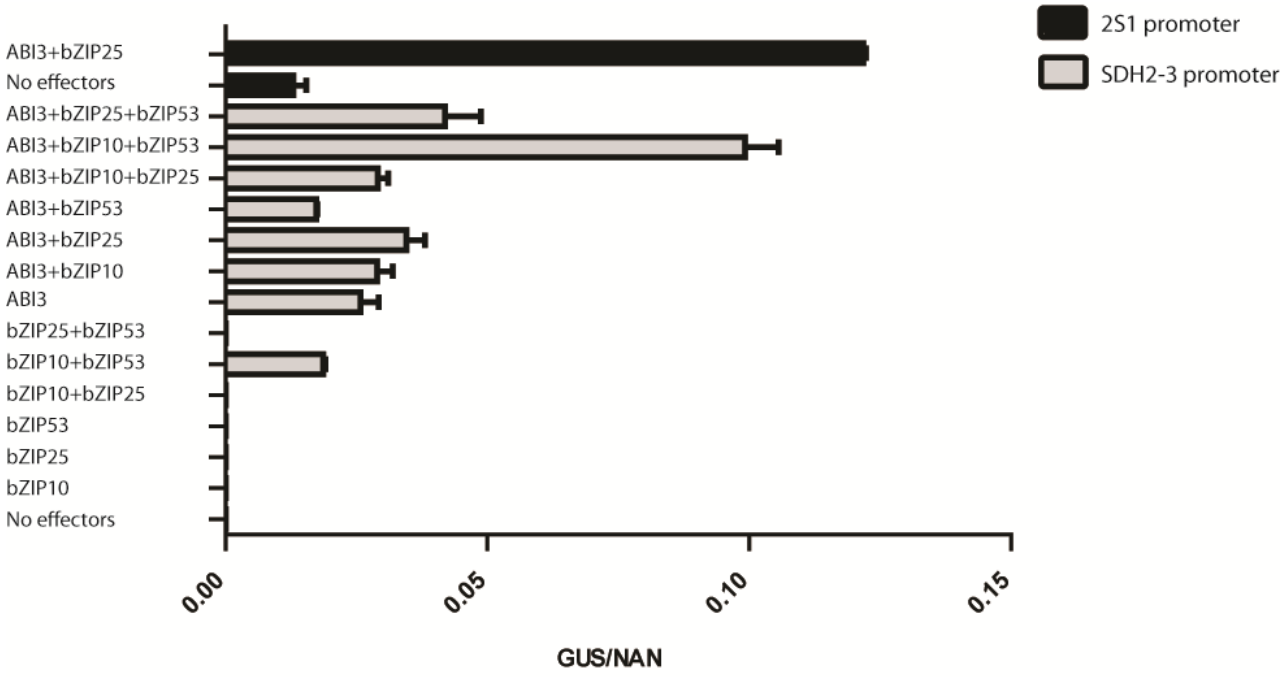
Roschzttardtz et al. (2009) described that bZIP and B3 transcription factors can bind *in vitro* regions of the *SDH2-3* promoter containing ABRE and RY elements, respectively, and that *SDH2-3* transcript levels are reduced in *abi3*, *lec2* and *fus3* mutants. However, evidence for direct activation of the *SDH2-3* promoter by either B3 domain and bZIP transcription factors is lacking.

Transient expression assays using the 2S1 albumin promoter, which also contains RY and ABRE elements, showed that bZIP10 and bZIP25 are involved in transcriptional activation in a concerted way with ABI3 (Lara et al., 2003). Additionally, bZIP53 (S1 bZIP group member) was shown to interact with both C group bZIPs (bZIP10 and bZIP25) and indirectly with ABI3 to regulate the transcriptional activation of seed maturation-specific promoters (Alonso et al., 2009). Moreover, we have already shown that it binds the *SDH2-3* promoter *in vitro* as a homodimer or heterodimer with C group bZIPs, especially bZIP10 (Roschzttardtz et al., 2009). These results allowed us to propose a model in which the activation of *SDH2-3* would require both bZIP (bZIP53 homodimers or heterodimers with either bZIP10 or bZIP25) and B3 (ABI3, FUS3 and LEC2) proteins.

To determine if B3 and particularly bZIP transcription factors are able to activate the *SDH2-3* promoter, we did transient expression assays in Arabidopsis leaf protoplasts (methods 3.5), transfecting different combinations of effector plasmids (which expression is driven by the CaMV35S promoter) for bZIP10, bZIP25, bZIP53 and ABI3 along with a vector construction containing a wild-type version of the -114/+49 *SDH2-3* promoter (figure 14). As a control, the 2S1 promoter was transfected. Single transfection of any of the bZIP factors did not produce any effect on *SDH2-3* promoter activation. However, when bZIP10 was transfected along with bZIP53 it activated transcription, contrary to bZIP25 with any of the other two. Additionally, single ABI3 transfection also did activate the promoter, as well as transfection along with any of the three bZIPs alone or in pairs. Furthermore, transfection with ABI3, bZIP10 and bZIP53 was the one with the greatest promoter activation. All the mentioned results show us that the *SDH2-3* promoter is under a redundant but at the same time spatially and temporally tight transcriptional regulation. Importantly, they describe for the first time that the SDH2-3 promoter is activated by bZIP transcription factors.

4.4. ABA and bZIP53 are involved in *SDH2-3* expression in dry seeds

bZIP53 has been characterized as an important transcriptional regulator in seed maturation (Alonso et al., 2009) and we have previously shown that bZIP53 binds *in vitro* to the *SDH2-3* promoter as homodimer or heterodimer with bZIP10 or bZIP25 (Roschzttardtz et al., 2009). To obtain further evidence for an *in vivo* role of bZIP transcriptions factors, we analyzed *SDH2-3* expression in dry seeds of *bzip10*, *bzip25* and



Student´s t-test		
Group 1	Group 2	
bZIP10+bZIP53	ABI3+bZIP10+bZIP25	0.01 < p < 0.05
bZIP10+bZIP53	ABI3+bZIP25	0.01 < p < 0.05
ABI3+bZIP53	ABI3+bZIP25	0.01 < p < 0.05
ABI3+bZIP53	ABI3+bZIP10+bZIP25	0.01 < p < 0.05
ABI3+bZIP10+bZIP53	All combinations	0.001 < p < 0.01

Figure 14. Transient expression assay using Arabidopsis leaf mesophyll protoplasts.

Protoplasts were transfected with the *SDH2-3* (grey bars) or *2S1* (black bars) promoters in a GUS-reporter vector, different combinations of vectors expressing different transcription factors and a transfection control vector containing NAN. Activity is expressed as relative GUS/NAN units. Each bar represents the mean ± SE of three independent transfections for *SDH2-3* and two for *2S1*. Significant differences are shown in the table below between different effector combinations. Transfections with no effectors, bZIP10, bZIP25, bZIP53, bZIP10+bZIP25 or bZIP25+bZIP53 were not considered in the Student´s t-test statistical analysis as these values were near zero.

bzip53 mutant lines (methods 3.3.2). Here we show that the accumulation of the *SDH2-3* transcript was significantly reduced in seeds lacking bZIP53, whereas *bzip10* and *bzip25* mutants did not show any significant difference as compared to wild-type plants (figure 15a). The latter could be explained by redundancy between both proteins. On the other hand, over-expressing lines of the three mentioned bZIPs did not show changes in the *SDH2-3* transcript level in dry seeds (figure 15b). These results point to a role of bZIP53 in the *in vivo* transcriptional regulation of *SDH2-3*. Regarding bZIP10 and bZIP25, although in protoplast transfection they have an effect on *SDH2-3* promoter activity, further work will be necessary to unravel their role in *SDH2-3* regulation, for instance, analysis of double *bzip10*/*bzip25* mutants.

The *cis*-acting ABA responsive element, or ABRE, is present in the *SDH2-3* promoter and we have determined its importance for proper transcriptional activity (Roschzttardtz et al., 2009). In order to evaluate the requirement for ABA in seed *SDH2-3* expression, we measured *SDH2-3* transcript levels in mutant lines with impaired ABA biosynthesis (figure 16). *aba2-1* and *aba2-3* are mutant lines in the ABA2 protein while *aba3-1* and *aba3-2* are for ABA3, responsible for the oxidation of xanthoxin and abscisic aldehyde during ABA biosynthesis, respectively (Barrero et al., 2006, Schwartz et al., 1997). Interestingly, the accumulation of *SDH2-3* mRNA was significantly reduced in all mutant lines, indicating the importance of ABA for *SDH2-3* transcription in seeds.

a)

b)

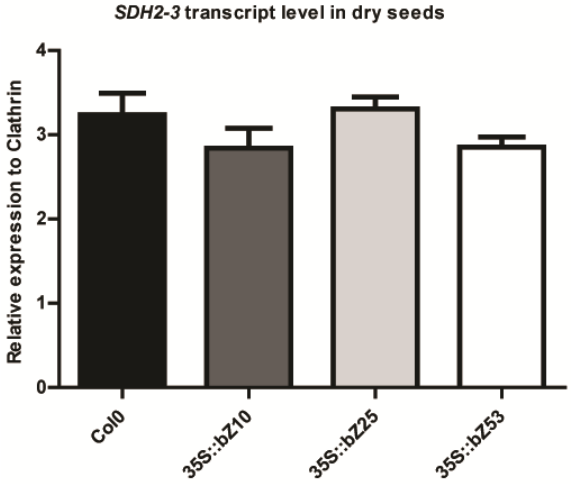


Figure 15. *In planta* regulation of the *SDH2-3* promoter by bZIP factors.

Expression of the *SDH2-3* gene in dry seeds of a) mutant and b) overexpressing lines of bZIP10, bZIP25 and bZIP53 transcription factors analyzed by RT-qPCR. Expression levels are given relative to the *CLATHRIN* gene for normalization. Values are means ± SE of three biological replicates. Asterisks indicate significant differences according to Student´s t-test (two asterisk for $0.001<p<0.01$).

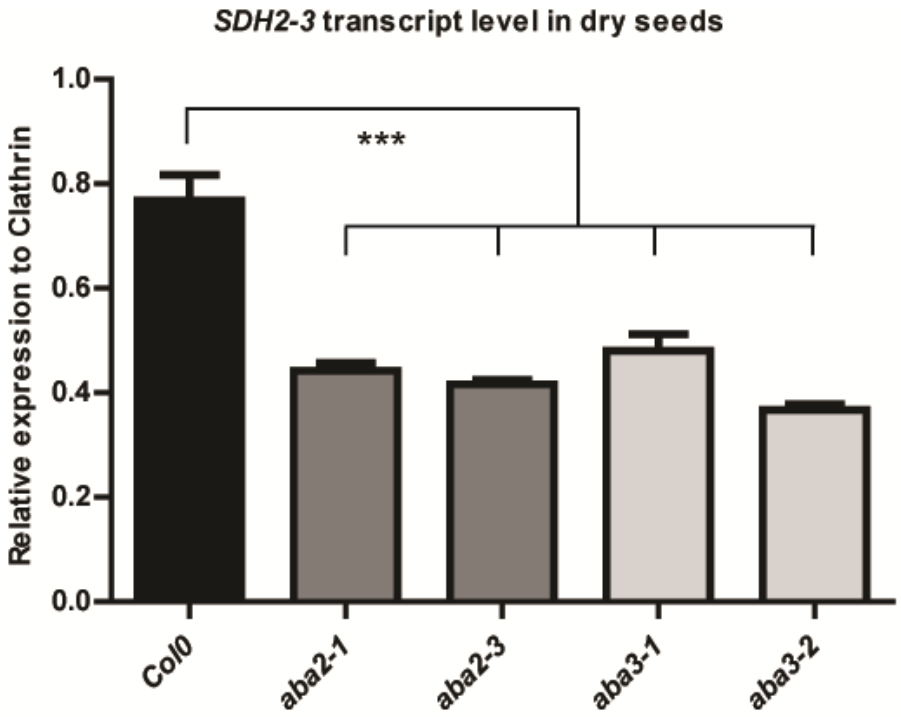


Figure 16. ABA involvement in *SDH2-3* transcription in mature seeds.

Expression of *SDH2-3* gene in dry seeds of Col-0, *aba2-1*, *aba2-3*, *aba3-1* and *aba3-2* lines. Transcript levels were analyzed by RT-qPCR and given relative to the *CLATHRIN* gene for normalization. Values are means of three biological replicates. Asterisks indicate significant differences according to Student´s t-test (three asterisks for $p<0.001$).

4.5. Epigenetic regulation of *SDH2-3* promoter

4.5.1. Effects of DNA methylation inhibition on seedling *SDH2-3* expression

SDH2-3 is not expressed in vegetative tissues, and DNA methylation may be involved in *SDH2-3* promoter inactivation. To address the possibility, our first approach was to determine how global DNA methylation inhibition affects *SDH2-3* promoter activity. For this, we used zebularine, a cell-permeable chemically stable cytidine analog shown to inhibit DNA methylation. Seeds from two independent transgenic lines with the -223/+49 *SDH2-3* promoter controlling the GUS reporter gene (1-4)1 and 2-1)3 lines), were sown on solid medium with or without 100 µM zebularine (methods 3.1). GUS expression was measured quantitatively in 15 day-old seedlings grown in standard conditions (methods 3.4.4). Interestingly, both lines showed a higher GUS activity when grown in the presence of zebularine, approximately 200 and 500 times for 1-4)1 and 2-1)3 lines, respectively (figure 17).

However, this strong activation of the *SDH2-3* promoter does not correlate with a markedly higher *SDH2-3* transcript level in zebularin treated seedlings. Indeed, a modest increase of 50% was found in one experiment by RT-qPCR (methods 3.3.2; figure 18). Nevertheless, it has to be taken in account that *SDH2-3* mRNA is barely detectable in 15 day-old seedlings. Therefore, our results may suggest post-trancriptional control of the *SDH2-3* mRNA level, likely at the level of transcript stability.

Previous work in our lab has shown that seedlings overexpressing ABI3 and treated with ABA, have increased *SDH2-3* transcript levels (Roschzttardtz et al., 2009). In order to see if ABA and methylation inhibition have a synergistic effect on *SDH2-3* expression, we

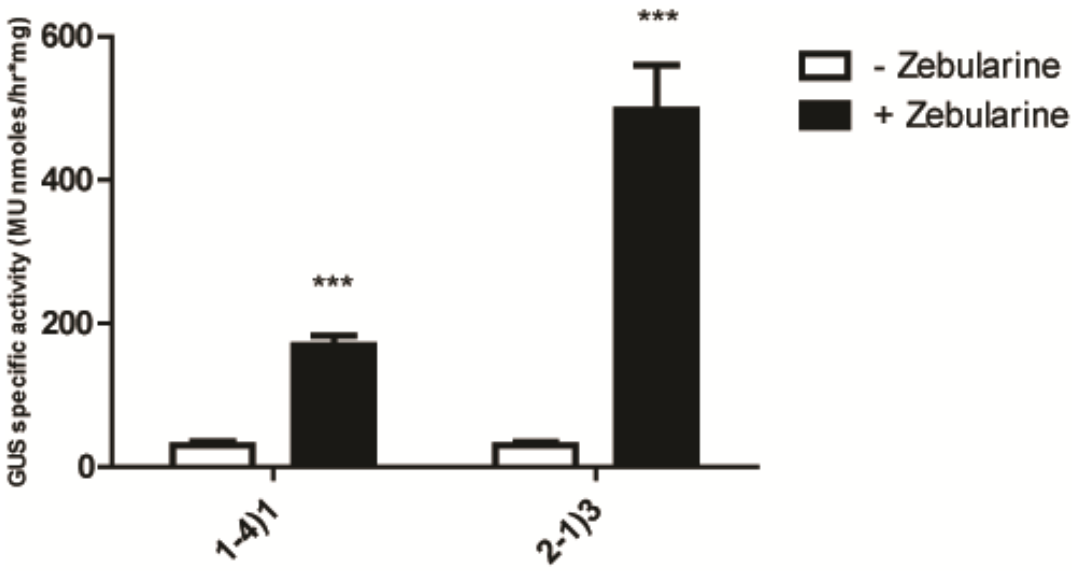


Figure 17. Zebularine effect on the activity of the *SDH2-3* promoter in seedings.

GUS activity was measured in 15 day-old Arabidopsis seedlings grown *in vitro* with (black bars) or without (white bars) 100µM zebularine. GUS activities were measured in protein extracts from 15 day-old seedlings and expressed as nmoles of 4-methylumbelliferone*h^{-1}*mg^{-1} of protein. Values are means ± SE of three biological replicates. Asterisks indicate significant differences according to Student´s t-test (three for $p<0.001$).

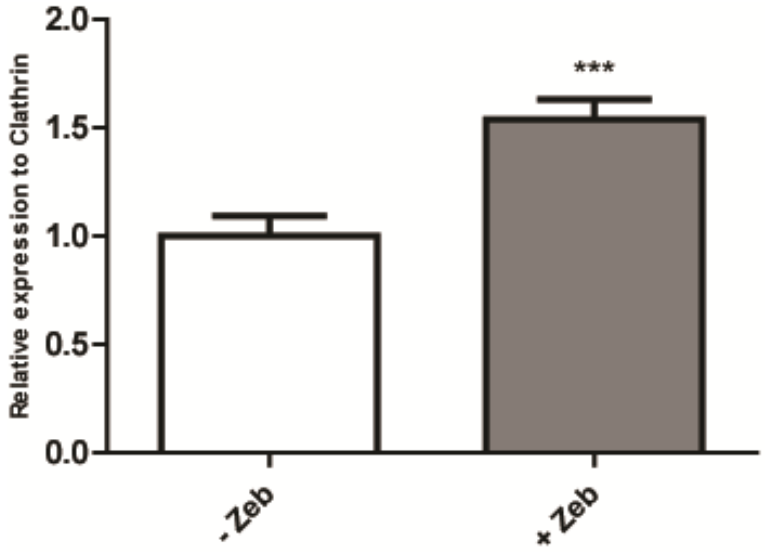


Figure 18. Zebularine effect on *SDH2-3* transcript levels in seedlings.

Expression of *SDH2-3* gene in zebularine-treated 15 day-old seedlings analyzed by RT-qPCR. Expression levels are given relative to the *CLATHRIN* gene for normalization, and the expression in control non-treated plants was set to 1 (white bar). Values are mean of three biological replicates ± SE. Asterisks indicate significant differences according to Student´s t-test (three asterisks for $p<0.001$).

treated 35S::ABI3 plants with or without 50 µM ABA and 100 µM zebularine (figure 19). We confirmed that ABA has a strong effect on *SDH2-3* mRNA level in these plants (x40 increase) and that zebularin has a modest effect in the presence of ABA (x1.7; p:0.032).

Altogether these results show that DNA methylation inhibition activates the *SDH2-3* promoter in seedlings but transcript levels do not increase accordingly, suggesting control of transcript half-life. Furthermore, our data do not allow differentiating between a direct effect (methylation of the *SDH2-3* promoter) or an indirect effect (see below).

4.5.2. Analysis of DNA methylation mutants

A different approach in order to analyze the influence of DNA methylation was undertaken by using mutant plants for DNA methyltransferases. DNA methylation in plants, unlike in other eukaryotic organisms, presents different methylation contexts. Aside the typical CG motif, there is also methylation in CHG and CHH (H being A, C or T). CG or canonical methylation is mediated by MET1 methyltransferase, while methylations in CHG and CHH are mediated by DRM1, DRM2 and CMT3. In order to determine how non-canonical methylation (in CHG and CHH) affects *SDH2-3* expression, we measured transcript levels in the *ddc* triple mutant (*drm1/drm2/cmt3*). This mutant showed a significant increase in *SDH2-3* transcript levels in 14 day-old seedlings when compared to wild type plants, and ABA was able to increase, although modestly, *SDH2-3* mRNA (figure 20).

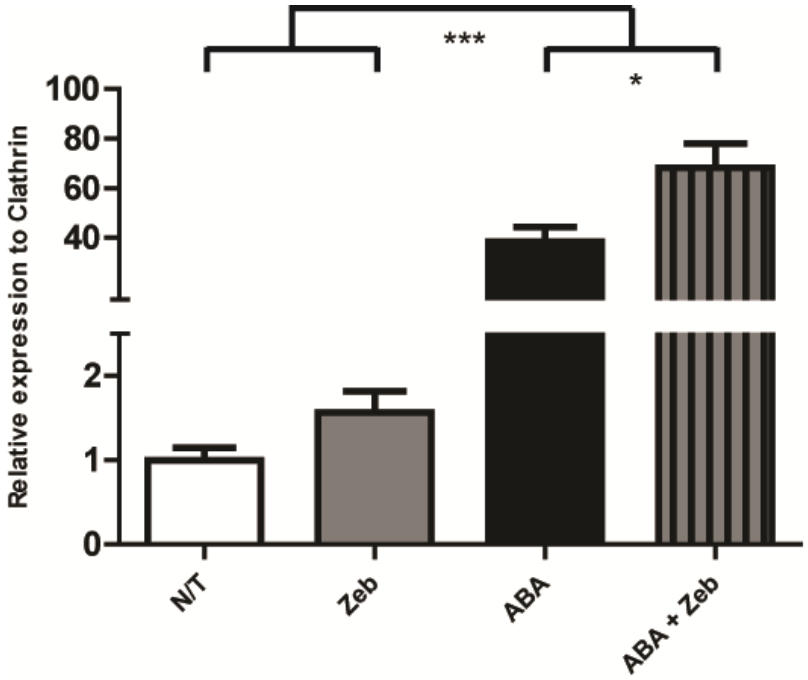


Figure 19. Zebularine and ABA effect on *SDH2-3* transcript levels in 35S::ABI3 seedlings.

Expression of *SDH2-3* in 15 day-old 35S::ABI3 seedlings treated with 100 µM zebularine (grey bar), 50 µM ABA (black bar) or both (vertical lines), analyzed by RT-qPCR. Expression levels are given relative to *CLATHRIN* for normalization, and the expression in control non-treated plants was set to 1 (white bar). Values are means of three biological replicates ± SE. Asterisks indicate significant differences according to Student´s t-test (one asterisk for $0.01<p<0.05$; three asterisks for $p<0.001$).

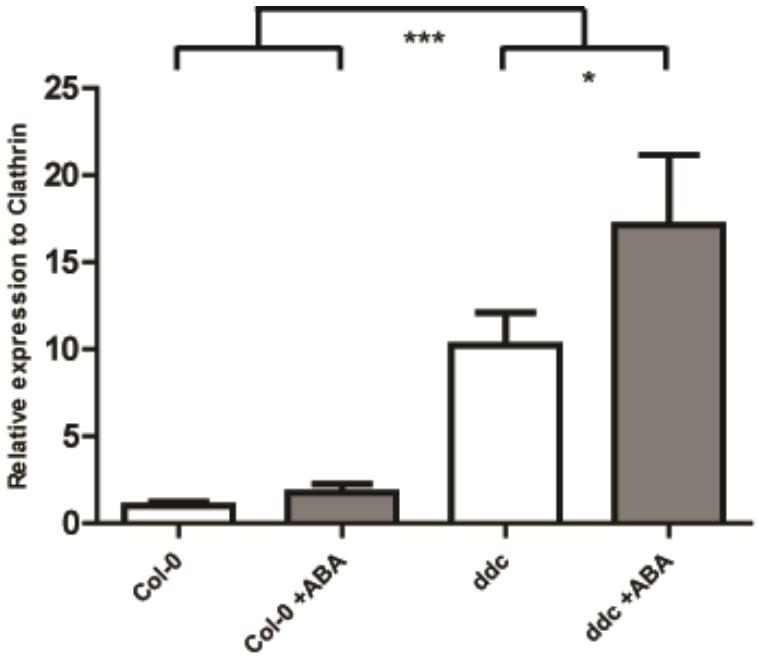


Figure 20. Expression of the *SDH2-3* gene in Col-0 and *ddc* mutant seedlings.

14 day-old seedlings incubated with (grey bars) or without (white bars) 50µM ABA. Expression levels were determined by RT-qPCR and are given relative to *CLATHRIN* for normalization. *SDH2-3* expression in non-treated Col-0 seddlings was set to 1. Values are means of three biological replicates ± SE. Asterisks indicate significant differences according to Student´s t-test (one asterisk for $0.01<p<0.05$; three asterisks for $p<0.001$).

On the other hand, heterozygous seedlings of the *met1-6* mutant, which has the canonical CG methylation inhibited, did not show any effect on *SDH2-3* transcript levels (figure 21). These results suggest that only non-canonical CHG/CHH methylation may be involved in *SDH2-3* gene silencing in seedlings.

Unexpectedly, *SDH2-3* expression in dry seeds of the *ddc* mutant is significantly reduced (figure 22a): a 50% decrease in seeds lacking CHG/CHH methylation was observed. In order to dismiss a pleiotropic effect, ABI3 transcript levels were measured and found to be similar in wild type and *ddc* seeds (figure 22b).

We were not able to analyze *SDH2-3* expression in *met1-6* seeds because homozygous *met1-6* plants, which can be identified by PCR genotyping, do not produce seeds.

4.5.3. Analysis of *SDH2-3* promoter methylation

The *SDH2-3* promoter is inactive in vegetative tissues. To analyze if DNA methylation may contribute to its silencing, genomic DNA was extracted from 14 day-old wild-type seedlings, and further treated with sodium bisulphite, which deaminates unmethylated cytosines (methods 3.6.1). These deaminated cytosines are later sequenced as thymines, while methylated cytosines are resistant to deamination and are sequenced as expected. We did not find any methylated C residue in the *SDH2-3* promoter sequence in seedlings (figure 23), suggesting that promoter methylation is not directly involved in *SDH2-3* promoter inactivation. Thus, the effect of zebularine on promoter activation (figure 17) and of the *ddc* mutant on *SDH2-3* transcript (figure 20) are likely indirect. As

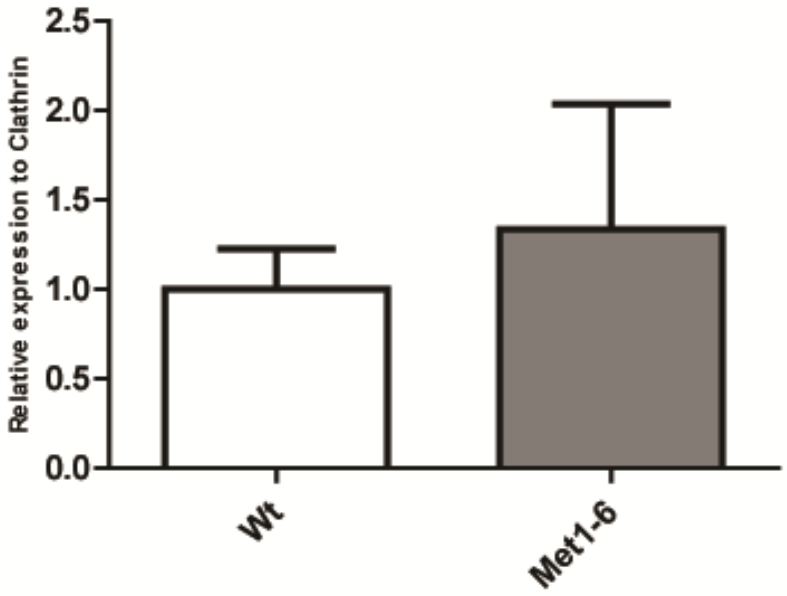
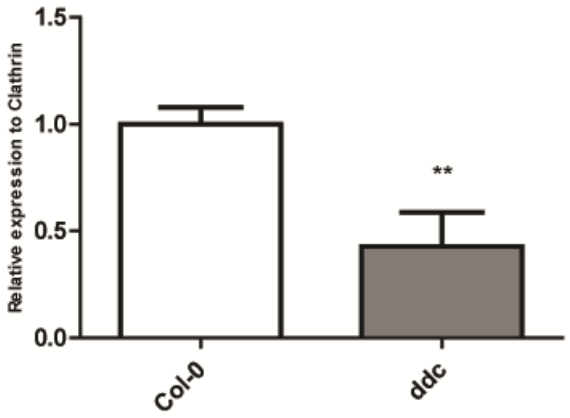


Figure 21. Expression of the *SDH2-3* gene in Col-0 and *met1-6* mutant seedlings.

Expression of *SDH2-3* gene in Col-0 (white bar) and *met1-6* (grey bar) 14 day-old seedlings. Expression levels were determined by RT-qPCR and are given relative to *CLATHRIN* for normalization. *SDH2-3* expression in non-treated Col-0 seedlings was set to 1. Values are means of three biological replicates ± SE.

a)



b)

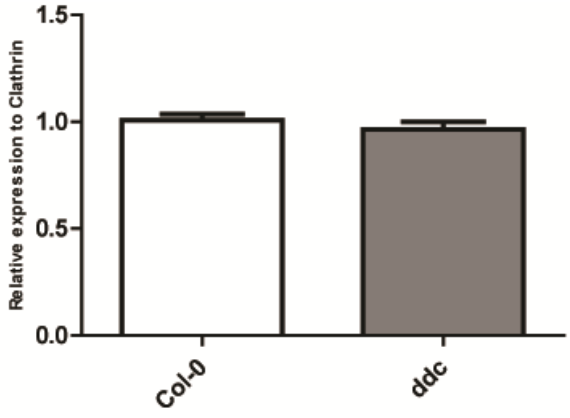


Figure 22. Expression of the *SDH2-3* gene in Col-0 and *ddc* mutant seeds.

Expression of a) *SDH2-3* and b) *ABI3* in Col-0 (white bars) and *ddc* (grey bars) dry seeds. Expression levels were determined by RT-qPCR and are given relative to *CLATHRIN* for normalization. Expression in Col-0 plants was set to 1 (white bar). Values are means of three biological replicates ± SE. Asterisks indicate significant differences according to Student´s t-test (two asterisks for $0.001<p<0.01$).

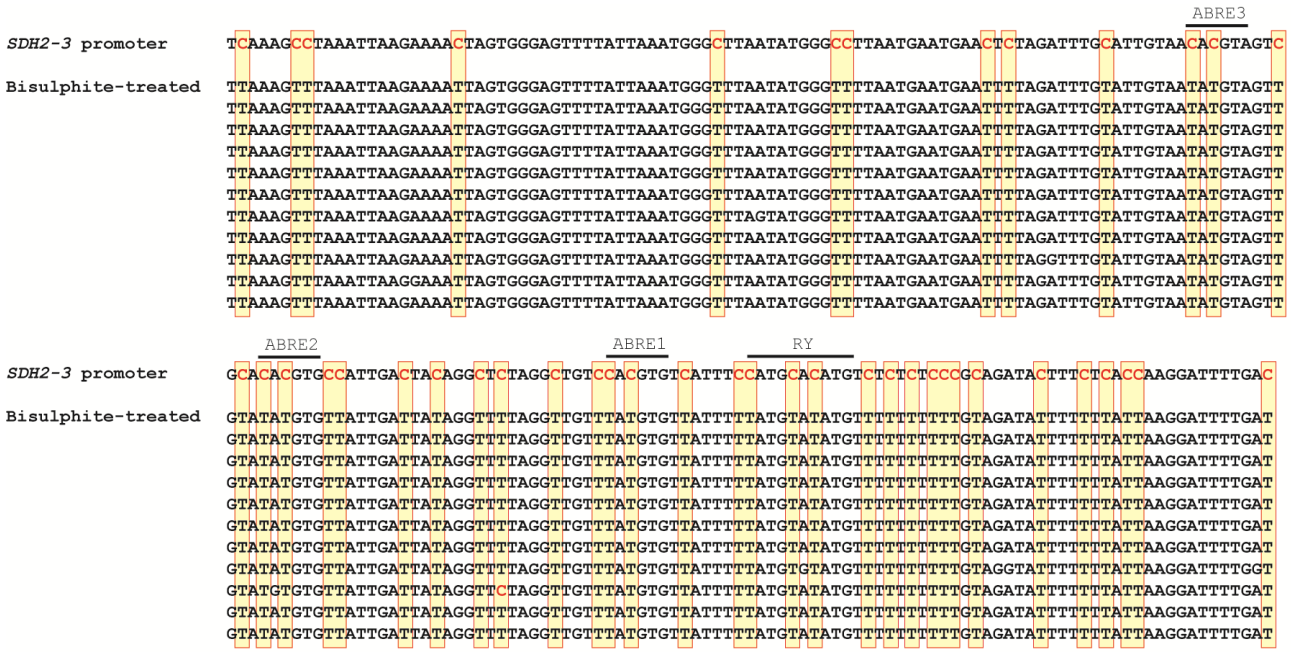


Figure 23. Sequencing of bisulphite treated Col-0 seedling promoter.

Non-treated and bisulphite-treated *SDH2-3* promoter sequences of 14 day-old Col-0 seedlings. All cytosines in the normal promoter are in red and C to T transformations are highlighted in yellow. Eleven clones from bisulphite-treated DNA were sequenced and found to have all C converted into T.

expected, analysis of the *SDH2-3* promoter in DNA extracted from tissues where it is active (seeds) showed no methylated residues (results not shown).

In conclusion, *SDH2-3* promoter methylation does not appear to have a role in repression of *SDH2-3* expression in vegetative tissue.

4.6. SDH2-3 and complex II roles during seed development, germination and postgerminative growth

4.6.1 Characterization of mutant *sdh2-3* seeds

We have shown that SDH2-3 is the main iron-sulfur subunit of complex II in mature seeds and that germination is retarded in *sdh2-3* mutants (Roschzttardtz et al., 2009). These results suggest a possible role during germination and early post-germinative growth; however it remains possible that this germination delay is due to problems in embryo development. As embryos mature, most of its metabolism is focused on the production of lipids and storage proteins, which will fuel germination and early posterior stages (Lin et al., 2006, Penfield et al., 2005, Weigelt et al., 2008). Thus, to evaluate if SDH2-3 is important for seed development, we measured the weight as well as protein and lipid content of mature seeds from wild-type and both *sdh2-3* mutant plants (dSpm and DsLox; methods 3.7.1 to 3.7.3). We found that dSpm and DsLox seeds were slightly (ranging from 5-15%) but significantly lighter that wild-type seeds in three experiments with different seeds batches. A representative experiment is shown in figure 24a. This reduced weight correlated well with lower protein content (figure 24b). Finally, lipid content appears to be slightly higher in the mutant seeds, although differences are statistically significant only for dSpm (figure 24c).

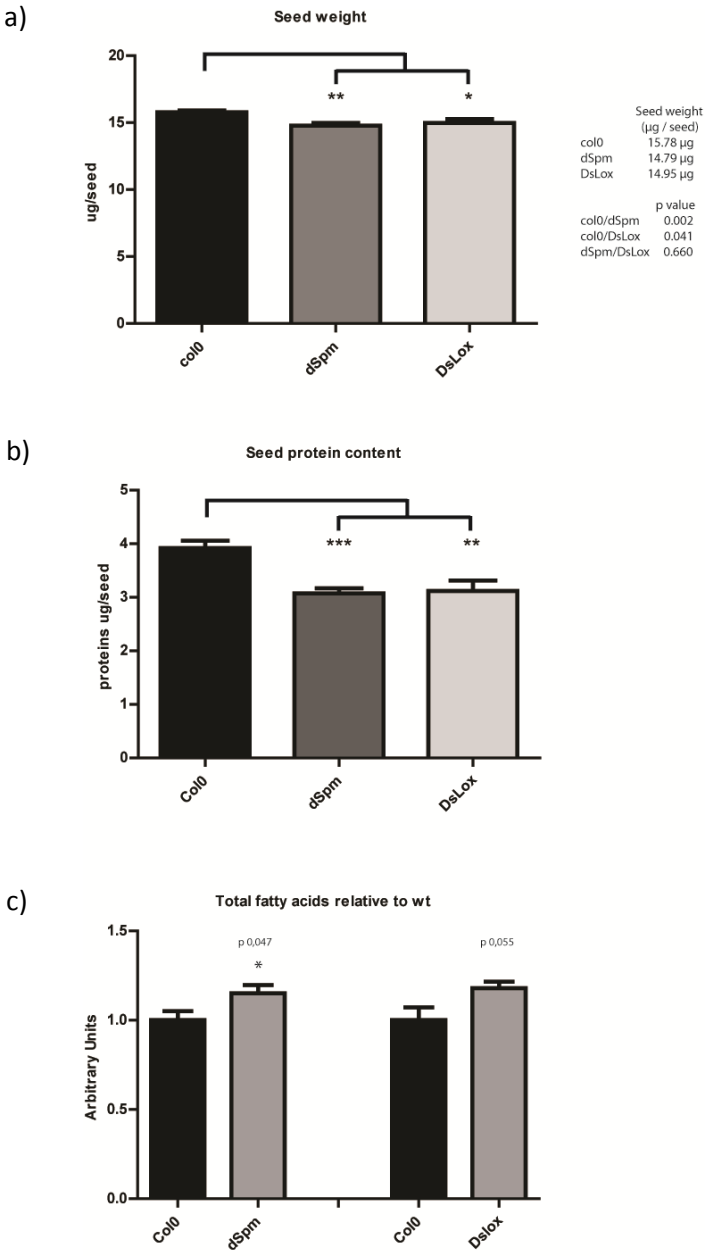


Figure 24. Effects of the *sdh2-3* mutation on seed weight, protein and lipid content.

a) Seed weight was measured in dry seeds of Col-0 and *sdh2-3* dSpm and DsLox mutant lines. Values are means ± SE of seven biological replicates, each containing around 200 seeds from a different plant. b) Total protein content per seed in Col-0, dSpm and DsLox seeds. Values are means ± SE of seven biological replicates. About 30 seeds were used for each extract preparation. c) Lipid content of mature seeds is given relative to the wild-type. Values are means ± SE of seven biological replicates. 50 seeds were used for each replicate. P value for each experiment (Col-0 versus dSpm or DsLox) is shown on the graph. Asterisks indicate significant differences according to Student´s t-test (one asterisk for $0.01<p<0.05$; two for $0.001<p<0.01$, and three for $p<0.001$).

Seed proteins were also analyzed by staining with aniline blue black (figure 25a) and SDS-PAGE (figure 25b): no obvious differences among genotypes were detected. Neither were clear differences in cell structure observed among genotypes by electronic microscopy (figure 25c; methods 3.7.4).

Altogether, these results indicate that *sdh2-3* mutants are slightly affected (slightly lower weight and protein content and slightly higher lipid content) and that SDH2-3 containing complex II may play a role during seed development. However, it is unlikely that these differences may explain such an early phenotype as the germination delay.

4.6.2. *sdh2-3* mutants have shorter hypocotyls in the dark

In darkness, seedlings undergo a process called skotomorphogenesis in which the hypocotyl elongates while maintaining the cotyledons closed and reducing root growth. This etiolated development is critical for seedlings growing in soil after germination in order to reach light and hence induce the photosynthetic apparatus to maintain autotrophic growth-associated processes (Gendreau et al., 1997). To investigate if SDH2-3 has a role in hypocotyl elongation, *sdh2-3* mutant seeds (dSpm and DsLox) were germinated in the dark and maintained in the absence of light for 3, 6 and 10 days (figure 26). No differences in hypocotyl length were found at three days in the absence of sucrose. In the presence of sucrose, which inhibits hypocotyl elongation at 3d, mutant hypocotyls are significantly shorter than wild-type ones. Between 3 and 6 days, elongation of mutant hypocotyls in the absence of sucrose is clearly reduced when compared to that of wild-type hypocotyls. The addition of sucrose, which stimulates hypocotyl growth

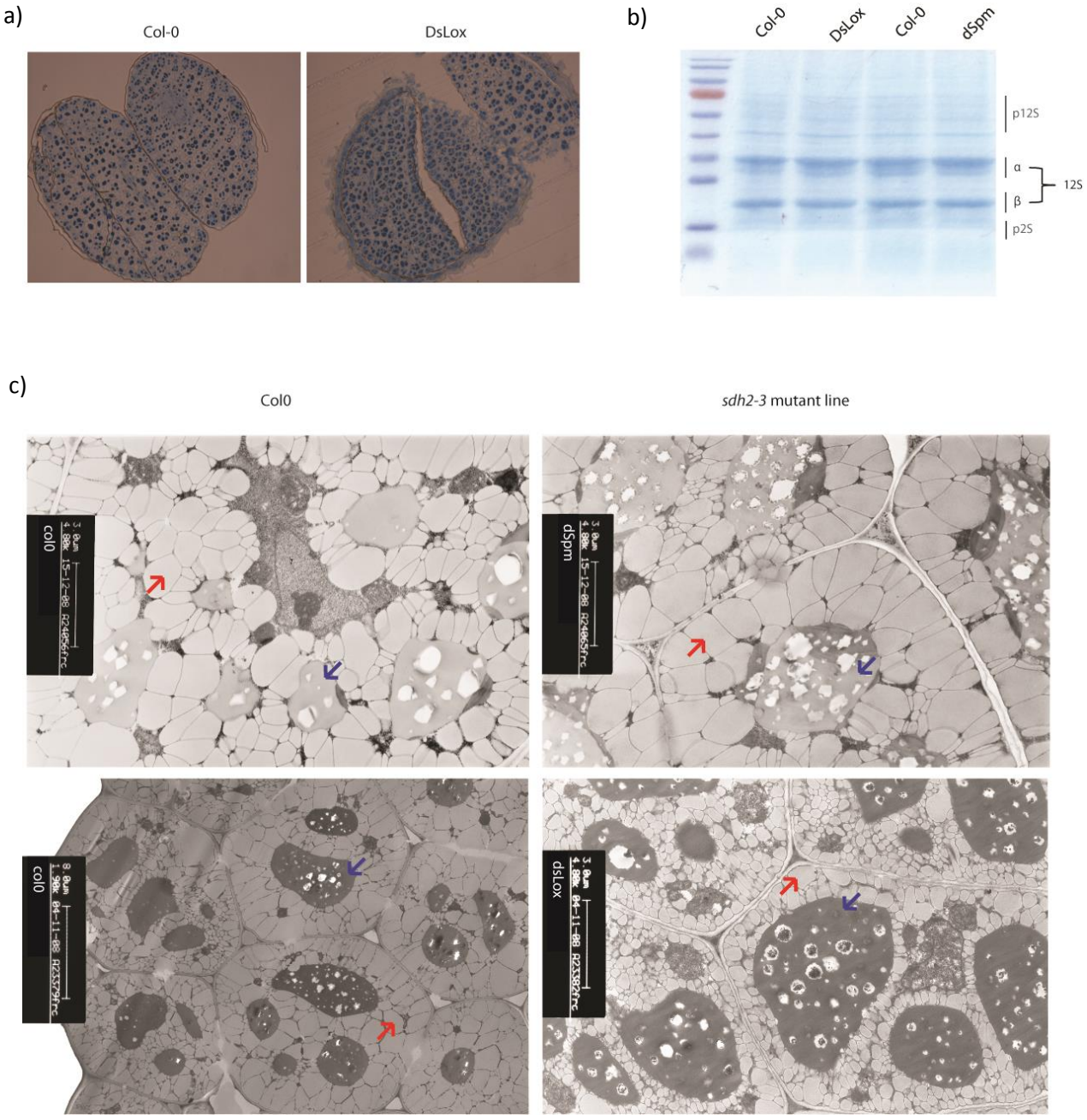


Figure 25. Effects of the *sdh2-3* mutation on protein profile and seed ultrastructure.

a) Total protein staining with aniline blue black of Col-0 (left) and DsLox (right). b) Total protein extracts from dry seeds of the indicated genotypes were analyzed by SDS-PAGE. Equal amounts of protein were loaded in each lane. Precursors for 2S and 12S albumins, and mature 12S protein weights are depicted on the side. c) Electronic microscopy of wild-type and *sdh*2-3 mutant lines (dSpm and DsLox). Lipid bodies are shown by red arrows and protein storage vesicles (PSV) by blue arrows.

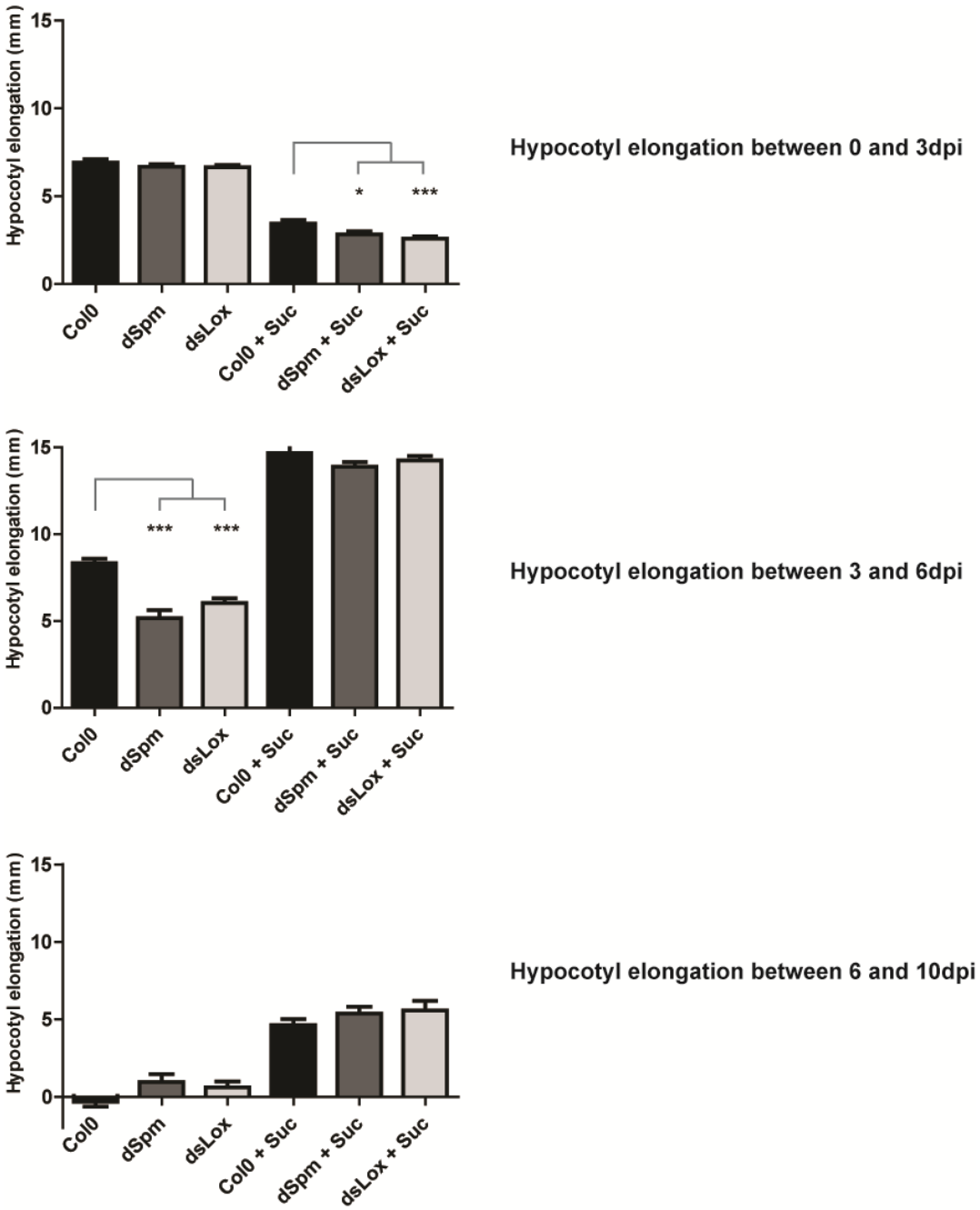


Figure 26. Effect of the *sdh2-3* mutation on hypocotyl length.

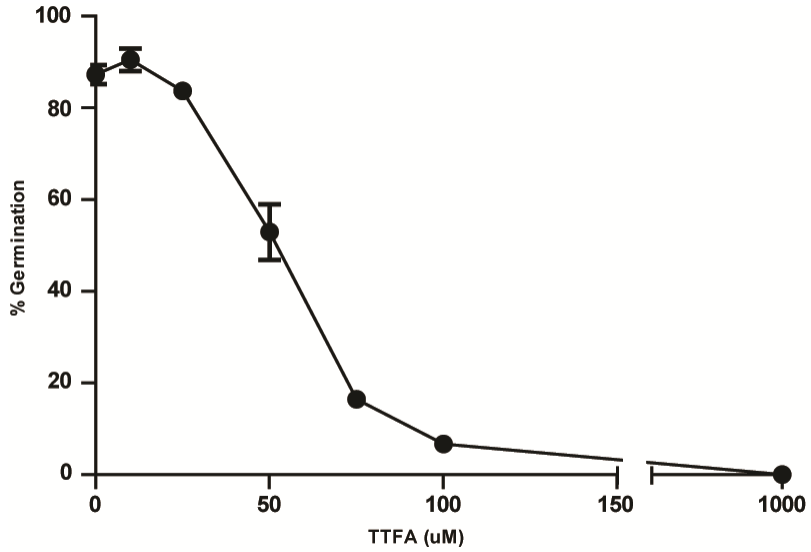
Three, six and ten day-old etiolated wild type (black bars) and *sdh2-3* dSpm (dark grey bars) and dsLox (light grey bars) mutant seedlings grown on media with or without 1% (w/v) sucrose. Plaques were scanned and hypocotyls were measured using the ImageJ software. Values are means ± SE of 47 hypocotyls from 4 plates at 3dpi and the differences between plates at 3 and 6dpi, and plates at 6 and 10dpi (all plates independent of each other). Asterisks indicate significant differences according to Student´s t-test (one for $0.01<p<0.05$; three for $p<0.001$).

between 3 and 6 days, restores the mutant growth defect. At 6 days, hypocotyl growth has ceased in the absence of sucrose since no significant differences were found in hypocotyl length between 6 and 10 days for any genotype. In contrast, hypocotyl continue to grow between 6 and 10 days in the presence of sucrose, and at day 10 there is no difference between wild-type and *sdh2-3* mutant hypocotyls. These results suggest a role for SDH2-3 in postgerminative processes in the dark.

4.6.3. Complex II is important for seed germination and plant establishment

We have determined that SDH2-3 plays an important role during germination (Roschzttardtz et al., 2009) and hypocotyl growth in the dark (figure 26). However, the lack of this isoform does not block germination nor hypocotyl elongation. Residual SDH activity in dry seeds, supported by SDH2-1 and/or SDH2-2, may be sufficient at early steps, until *SDH2-1*/*SDH2-2* expression is induced (1 day post imbibition; Elorza et al. 2006). In order to evaluate if complex II is essential for germination and postgerminative growth, we germinated and grew seeds in the presence of Thenoyltrifluoroacetone (TTFA), a specific complex II inhibitor which blocks the respiratory chain (methods 3.7.5). We found that 50 µM of TTFA inhibited 50% of germination at 48 hours (figure 27a), however after 10 days almost all seeds have germinated, even at 150 µM (figure 27b). On the other hand, cotyledon expansion, seedling establishment and hypocotyl elongation in the dark were completely blocked at 150 µM, 75 µM and 50 µM, respectively (figure 27b). Thus, postgerminative growth and seedling establishment appear to be more sensitive to TTFA, being completely blocked at TTFA concentrations that only delay germination. These

a)



b)

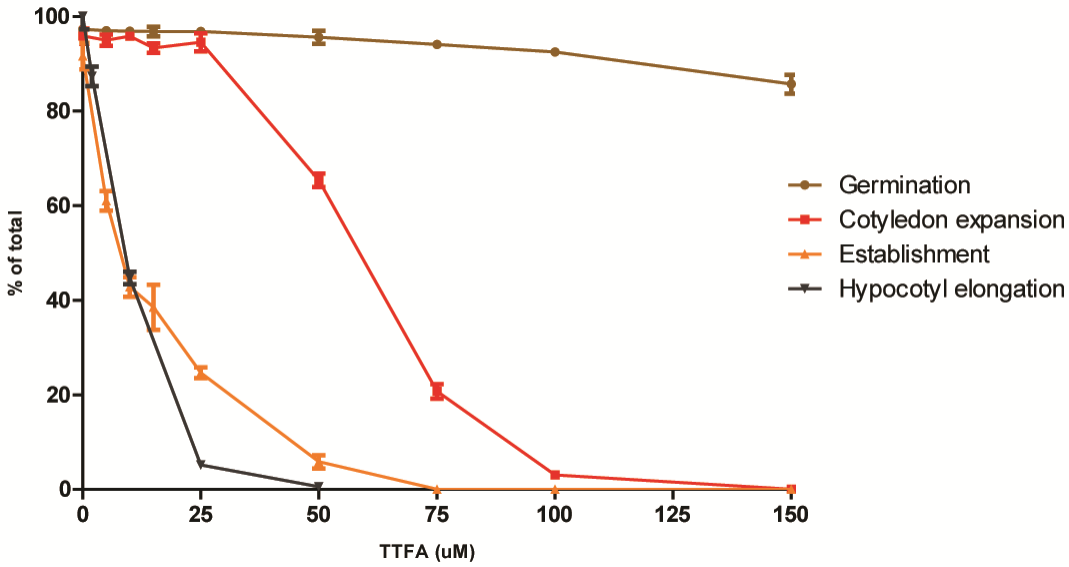


Figure 27. TTFA effect on germination and postgerminative growth

a) Germination at 2 days post stratification. Three biological replicates each of 66-85 stratified seeds were sown on 0.5X MS plates containing different TTFA concentrations. After two days at 22°C under a 8 h light / 16 h dark cycle, radicle emergence was scored. b) Germination (radicle emergence), cotyledon expansion, seedling establishment (presence of true leaves) and hypocotyl length were evaluated at 10 days post stratification. For germination, cotyledon expansion and seedling establishment, incubations were performed at 22°C under a 16 h light / 8 h dark cycle. For hypocotyl elongation, incubations were carried out at 22°C in the dark. Values are means ± SE of three biological replicates, each containing 75-250 seeds.

results confirm a role of SDH in germination, as suggested before by the *sdh2-3* mutants, and points to an essential SDH role in seedling establishment.

4.6.4. Effects of exogenous succinate and fumarate on germination

Succinate dehydrogenase or mitochondrial complex II catalyzes in the Krebs cycle the oxidation of succinate to fumarate. Work in our laboratory has determined that SDH2 malfunctioning in seeds leads to succinate accumulation after two days of imbibition (Fuentes, 2011). To determine how exogenous succinate can influence germination, we germinated Col-0 and *sdh2-3* mutant seeds in the presence of different concentrations of this metabolite (figure 28a). A germination delay was seen only with 25 and 50 mM of succinate, while lower physiological concentrations did not show any measurable effect or even accelerated germination. Additionally, cotyledon aperture was scored at 2 days post-stratification showing that this process is also affected at 25 and 50 mM succinate (figure 28b). Thus, succinate accumulation in *sdh2-3* mutants cannot explain the delay in germination.

On the other hand, we wanted to evaluate if exogenous fumarate can compensate for the lack of a normal functioning complex II in seeds. In order to achieve this, Col-0 and DsLox *sdh2-3* mutant seeds were sown on plates containing different fumarate concentrations (0, 5 and 25 mM) and germination was scored between 16 and 46 hours post-stratification. Mutant seeds grown in the absence of fumarate show the expected germination delay which is lost after 46 hours post-stratification (figure 29a). The presence of 5 mM fumarate abolishes the difference between wild-type and mutant seeds

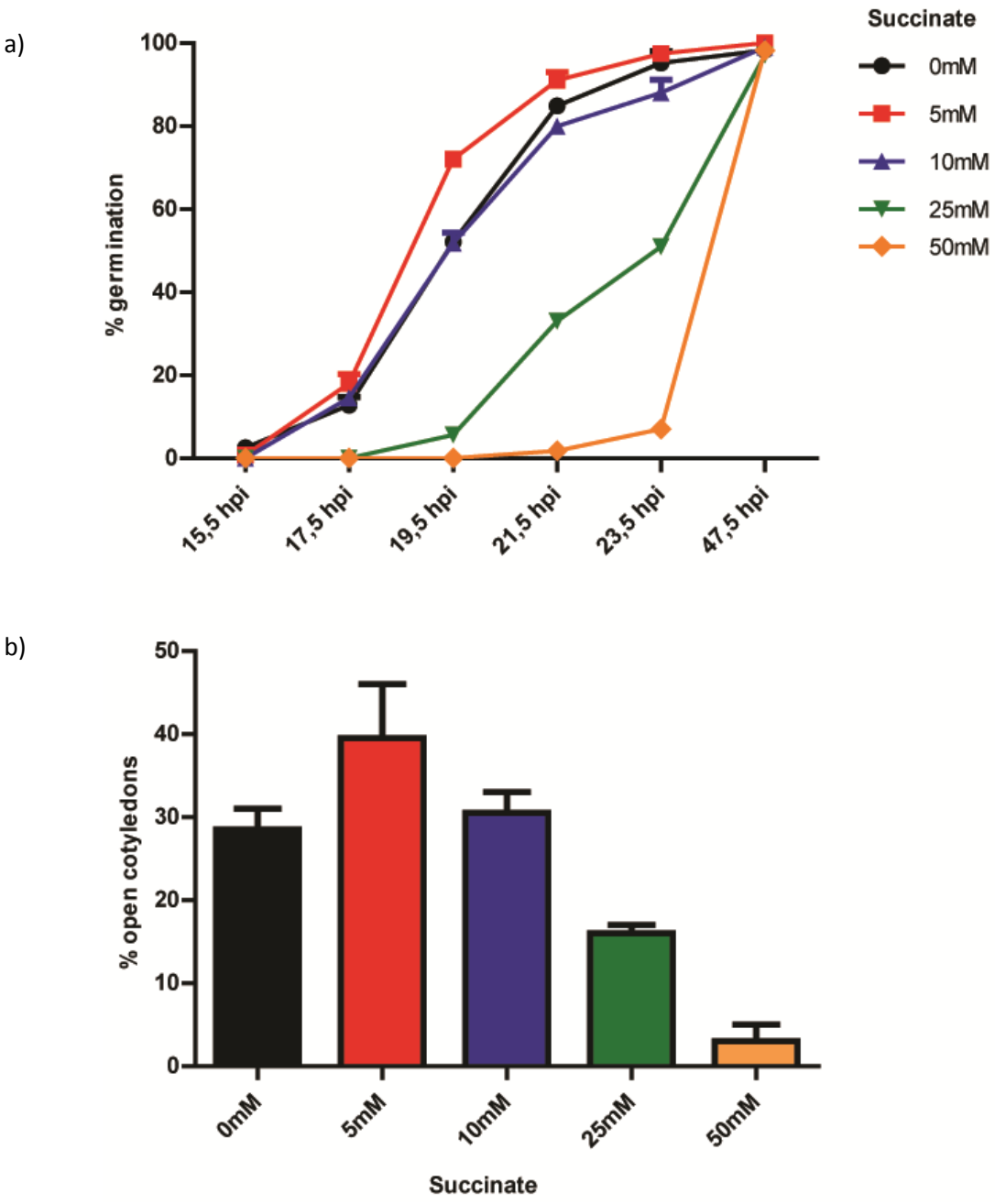


Figure 28. Effects of exogenous succinate on germination and cotyledon expansion.

a) Effect of different succinate concentrations on germination. Germination was scored as radicule protrusion. b) Succinate effect on cotyledon expansion in Arabidopsis. Cotyledon aperture was measured 2 days post stratification. Values are means ± SD of two biological replicates, each containing 52-69 seeds. Asterisk indicate differences with 0mM succinate according to Student´s t-test ($0.01<p<0.05$).

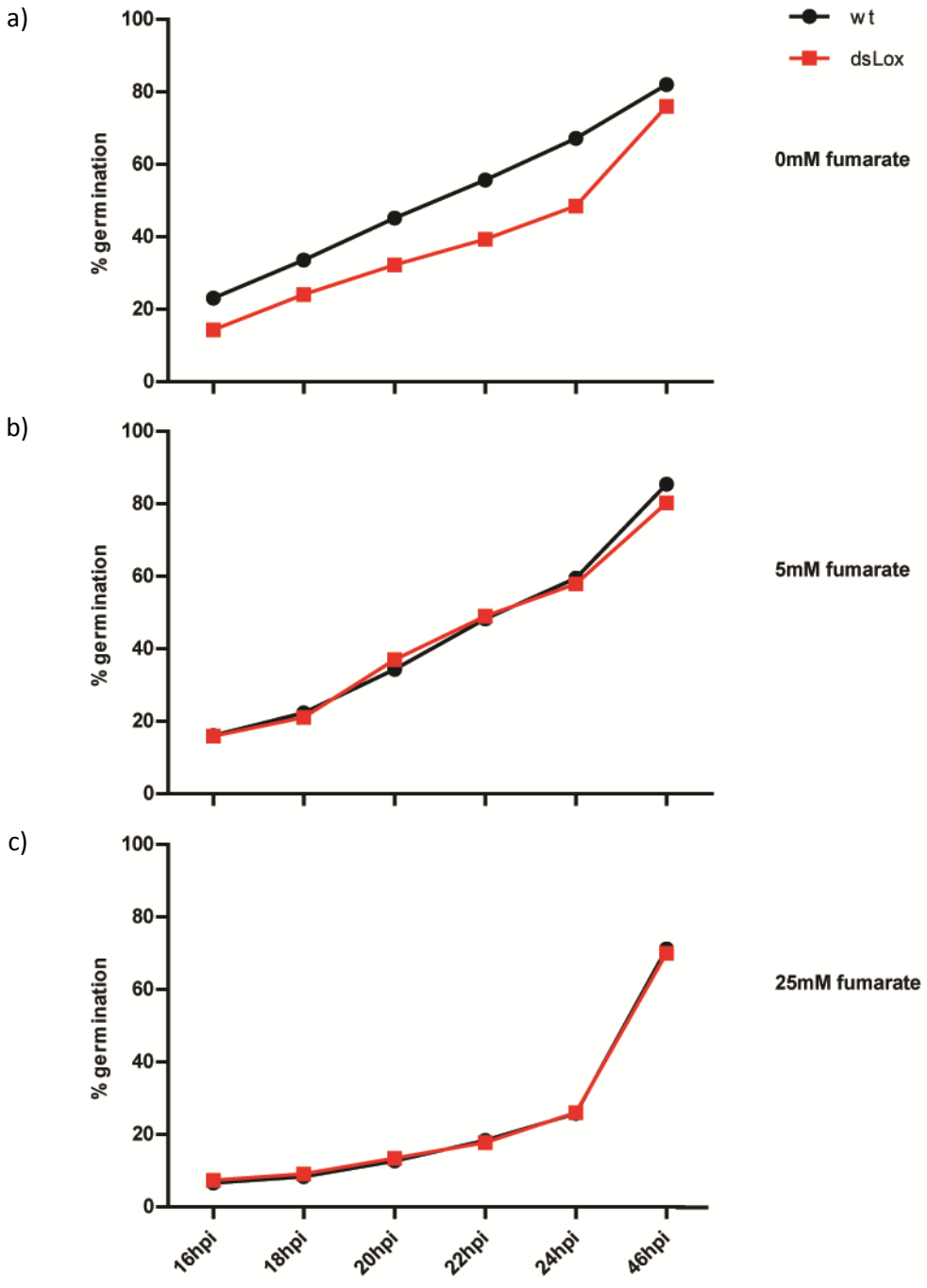


Figure 29. Effect of exogenous fumarate on germination.

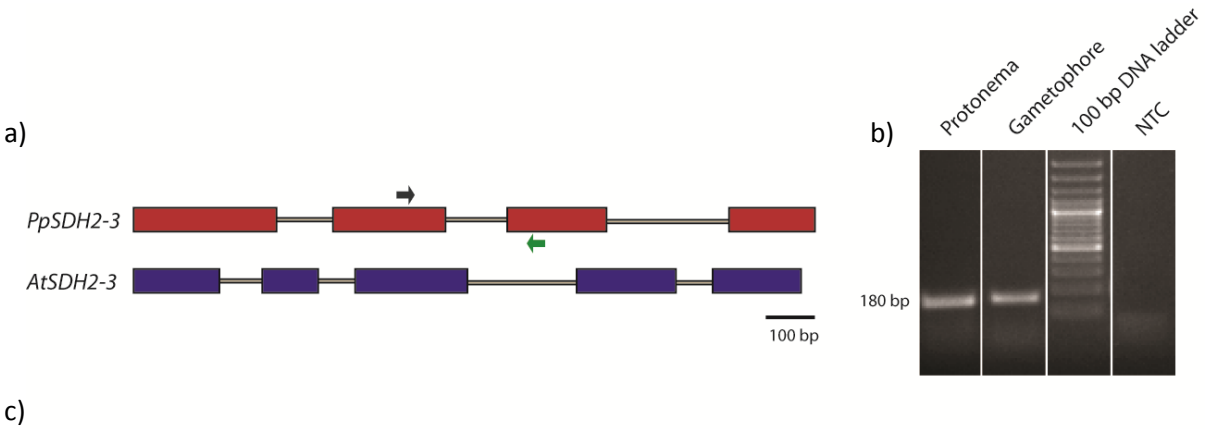
Effect on germination in Arabidopsis seeds of a) 0 mM, b) 5 mM and c) 25 mM fumarate . Germination was scored as the radicule emergence and measured between 15 hours and 45 hours post stratification. Values are means ± SE of three biological replicates, each containing 43-59 seeds.

(figure 29b), while 25 mM of fumarate delays germination in both wt and mutant seeds, especially in the first 24 hours (figure 29c). A detailed statistical analysis shows that germination of Col-0 seeds is inhibited by 5 mM fumarate, while germination of the mutant seeds is not affected by the metabolite (p of 0.01 and 0.02 for 16 and 20 hours for Col-0 seeds, respectively).

4.7. *SDH2-3*-like gene in *Physcomitrella patens*

The existence of a *SDH2-3*-like gene in Physcomitrella is of great interest to our laboratory. Until now, this type of gene has been found only in angiosperms, being expressed in seeds. Surprisingly, *in silico* analysis of the moss *Physcomitrella patens* genome, using the Joint Genome Institute (JGI) database, uncovered two types of *SDH2* genes encoding putative iron-sulfur proteins: a AtSDH2-1-like protein and a AtSDH2-3-like protein, which we referred to from now on as PpSDH2-1 and PpSDH2-3, respectively. The existence of an angiosperm seed-specific subunit in this moss, despite they do not possess a seed stage, captured strongly our attention. Analysis of the exon/intron structure show important differences between PpSDH2-3 and AtSDH2-3 (figure 30a), however, they have near 65% identity regarding the protein sequence (figure 30c).

We were able to detect through RT-PCR the presence of the *PpSDH2-3* transcript in two tissues: the gametophore and the protonema (methods 3.9 and 3.3.2). Both belong to the haploid phase of this moss, which is the predominant stage in the Physcomitrella life cycle (figure 30b).



c)

```
AtSDH2-3        MSS--VLRLLGRRICNPAAEKVRLSSSLSGGGDFPILNGHKAAQDLSKD-TLKSQDITKE 57
PpSDH2-3        MAHRKTLPVLLQSIKSYDAVSGHHVRNMSMLPNLKVDDPRHKDVCITKDNVLGDQKVAKK 60
                *:   .* :* : * .  * . :   .:*   :: : : ::    ::** .* .*.::*:

AtSDH2-3        KEGQHK-EVKKEFKIYRWNPDKPNSKPFLQSFFVDLSSCGPMVLDVLQKIKAEDDASLSY 116
PpSDH2-3        QAGISKSTTTKEFKIYRWNPDN-GGKPYLKSYFVDISKCGPMI-----------DPTLVY 108
                : *  *  ..***********: ..**:*:*:***:*.****:          *.:* *

AtSDH2-3        RRSCREGICGSCSMNIDGTNTVACLKPINPNTSKPTIITPLPHMYVIKDLVVDLTNFYQQ 176
PpSDH2-3        RRSCREGICGSCSMNIDGINTVACLCSVNMDPKHVTTITPLPHMYVIKDLVVDMTQFCQQ 168
                ***************** ****** .:* :..: * ****************:*:* **

AtSDH2-3        YKSMEPWLKTRKPPKDGREHRQSPKDRKKLDGLYECILCACCTTSCPSYWWNPEEFPGPA 236
PpSDH2-3        YKSVEPWLKTRKPAPENKEYLMSKKDRKKLDGLYECILCACCNTACPSYWWNQEKFLGPA 228
                ***:*********. :.:*:  * *****************.*:******* *:* ***

AtSDH2-3        ALL--QAYRWISDS---RDEYREERLQAITESETKVYRCRAIKNCTATCPKGLNPASAIL 291
PpSDH2-3        AFISVEVQERVAKLNLVRDEYTLERIDYLDN-EFKLGRCHTIMNCARTCPKSLNPGKAIA 287
                *::  :. . ::.    ****  **:: : : * *: **::* **: ****.***..**

AtSDH2-3        KMKSKHLLSDPLVRTESV 309
PpSDH2-3        QIRRMQVTKFFSDRPDMI 305
                :::  :: .    *.: :
```

Figure 30. *PpSDH2-3* structure and expression.

a) Schematic representation of *PpSDH2-3* and *AtSDH2-3* exon/intron structure. b) *PpSDH2-3* transcript expression in protonema and gametophore tissues. RT-PCR analysis was performed on total RNA. Thirty-five amplification cycles were used and primers are depicted in a) by a black (RT-ppsdh2-3-F) and a green (RT-ppsdh2-3-R) arrow. NTC: non template control. c) Comparison between PpSDH2-3 and AtSDH2-3 amino acid sequences. An asterisk indicates positions which have a single, fully conserved residue. A : (colon) indicates conservation between groups of strongly similar properties (scoring > 0.5 in the Gonnet PAM 250 matrix). A . (period) indicates conservation between groups of weakly similar properties (scoring =< 0.5 in the Gonnet PAM 250 matrix).

Given that mosses do not produce seeds or any homologous structure, the next step was to determine if abiotic stresses mimicking seed development could have an impact on *PpSDH2-3* transcript levels. Interestingly, only osmotic stresses (600 mM manitol or sorbitol), but not saline (250 mM NaCl), increased *PpSDH2-3* levels after a 24h treatment (figure 31). The *PpSHP1* gene was used as a positive stress control, its transcript levels have been described to increase in all the abiotic stresses mentioned before (Kroemer et al., 2004). Is worth noting that transcript increases are greater in protonema tissue than in gametophore.

On the other hand, ABA (100 μM) or desiccation did not change significantly the *PpSDH2-3* transcript level (figure 32). The *PpSHP1* control was also used, showing that its transcript level increases with both ABA and desiccation.

We also evaluated if the *PpSDH2-3* promoter is active in Arabidopsis, particularly in seeds. For this, we transformed Col-0 Arabidopsis plants (methods 3.4.3) with a construct containing 1000 bp of the *PpSDH2-3* promoter upstream of the GUS reporter gene (methods 3.4.1). GUS qualitative staining experiments showed weak, if any, expression in seeds. This expression seems to slightly increase when imbibed 1 day, and then decreased 4 days after imbibition (figure 33). Weak GUS expression was also detected in pollen, a high energy-consuming tissue that is dependent on mitochondrial activity. Thus, we can conclude that the Physcomitrella *SDH2-3* promoter has not significant activity in Arabidopsis.

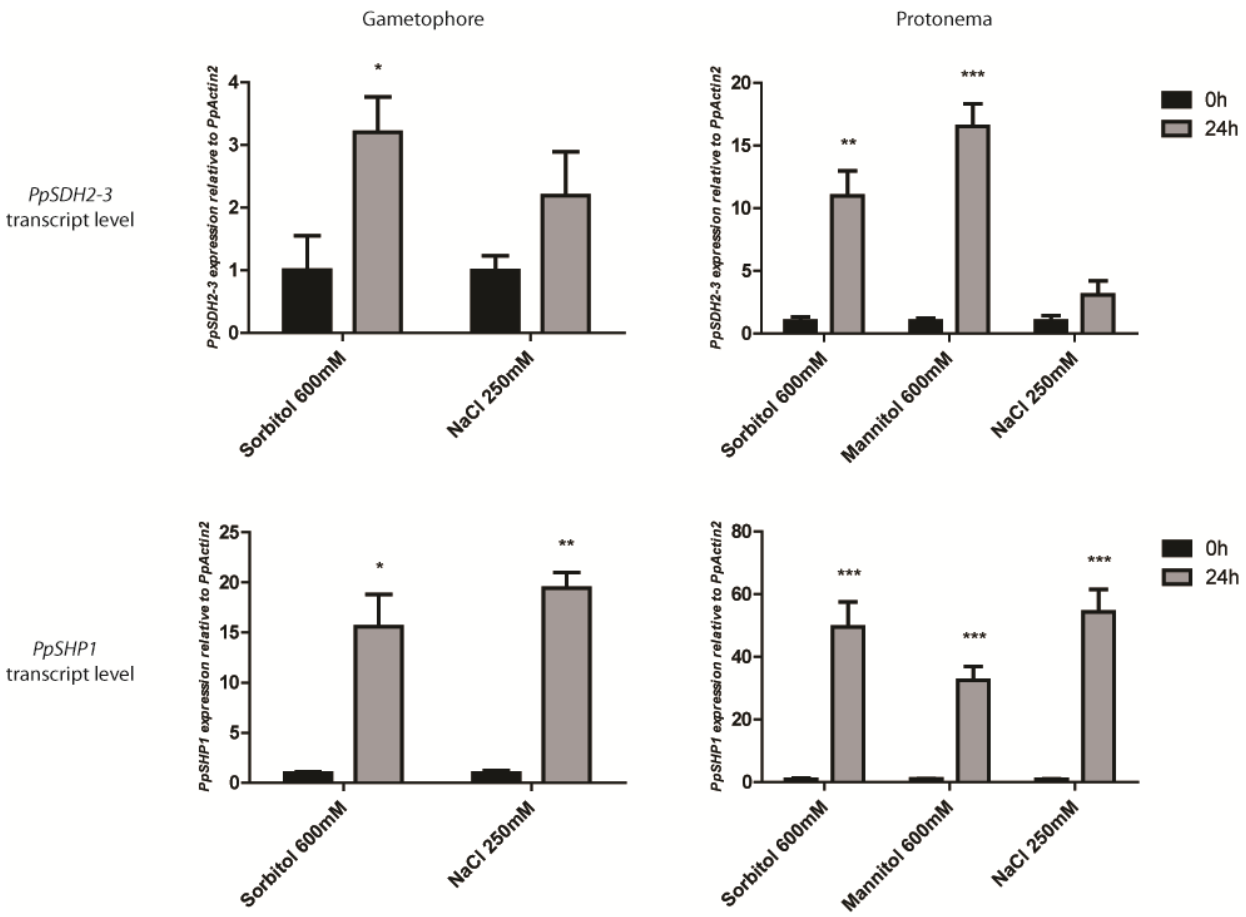


Figure 31. Effects of osmotic and saline stress on *PpSDH2-3* expression.

PpSDH2-3 (upper panels) and *PpSHP1* (bottom panels) transcript levels in gametophore (left) and protonema (right) tissues. Physcomitrella samples were subjected for 24 hours to osmotic stress (600mM sorbitol or mannitol) or high-salt stress (250mM NaCl). RT-qPCR analysis was performed on total RNA from these tissues. Expression levels are given relative to the *PpACTIN2* gene for normalization. Values are means ± SE of three biological replicates. Asterisks indicate significant differences according to Student´s t-test (one for $0.01<p<0.05$; two for $0.001<p<0.01$; three for $p<0.001$).

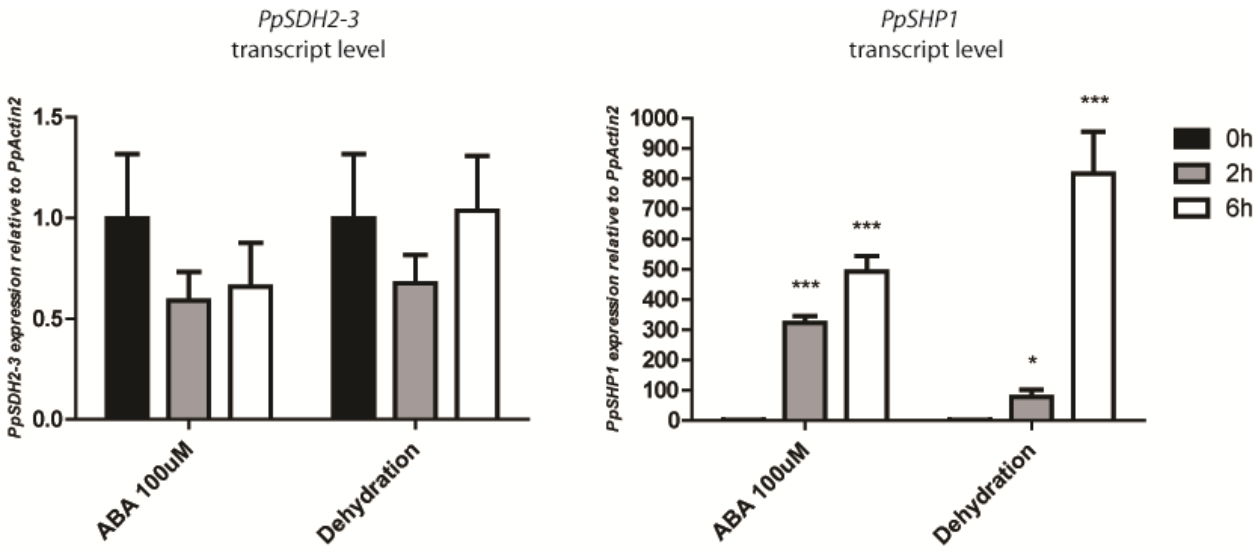


Figure 32. Effects of ABA and dehydration stress on *PpSDH2-3* expression.

PpSDH2-3 (left) and *PpSHP1* (right) transcript levels in protonema tissue. Physcomitrella samples were subjected for 0, 2 and 6 hours to 100uM ABA or dessication treatments. RT-qPCR analysis was performed on total RNA from this tissue. Expression levels are given relative to the *PpACTIN2* gene for normalization. Expression in the absence of treatments was set to 1.0. Values are means ± SE of three biological replicates. Asterisks indicate significant differences according to Student´s t-test (one for $0.01<p<0.05$; three for $p<0.001$).

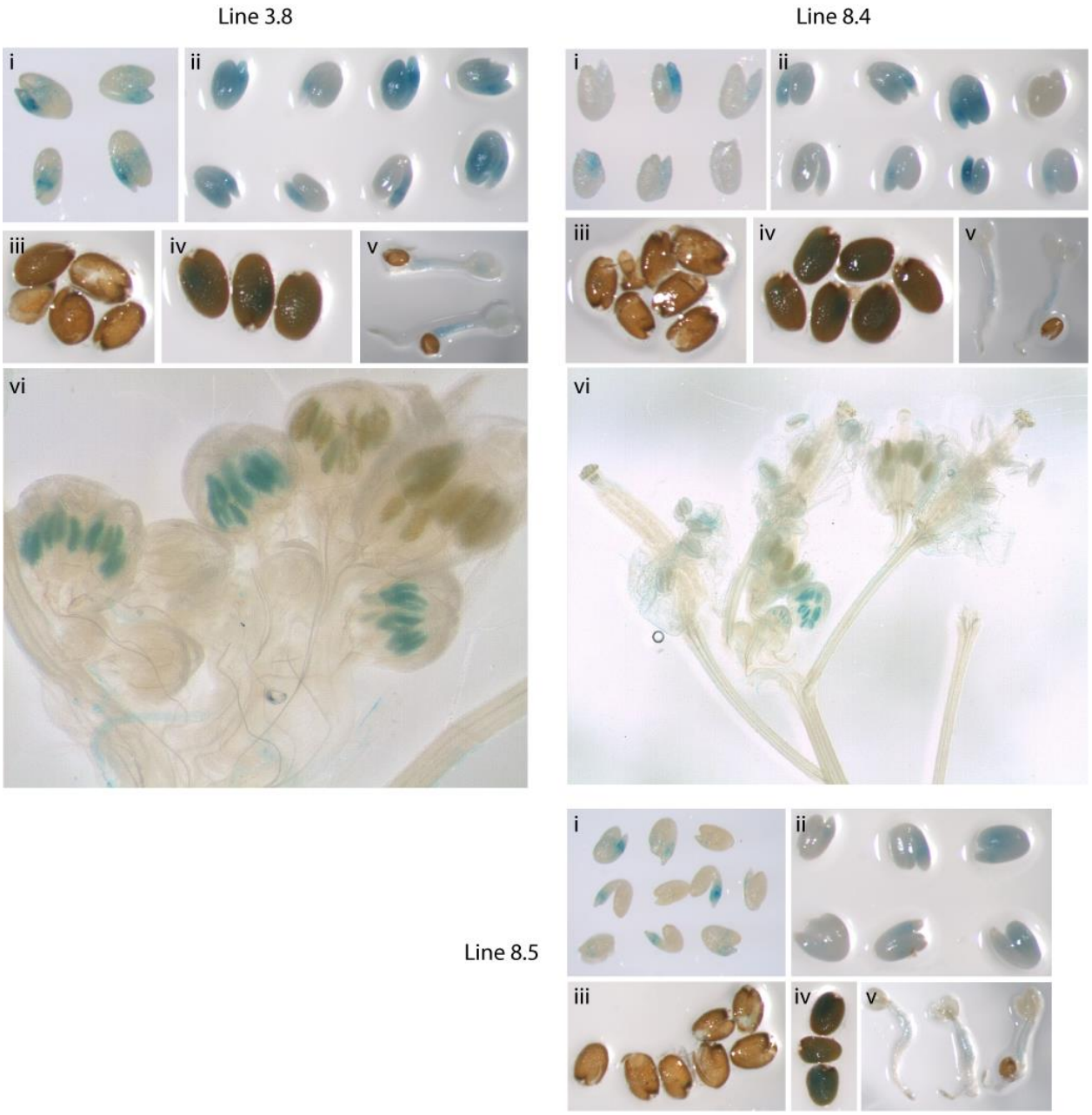


Figure 33. *PpSDH2-3* promoter expression in Arabidopsis.

GUS staining was performed overnight as described in the methods section on: i) embryos extracted from dry seeds ii) embryos extracted from seeds imbibed for 24 h iii) testa plus endosperm obtained from 24 h-imbibed seeds iv) whole 24 h-imbibed seeds v) 4 day-old seedlings vi) flowers. Three independent transgenic lines (3.8, 8.4 and 8.5) were analyzed.

In conclusion, *Physcomitrella patens* contains an *SDH2-3*-like gene whose expression is induced by osmotic stress but not by saline stress or ABA. This gene may be related to an ancestor gene that evolved to give rise to the angiosperm embryo-specific complex II iron-sulfur subunit.

4.8. Yeast SDHB mutant complementation with Arabidopsis and Physcomitrella SDH2 isoforms

Steinmetz et al. (2002) in a screen looking for mitochondrial related proteins involved in human diseases, reported that SDHB yeast mutants (AtSDH2 homolog) have an impaired growth (fitness) in non-fermentative substrates (glycerol, ethanol and lactate) when compared to growth in a fermentative sugar (glucose). We took advantage of this phenotype and used this yeast SDHB mutant as a model to study if Arabidopsis SDH2 isoforms can complement this mutation, and to determine if there is any difference in the fitness among the three. Indeed, although we have clearly shown that SDH2-1/2-2 and SDH2-3 are differentially expressed in Arabidopsis, evidence is lacking for functional differences between complex II containing either SDH2-1/2-2 or SDH2-3. Thus, we propose to use the yeast model to analyze SDH2 function.

Here we show that both a and α strains of this yeast mutant grew normally with glucose as substrate, but had an impaired growth in a non-fermentative media containing glycerol, as expected (figure 34). The three Arabidopsis (*AtSDH2-1*, *AtSDH2-2* and *AtSDH2-3*) and the two Physcomitrella (*PpSDH2-1* and *PpSDH2-3*) isoforms were cloned into a modified pYES2 yeast expression vector, containing the ADH1 strong promoter controlling

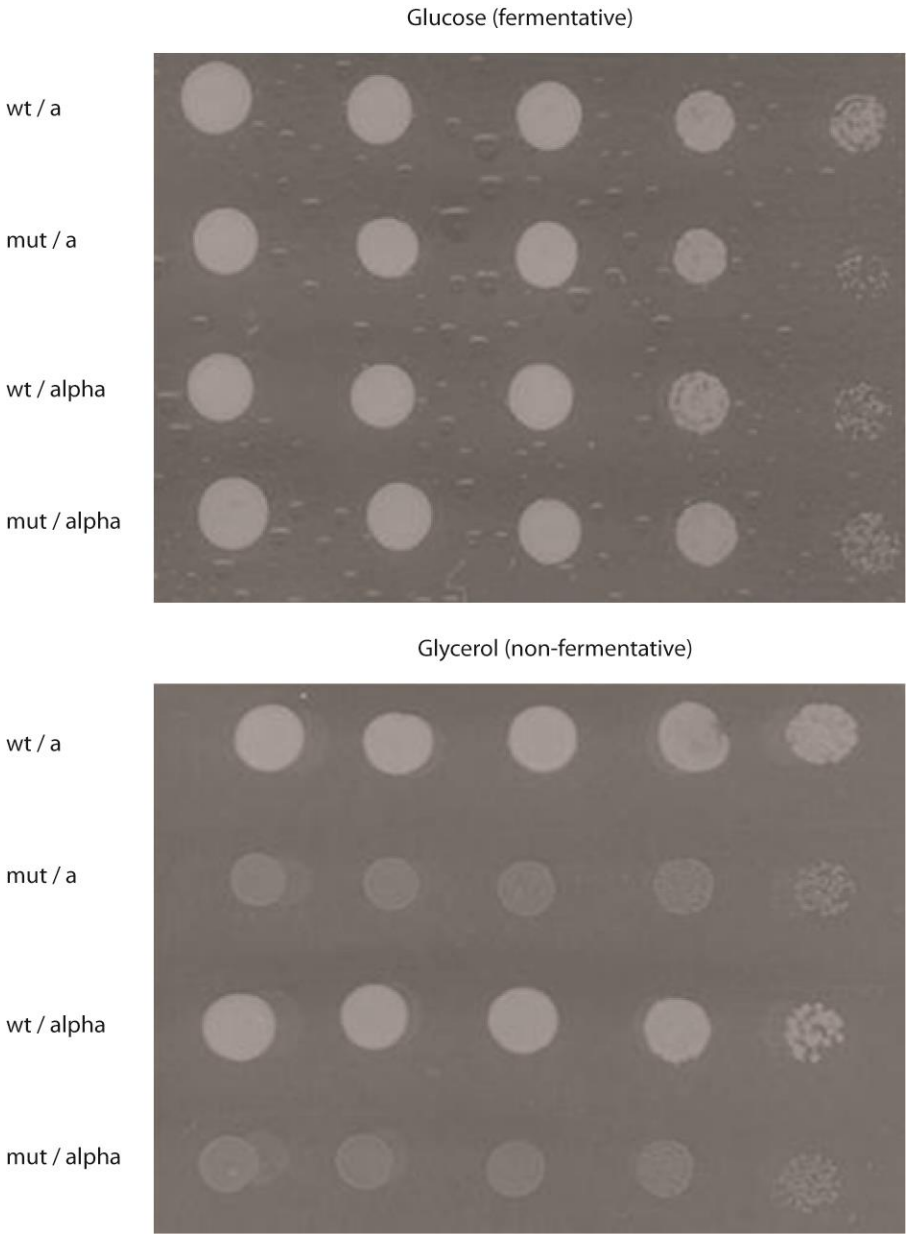


Figure 34. Yeast *sdhb* mutant grown in fermentative and non-fermentative media.

Wild type and mutant *sdhB* yeast strains (a and alpha) grown on solid media with glucose (left, fermentative) or glycerol (right, non-fermentative). Serial 1:10 dilutions are shown in both panels. Photos were taken after 2 days growing at 30°C. wt: wild type, mut: mutant.

the mentioned genes. Wild type and mutant *sdhb* strains were transformed and grown with glycerol and ethanol, two non-fermentative substrates. Neither transformation with the Arabidopsis nor Physcomitrella isoforms gave a complementation phenotype (figure 35). One possible explanation for this lack of complementation may be an impaired plant targeting peptide recognition by yeast mitochondria.

Yeast SDHB mitochondrial targeting peptide has been experimentally characterized and shown to comprise 20 aminoacids from the N-terminus (http://www.yeastgenome.org/). On the other hand, Arabidopsis isoforms targeting peptides were determined *in silico* by several programs (figure 36a). New constructs were made in which the target peptide of AtSDH2-1, AtSDH2-3 and PpSDH2-3 was exchanged for the one in SDHB (figure 36b, marked by the red arrow).

Wild type and mutant yeast strains were transformed with these chimeric iron-sulfur proteins and grown with glycerol as substrate, but no complementation phenotype was observed (figure 37). Thus, our attempts to analyze the function of Arabidopsis SDH2 isoforms in yeast were unsuccessful. We presume that Arabidopsis SDH2 subunits were unable to interact properly with the other yeast SDH subunits in order to be incorporated in a functional complex II.

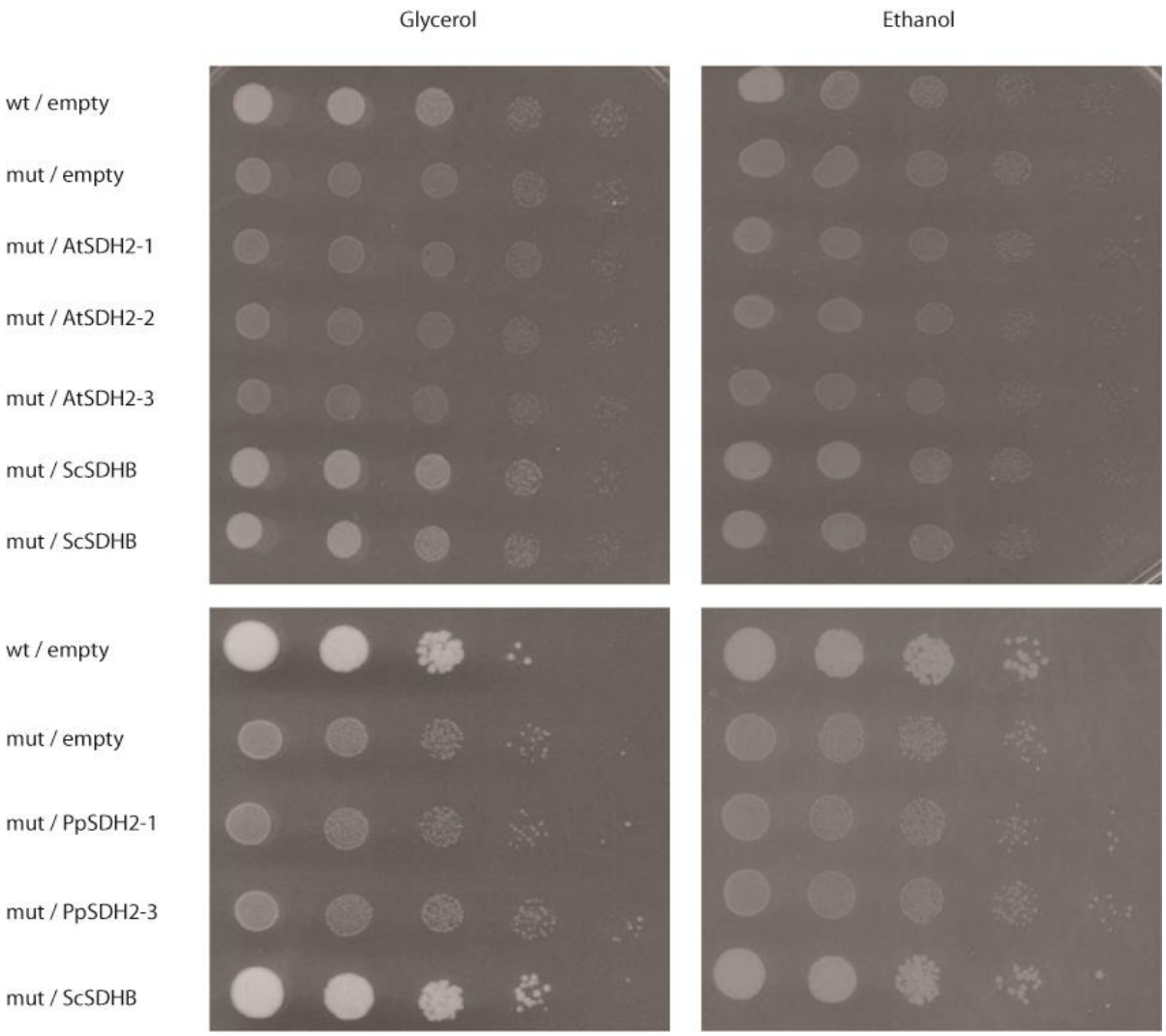


Figure 35. Yeast *sdhb* mutant complementation with Arabidopsis isoforms.

Wild type and mutant *sdhB* yeast strains grown with glycerol (left panels) or ethanol (right panels). Transformed strains with vectors containing different genes are shown. Serial 1:10 dilutions are shown in all panels. Photos were taken after 2 days growing at 30°C. wt: wild type, mut: mutant.

a)

```
ScSDHB      MLNVLLRRKAFCLVTKKGMATATTAAATHTPRLKTF  -19
AtSDH2-1    MASGLIGRLVGTKPSKLATAARLIPARWTSTGAEAETKASSGGGRGSNLKTF     -28
AtSDH2-2    MAFGLIGRVVGTKSSRLSTAARLIPARWTSTGSEAQSKASTGGGGASLKTF -28
AtSDH2-3    MSSVLRLLGRRICNPAAEKVRLSSSLSGGGDFPILNGHKAAQDLSKDTLKSQDITKEKEGQHKEVKKEF    -22
PpSDH1-1    MAAITRAASASGRAKGILNSYFLRSVSTTSASSTALKSSTEQSDPSPGITATKEPNIKKF               -37
PpSDH2-3    MAHRKTLPVLLQSIKSYDAVSGHHVRNMSMLPNLKVDDPRHKDVCITKDNVLGDQKVAKKQAGTSKSTTTKEF -28
```

b)

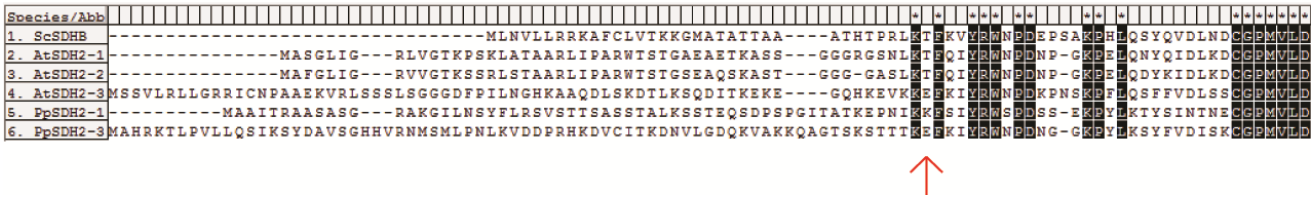


Figure 36. Yeast/Arabidopsis chimeric constructions.

a) Experimental or predicted targeting peptide cleavage sites for yeast, Arabidopsis and Physcomitrella iron-sulfur proteins (yellow). b) Sequence alignment of yeast, Arabidopsis and Physcomitrella iron-sulfur proteins. Conserved aminoacids are shown in black. cDNAs encoding chimeric iron-sulfur proteins were made using the yeast 35 N-terminal aminoacid sequence (until the red arrow, including it) fused to the downstream sequence of Arabidopsis and Physcomitrella SDH2 isoforms.

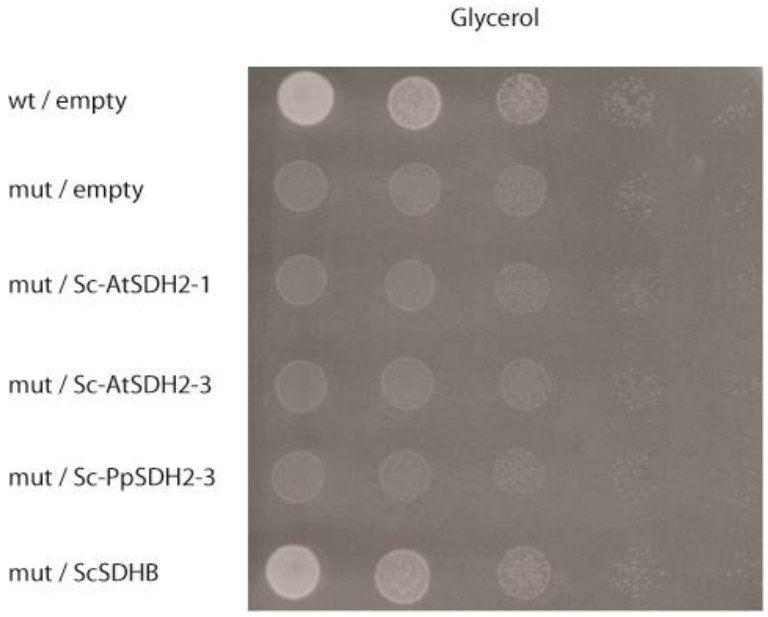


Figure 37. **Yeast *sdhb* mutant complementation with chimeric constructions.**

Wild type and mutant *sdhB* yeast strains grown with glycerol. Transformed strains with vectors containing different chimeric constructs are shown. Serial 1:10 dilutions are shown. Photos were taken after 2 days growing at 30°C. wt: wild type, mut: mutant, Sc: yeast targeting peptide sequence, At or Pp: Arabidopsis or Physcomitrella remaining protein, respectively.

5. DISCUSSION

SDH2-3 is the first described TCA gene to be expressed only in seeds during maturation. Our laboratory has undertaken the task of uncovering its transcriptional regulation, as it presents a novel expression pattern for a mitochondrial protein. In addition, previous work in our laboratory determined that *sdh2-3* mutants show a delay in germination. Our aim is to determine if this phenotype can be explained by defects during previous stages, as maturation, or if it is due to a defective SDH complex during germination. Furthermore, we look forward to uncover a role for this gene during postgerminative growth as plant establishment is probably one of the most important stages in the life of a plant. Moreover, until now *SDH2-3*-like genes were found only in seed plants. However, *Physcomitrella patens*, a moss which does not possess a seed-like structure, contains a *SDH2-3*-like gene, besides the usual *SDH2-1*/*SDH2-2* type of gene. We aim to characterize the expression of the Physcomitrella *SDH2-3* gene in order to gain insight on the evolution of *SDH2-3*-like genes.

5.1. Regulation of *SDH2-3* expression

5.1.1 Promoter analysis

Our laboratory previously determined an active *SDH2-3* promoter between -223 and +49 from the transcription start site (Elorza et al., 2006). Individual mutation of three ABRE elements and a RY element found between -114 and -32, caused significant reductions in promoter activity, indicating an active role for these elements in *SDH2-3* expression (Roschzttardtz et al., 2009). However, fusion of this fragment to a CaMV35S

minimal promoter showed no activity, suggesting an important role for the region upstream of the ABRE3 element (-223 to -114) and/or the region downstream of the RY element (-32 to +49). We demonstrated here that only this second region (-32 to +49) is needed for promoter activity (figure 11). Indeed, deletion of the region upstream of the ABRE3 element (-223 to -114) did not affect it at all (figure 11; construct -114/+49). This allowed us to establish the minimal functional *SDH2-3* promoter between -114 and +49. Afterwards, in order to determine important regions for transcriptional activity of the *SDH2-3* promoter, we evaluated the 5´UTR (+1 to +49), exchanging it for a known functional 5´UTR from the CaMV 35S promoter of the pBI121 plasmid, similar in length (41 bp). No promoter activity was observed with this construct, leading to the conclusion that the *SDH2-3* 5´UTR is essential and suggesting that it may contain *cis* elements involved in promoter activity. It is important to add that the *SDH2-3* gene is TATA-less and has a relaxed form of the initiator (Inr) motif (Y<u>R</u>, with the transcription start site underlined), a typical feature of TATA-less promoters (Yamamoto et al., 2009). However, *SDH2-3* has a 49 bp 5´UTR, significantly shorter than the 138 bp found in most TATA-less promoters in Arabidopsis (Molina & Grotewold, 2005). It has been suggested that promoters of this type have longer 5´UTR than TATA-containing promoters, as a way to provide additional features in order to assembly the pre-initiation complex (PIC). Nevertheless, our results indicate that 49 bp of the 5´UTR are sufficient, in combination with 114 bp upstream of the transcription start site, to provide the necessary elements for transcriptional activation.

Further *in silico* analysis of the -114/+49 region of the *SDH2-3* promoter, revealed the existence of putative *cis* elements that caught our attention: an auxin-responsive element (AuxRE) and a DOF transcription factor binding site, located 2 and 21 bp downstream of the RY element, respectively. Auxin has been involved in the regulation of diverse seed-related processes, making this element a good candidate as transcriptional regulator of the *SDH2-3* gene (Friml, 2003). On the other hand, several DOF transcription factors have been involved in SSP genes transcriptional regulation in monocots, binding the prolamin box containing the typical AAAG consensus sequence, also found in the *SDH2-3* promoter (Diaz et al., 2005, Diaz et al., 2002, Marzabal et al., 2008). Up to date, no DOF transcription factors have been involved in seed development in dicotyledonous species. Plants transformed with constructs containing a mutated version of the AuxRE element, the DOF element or both showed a mild reduction in GUS gene expression, however differences were not statistically significant (figure 12). Data obtained from this experiment showed a normal distribution (Shapiro-Wilk test), however they were treated as the opposite as plant transformations are known to have an enormous variation due to the *position effect* of the construct insertion in the genome (Nap et al., 1993). Non-parametric tests (Krustall-Wallis and Mann-Whitney) and also Box-Cox data transformation followed by parametric tests (ANOVA) showed no significant differences between mutated and wild-type promoters. Thus, both AuxRE and DOF elements appear to have a minor role, if any, in the *SDH2-3* transcriptional regulation in seeds. Is worth noting that the AuxRE element is only 2 bp downstream of the RY element, which is composed of alternate purine-pyrimidine nucleotides with a CATGCA consensus motif.

Interestingly, the following four nucleotides downstream of the RY element are also purine-pyrimidine pairs (CATG), the last two nucleotides being part of the putative AuxRE element. This raises the possibility that this two purine-pyrimidine pairs could be an extension of the RY element rather than part of an active AuxRE element, therefore explaining the mild reduction in GUS activity in the AuxRE mutant by mutation of the last nucleotide of an extended RY element. In summary, we have been able to define more precisely the promoter region required for high SDH2-3 expression in the embryo (-114 to +49) and or results suggest an important role for the region encoding the 5´UTR. However, further work is necessary to identify the *cis* elements present between -32 and -1 and/or between +1 and +49.

We have previously determined the importance of the ABRE and RY elements for *SDH2-3* transcriptional activation as embryos transformed with promoter constructs containing specific mutations in these elements show a lower GUS activity than constructs with the wild type promoter (Roschzttardtz et al., 2009). However, there is no information on how they interact during embryogenesis. Here we demonstrate a module-specific expression patterning involving the ABRE and RY elements as seen by qualitative GUS staining (figure 13). Individual mutations in the three ABRE elements mostly affected the hypocotyl region staining, being ABRE2 the mutation with the highest effect. Additionally, the triple ABRE mutant resembled the ABRE2 mutation, suggesting that the latter is predominant over the other two. Moreover, this staining pattern is strongly correlated with the GUS activity quantification as ABRE2 and the triple mutant showed the most drastic reductions (Roschzttardtz et al., 2009). On the other hand, mutation in the RY

element produced a mild decrease in GUS staining in the whole embryo, except in the radicle region suggesting that the RY element could play a dual role depending on the embryo tissue. It could be necessary for promoter activation in hypocotyl and cotyledons but play no role or a negative role in the radicle. These results are in agreement with those obtained with the *PHAS* promoter, another seed-specific gene, in both *Arabidopsis thaliana* and *Phaseolus vulgaris*. In this case, individual mutation of three distal RY elements produced an increase in promoter activity in the radicle, while mutation of a proximal RY element does not appear to have any effect on it (Chandrasekharan et al., 2003). Additionally, no apparent changes in GUS expression are described in the hypocotyl and cotyledon regions. Moreover, mutation of the four RY elements in the *PHAS* promoter produces an 89% loss in GUS expression in the whole embryo, indicating a synergistic interplay between them. Additional evidence supporting a negative role in the embryo for the RY element comes from *fus3* mutant lines. FUS3 is a B3 type of transcription factor which bind the RY element and is highly expressed in the radicle (Parcy et al., 1994). Hypocotyls of *fus3* mutant seeds have extra files of cells compared to wild-type seeds, suggesting a repressive role of this transcription factor in cell duplication (Raz et al., 2001). This could somehow suggest a negative role of the RY element through its interaction with FUS3, nevertheless, more experiments are needed to address this issue.

5.1.2. Transcription factors involved in *SDH2-3* regulation

As previously reported (Roschzttardtz et al., 2009), ABRE and RY elements are major regulators of the *SDH2-3* gene in seeds and these motifs are *in vitro* targets of bZIP

and B3 domain transcription factors, respectively. In order to gain further insight into the mechanism involved in *SDH2-3* gene regulation, we did *in vivo* transient expression assays using Arabidopsis mesophyll protoplasts, which we transfected with different combinations of the mentioned transcription factors (ABI3, bZIP10, bZIP25 and bZIP53; figure 14). Single bZIP transcription factors were unable to activate the promoter, and only the bZIP10+bZIP53 combination was able to induce promoter expression. This is the first evidence of bZIP transcription factors directly involved in the activation of the *SDH2-3* promoter. On the other hand, single ABI3 transfection did activate the promoter, although this activation was not significantly different to transfections of ABI3 along with different combinations of bZIP factors (except for the ABI3+bZIP10+bZIP53 transfection, which was significantly higher). Promoter transcription by ABI3 and bZIP factors has been described in other seed-specific promoters (Lara et al., 2003). ABI3 is known to bind weakly to the RY element *in vitro* (Suzuki et al., 1997), but also it appears to be tethered to a promoter through interaction with a group C bZIP factor, such as bZIP10 or bZIP25 (Lara et al., 2003). We have previously shown that ABI3 is important *in vivo* for *SDH2-3* expression in seeds, since *abi3* mutants have reduced transcript levels, and that ABI3 binds *in vitro* to the promoter. Now we have confirmed that ABI3 likely regulates directly *SDH2-3* promoter, in coordination with bZIP factors. We also found that transfection of bZIP53 (S1 subgroup bZIP) along with bZIP10 (C subgroup bZIP) resulted in increased promoter transcription, consistent with the fact that the bZIP10/bZIP53 heterodimer showed a stronger *in vitro* binding compared to the bZIP25/bZIP53 heterodimer (Roschzttardtz et al., 2009). The latter shows that, although ABI3 is essential in dry seeds, transfection of bZIP10 and

bZIP53 can activate the promoter without an apparent need of a B3 domain transcription factor. This conclusion on the *in vivo* role of bZIP factors is reinforced by the observation that *SDH2-3* transcript levels are significantly reduced in the *bzip53* mutant dry seeds (figure 15a). In contrast, *bzip10* and *bzip25* mutants did not show a reduction in *SDH2-3* transcript levels, suggesting redundancy between them and/or with other bZIP transcription factors.

Altogether, our results show that ABI3 and bZIP10 plus bZIP53 regulate SDH2-3 promoter activity, likely acting through RY and ABRE *cis* elements. As already mentioned, further work is needed to identify additional *cis* elements between -32 and +49, and also the transcription factors binding them. In addition, more detailed analysis, for instance in the transient assay, will be required to analyze interactions between transcription factors.

5.1.3. ABA is required for proper *SDH2-3* expression

Although the sesquiterpenoid ABA is a phytohormone involved in the regulation of several physiological processes, including seed development and maturation, there is little evidence on how it affects *SDH2-3* transcription. In order to determine the role of ABA in *SDH2-3* transcription in dry seeds we used four ABA biosynthesis mutants (*aba2-1*, *aba2-3*, *aba3-1* and *aba3-2*) and measured *SDH2-3* transcripts levels. The four of them showed a significant decrease in transcript levels in dry seeds, demonstrating that ABA regulates *SDH2-3* transcription (figure 16). The plant phytohormone ABA is known for its importance in SSP and LEA gene transcription during seed maturation (Leung & Giraudat, 1998, Rohde et al., 2000). These results confirm that the *SDH2-3* promoter behaves as other seed-

specific promoters that contain the ABRE element. To our knowledge, this is the first report describing regulation mediated by ABA of a mitochondrial electron transport chain gene. However, it has to be pointed out that neither ABA nor drought or saline stress are able to induce *SDH2-3* expression in vegetative tissues (Roschzttardtz, unpublished results). Similar results have been obtained with other SSP proteins (not LEA proteins) and are likely due to the lack of the B3 domain transcription factors (ABI3, LEC2 and FUS3) in vegetative tissues.

Results presented in this thesis and those of previous studies (Roschzttardtz et al., 2009), allows us to propose a model for *SDH2-3* promoter transcriptional activation in seeds. Promoter activation would require ABI3 and bZIP (bZIP53 and bZIP10) transcription factors. ABI3 could bind the RY element, although it could also be tethered to the promoter through interaction with bZIP10 (Lara et al., 2003). We also found that the hormone ABA plays an important role in transcription in seeds, probably mediated by the ABRE elements, targets of bZIP homo and heterodimers. From here, we can infer that *SDH2-3* transcriptional regulation is highly dynamic and redundant, but at the same time spatially and temporally restricted. This is the first example of a TCA promoter bearing a seed-specific transcriptional regulation.

5.1.4. Effect of DNA methylation in *SDH2-3* transcriptional regulation

Epigenetic factors were also analyzed in order to understand *SDH2-3* transcriptional regulation. Interestingly, zebularine-mediated methylation inhibition caused significant promoter activation in vegetative tissue as seen by GUS reporter

expression (200 and 500 fold increase; figure 17). However, this activation was not reflected at the transcript level, which showed only a very modest 1.5-fold increase (figures 17 and 18). Given the low transcript level in normal conditions, the biological relevance of this increase remains in doubt. Altogether, these results suggest that the *SDH2-3* transcript level decline in vegetative tissue could be due to diminished promoter activity and lowered transcript stability, although the latter remains to be tested.

Additionally, evidence that *non-canonical* methylation in CHG and CHH motifs could be participating in turning off the *SDH2-3* gene in vegetative tissue was obtained using the *ddc* triple mutant, which lack a functional version of the three methyltransferases involved in CHG and CHH methylation (DRM1, DRM2 and CMT3). *SDH2-3* transcript levels in vegetative tissue of this mutant showed a significant 10-fold increase (figure 20). On the other hand, a mutant in the *MET1* gene, involved in *canonical* CG methylation did not affect *SDH2-3* transcript levels in seedlings (figure 21). Furthermore, *ddc* mutant seeds showed a 50% decrease in *SDH2-3* transcript levels, contrary to what was observed in seedlings (figure 22).

We have determined the methylation status of the proximal *SDH2-3* promoter in seedlings, showing no methylation at all (figure 23). The same was observed in the promoter of seeds (results not shown). Thus, the role of promoter methylation in *SDH2-3* silencing suggested by the increase in *SDH2-3* transcript levels in the mutant *ddc*, should be indirect, if any. Pleiotropic effects due to defects in global DNA methylation may explain our results. Moreover, methylome analysis of 5 week-old plants shows a complete lack of methylation in *SDH2-3*, *ABI3*, *LEC2*, *FUS3* and *bZIP53* promoter regions. However,

bZIP25 shows strong methylation in the promoter region (Zhang et al., 2006). Further work is needed in order to determine if this gene is related to the *SDH2-3* transcript increase in the mutant *ddc*. Another possible explanation not analyzed here, could involve gene-body methylation, which has been described as an important transcriptional regulation mechanism in many plant genes (Aceituno et al., 2008). Altogether these results show that DNA methylation has contradictory effects on *SDH2-3* transcript levels, depending on the developmental stage. Nonetheless, more studies are needed to determine how it affects promoter activation in vegetative and embryo tissues.

5.2. SDH2-3 and complex II role during seed maturation, germination and postgerminative growth

Previous work in our laboratory showed that SDH2-3 is the main iron-sulfur subunit of complex II in dry seeds and that *sdh2-3* mutants have a delay in germination (Roschzttardtz et al., 2009). Here we add evidence supporting that SDH2-3 influences seed development, as both mutant lines showed a decrease in seed weight (figure 24a). Interestingly, protein content also showed a reduction in both mutants (figure 24b) while lipid content does not show any biologically significant variation (figure 24c). This is an interesting feature since seed metabolism is directed during maturation towards the formation of seed storage proteins (SSP), late embryogenesis abundant (LEA) proteins and lipids (Hughes & Galau, 1989, Lin et al., 2006, Parcy et al., 1994, Penfield et al., 2005, Weigelt et al., 2008). Thus, the decrease in protein content would explain the reduction in total weight. These results may suggest that the delay in germination could be due to

defects in seed maturation, specifically in storage protein production. This new role associated to total protein content go against what was believed to be the role of mitochondria in seed development. Baud et al. (2008) suggested that a full TCA cycle is absent and that flux through mitochondria was devoted to the formation of citrate, which is exported to the cytosol and used in the elongation of fatty acids. Our results suggest that the role of mitochondria should be reconsidered, as it may carry out other important metabolic tasks during this stage, providing for instance ATP and/or carbon skeletons for aminoacid and protein synthesis.

On the other hand, germination and seedling establishment are known critical stages in plant life cycle. In order to reach a sustainable growth, seedlings must switch from heterotrophic metabolism to photoautotrophism. Arabidopsis is an oilseed plant that converts storage reserves such as fatty acids into acetyl-CoA through the β-oxidation process, which in turn is converted to succinate by the glyoxylate cycle and ultimately to sucrose by SDH and gluconeogenesis (Eastmond & Graham, 2001, Penfield et al., 2005). After germination, growth in the dark (skotomorphogenesis) depends largely on β-oxidation and the glyoxylate cycle, suggesting that complex II could have an important role. Consistent with this role, we showed that *sdh2-3* mutants have impaired hypocotyl growth in the dark between 3 and 6 days in the absence of sucrose, and that exogenous sucrose complements this defect (figure 26). Unexpectedly, we found that in the presence of sucrose initial hypocotyl elongation (between 0 and 3 days) was inhibited and slower in the *sdh2-3* mutants than in wild-type, suggesting that at this early stage complex II may be important for ATP production through substrate respiration rather than for

gluconeogenesis. Interpretation of these experiments in terms of complex II role during postgerminative growth is complicated by the fact that during this period SDH2-3 is probably being replaced by SDH2-1/SDH2-2 in wild-type plants, and in *sdh2-3* mutants newly complex II is being synthesized. To address this issue we used TTFA, a well-known complex II inhibitor, and found that it abolishes hypocotyl elongation and seedling establishment (Figure 27). Altogether, these results provide compelling evidence for an essential role of complex II (and TCA cycle) in postgerminatve growth and seedling establishment. TTFA delayed but did not block germination (except at very high concentrations, 0.5-1 mM). This effect is similar to what we observed for *sdh2-3* mutants, and thus strongly suggests that a SDH2-3 containing complex II, although not essential, is important for an efficient germination. These results are also consistent with succinate-dependent O_2 consumption by mitochondria at early stages after seed imbibition (Benamar et al., 2008, Logan et al., 2001).

Wild seeds in nature generally germinate underground, in conditions where they lack direct sunlight. A seed with a non-functional SDH2-3 would be in disadvantage over wild-type seeds, which would elongate their hypocotyls further until they reach light in order to promote photoautotrophic growth. SDH2-3 gives an important advantage to the plant in energy-consuming processes such as germination and early stages towards seedling establishment. This work shows the importance that this non-essential gene can have in critical stages of plant development.

Recently our laboratory showed that wild-type seeds imbibed for 48 hours have a reduction in succinate levels (Fuentes, 2011). Wild-type and mutant *sdh2-3* dry seeds have

similar succinate levels, however its decrease at 48 h post imbibition is significantly lower in mutant seeds when compared to wild-type ones. This result is consistent with the proposed role of SDH2-3 containing complex II at very early stages after imbibition (see above). To analyze if exogenous succinate or fumarate can mimic (succinate) or complement (fumarate) the mutant phenotype, experiments were performed with concentrations ranging from 0 to 50 mM. No obvious effects were observed, except at very high concentrations of succinate and fumarate, which inhibit germination and cotyledon expansion. It is worth mentioning that 50 mM of succinate is near three orders of magnitude higher than the internal seed concentration, and that 5 mM fumarate is approximately 5 times the seed fumarate concentration (Angelovici et al., 2010, Chia et al., 2000).

In both cases, an excess of succinate and fumarate produced detrimental consequences in wild-type seeds. Phenotypes obtained by mutation of *SDH2-3* or by complex II inactivation can hardly be attributed to an excess of succinate due to a decrease in its oxidation, nor its mutant phenotype bypassed by an exogenous source of fumarate. These results are consistent with a role of SDH2-3 containing complex II in providing electrons for ATP synthesis and/or carbon skeletons for anabolic pathways at early stages after imbibition.

5.3. *SDH2-3*-like gene in Physcomitrella patens

The existence of a *SDH2-3*-like gene in the moss *Physcomitrella patens* has drawn our attention because *SDH2-3* has been described as a seed-specific expressed gene in

angiosperms, and mosses do not have this kind of structure. The sequenced Physcomitrella genome showed two ORFs with homology to *SDH2* genes, one encoding a *SDH2-1/SDH2-2*-like gene and another encoding a *SDH2-3*-like gene. Both have a predicted mitochondrial destination. Here we have confirmed by RT-PCR that the *SDH2-3*-like gene is expressed (figure 30b). Interestingly, some parallels have been proposed between seed-related processes and Physcomitrella biology. For instance, ABA responses have been described, uncovering the existence of three isoforms of an ABI3-like protein (named *PpABI3A*, *PpABI3B* and *PpABI3C*) in Physcomitrella. Furthermore, only *PpABI3A* can partially complement the *abi3* Arabidopsis mutant (Marella et al., 2006). Given these data, we analyzed *PpSDH2-3* gene expression in gametophore and protonema tissues subjected to different kind of stresses, mimicking what might be found in seeds (high osmotic potential, desiccation and ABA content increases; figures 30 and 31). Surprisingly, we observed an increase in the *PpSDH2-3* transcript level only with osmotic stress (mannitol and sorbitol). No apparent effect was seen with desiccation, ABA and high salt concentration. It is worth noting that Arabidopsis *SDH2-3* is not induced in vegetative tissue by either osmotic or saline stress, or ABA treatment (unpublished results). All the above suggests that the transcriptional regulation of this gene in Physcomitrella evolved in an independent way compared to Arabidopsis. Consistent with this assumption, the Physcomitrella *SDH2-3* promoter lacks significant activity in Arabidopsis, either in seeds or vegetative tissue (figure 33). These results suggest that the *PpSDH2-3* promoter behaves in a different way than the Arabidopsis one. However, the PpSDH2-3 protein may be

related to an ancient SDH2 isoform which became adapted to seed physiology during angiosperm evolution.

5.4. Yeast *sdhb* mutant complementation with Arabidopsis and Physcomitrella isoforms

SDH2-3 expression in osmoticly stressed *Physcomitrella patens* tissue and *Arabidopsis thaliana* seeds may suggest that complex II harboring this subunit should be more resistant to water deficit. In a first attempt to find differences in the properties of the three Arabidopsis and the two Physcomitrella SDH2 proteins, we try to complement yeast strains harboring a mutated SDHB gene (SDH2 homolog) that have impaired fitness in non-fermentative substrate (Steinmetz et al., 2002). Our aim was to determine if there is any difference between the three Arabidopsis and the two Physcomitrella isoforms at a functional level, subjecting for instance the different complemented yeasts to different types of stresses and determining how the SDH2 isoforms affect yeast strain fitness. Yeast SDHB mutants were transformed with the three Arabidopsis SDH2 and two Physcomitrella isoforms, but none of the constructs was able to restore normal growth in non-fermentative media (figure 35). Transcription of the SDH2 isoforms was confirmed by RT-PCR, and complementation using the yeast SDHB gene re-established normal growth, indicating that transformation and the vector were correct. One plausible explanation for the lack of complementation would be that the Arabidopsis and Physcomitrella isoforms are being misprocessed and probably not delivered to mitochondria. In order to rule out this possibility, we exchanged the Arabidopsis targeting peptide region for the one present in the yeast *SDHB* gene. The yeast targeting peptide has been experimentally

determined to be 20 amino acids long (http://www.yeastgenome.org/), while targeting peptides from Arabidopsis and Physcomitrella isoforms were determined *in silico*. The SDHB targeting peptide region used had a length of 35 aminoacids, in case the processing was dependent on the sequence-context. The exchanged regions of *AtSDH2-1* and *AtSDH2-3* covered the N-terminal sequence until the KIF and KEF aminoacids motifs, respectively (figure 36b). For a second time, no complementation was observed, suggesting that either the protein is not being properly translated or they are not able to integrate into the yeast complex II (figure 37). Future experiments should include complementation of Arabidopsis mutants with its own subunits, for instance *sdh2-3* mutant complemented with *SDH2-1* under the control of the *SDH2-3* promoter. On the other hand, *sdh2-1* and *sdh2-2* mutant lines do not show any apparent phenotype, probably due to redundancy between both genes, indicating that they are not suitable for complementation assays. Attempts in our laboratory have been made in order to produce a double *sdh2-1/sdh2-2* homozygous mutant without any success, indicating that the presence of at least one of these two genes is essential. Indeed, plants bearing one single wild-type allele from either *SDH2-1* or *SDH2-2* genes have been obtained, but not yet studied. Future studies should consider also complex purification with different isoforms in order to determine kinetics properties. More work is needed to determine functional differences between the different Arabidopsis SDH2 isoforms.

6. CONCLUSIONS

Concerning *SDH2-3* expression

- We defined a new minimal promoter region (-114 to +49) for embryo expression and found that the region encoding the 5´UTR is essential for proper transcription.
- We demonstrate that ABA, ABI3 and bZIP transcription factors regulate *SDH2-3* transcription.
- We demonstrate that *SDH2-3* promoter inactivation in vegetative tissues is not due to its methylation, and that global DNA methylation inhibition may indirectly activate *SDH2-3* expression.

Concerning SDH2-3 and complex II physiological roles

- We show that SDH2-3 containing complex II may have a role in seed development since *sdh2-3* mutant seeds have reduced weight and protein content.
- We demonstrate that SDH2-3 containing complex II is important at early steps during germination and postgerminative growth since *sdh2-3* mutants show germination delays and inhibited hypocotyl elongation in the dark.
- We show that complex II is required for postgerminative growth and seedling establishment (through the analysis of TTFA effects).
- We identified an *SDH2-3*-like gene in *Physcomitrella patens*, which is expressed during osmotic stress and may be related to the angiosperm *SDH2-3* ancestor.

7. REFERENCES

Aceituno FF, Moseyko N, Rhee SY, Gutierrez RA, 2008. The rules of gene expression in plants: organ identity and gene body methylation are key factors for regulation of gene expression in Arabidopsis thaliana. *BMC Genomics* **9**, 438.

Adams S, Vinkenoog R, Spielman M, Dickinson HG, Scott RJ, 2000. Parent-of-origin effects on seed development in Arabidopsis thaliana require DNA methylation. *Development* **127**, 2493-502.

Agarwal P, Kapoor S, Tyagi AK, 2011. Transcription factors regulating the progression of monocot and dicot seed development. *Bioessays* **33**, 189-202.

Al-Ani A, Bruzau F, Raymond P, Saint-Ges V, Leblanc JM, Pradet A, 1985. Germination, respiration, and adenylate energy charge of seeds at various oxygen partial pressures. *Plant Physiol* **79**, 885-90.

Alonso R, Onate-Sanchez L, Weltmeier F*, et al.*, 2009. A pivotal role of the basic leucine zipper transcription factor bZIP53 in the regulation of Arabidopsis seed maturation gene expression based on heterodimerization and protein complex formation. *Plant Cell* **21**, 1747-61.

Angelovici R, Fait A, Fernie AR, Galili G, 2010. A seed high-lysine trait is negatively associated with the TCA cycle and slows down Arabidopsis seed germination. *New Phytol* **189**, 148-59.

Barrero JM, Rodriguez PL, Quesada V, Piqueras P, Ponce MR, Micol JL, 2006. Both abscisic acid (ABA)-dependent and ABA-independent pathways govern the induction of NCED3, AAO3 and ABA1 in response to salt stress. *Plant Cell Environ* **29**, 2000-8.

Baud S, Dubreucq B, Miquel M, Rochat C, Lepiniec L, 2008. Storage reserve accumulation in Arabidopsis: metabolic and developmental control of seed filling. *Arabidopsis Book* **6**, e0113.

Beevers H, 1980. *The role of the glyoxylate cycle in The Biochemistry of Plants*. Academic Press.

Benamar A, Rolletschek H, Borisjuk L*, et al.*, 2008. Nitrite-nitric oxide control of mitochondrial respiration at the frontier of anoxia. *Biochim Biophys Acta* **1777**, 1268-75.

Benamar A, Tallon C, Macherel D, 2003. Membrane integrity and oxidative properties of mitochondria isolated from imbibing pea seeds after priming or accelerated ageing. *Seed Science Research* **13**, 35-45.

Bewley JD, 1997. Seed Germination and Dormancy. *Plant Cell* **9**, 1055-66.

Bradford MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54.

Bronner R, 1975. Simultaneous demonstration of lipids and starch in plant tissues. *Stain Technol* **50**, 1-4.

Busk PK, Pages M, 1998. Regulation of abscisic acid-induced transcription. *Plant Mol Biol* **37**, 425-35.

Clark SJ, Statham A, Stirzaker C, Molloy PL, Frommer M, 2006. DNA methylation: bisulphite modification and analysis. *Nat Protoc* **1**, 2353-64.

Clough SJ, Bent AF, 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* **16**, 735-43.

Cove D, 2005. The moss Physcomitrella patens. *Annu Rev Genet* **39**, 339-58.

Chandrasekharan MB, Bishop KJ, Hall TC, 2003. Module-specific regulation of the beta-phaseolin promoter during embryogenesis. *Plant J* **33**, 853-66.

Chen Y, Vaidyanathan S, 2012. A simple, reproducible and sensitive spectrophotometric method to estimate microalgal lipids. *Anal Chim Acta* **724**, 67-72.

Chia DW, Yoder TJ, Reiter WD, Gibson SI, 2000. Fumaric acid: an overlooked form of fixed carbon in Arabidopsis and other plant species. *Planta* **211**, 743-51.

Diaz I, Martinez M, Isabel-Lamoneda I, Rubio-Somoza I, Carbonero P, 2005. The DOF protein, SAD, interacts with GAMYB in plant nuclei and activates transcription of endosperm-specific genes during barley seed development. *Plant J* **42**, 652-62.

Diaz I, Vicente-Carbajosa J, Abraham Z, Martinez M, Isabel-La Moneda I, Carbonero P, 2002. The GAMYB protein from barley interacts with the DOF transcription factor BPBF and activates endosperm-specific genes during seed development. *Plant J* **29**, 453-64.

Eastmond PJ, Germain V, Lange PR, Bryce JH, Smith SM, Graham IA, 2000. Postgerminative growth and lipid catabolism in oilseeds lacking the glyoxylate cycle. *Proc Natl Acad Sci U S A* **97**, 5669-74.

Eastmond PJ, Graham IA, 2001. Re-examining the role of the glyoxylate cycle in oilseeds. *Trends Plant Sci* **6**, 72-8.

Ehlert A, Weltmeier F, Wang X*, et al.*, 2006. Two-hybrid protein-protein interaction analysis in Arabidopsis protoplasts: establishment of a heterodimerization map of group C and group S bZIP transcription factors. *Plant J* **46**, 890-900.

Elorza A, Leon G, Gomez I*, et al.*, 2004. Nuclear SDH2-1 and SDH2-2 genes, encoding the iron-sulfur subunit of mitochondrial complex II in Arabidopsis, have distinct cell-specific expression patterns and promoter activities. *Plant Physiol* **136**, 4072-87.

Elorza A, Roschzttardtz H, Gomez I*, et al.*, 2006. A nuclear gene for the iron-sulfur subunit of mitochondrial complex II is specifically expressed during Arabidopsis seed development and germination. *Plant Cell Physiol* **47**, 14-21.

Ezcurra I, Wycliffe P, Nehlin L, Ellerstrom M, Rask L, 2000. Transactivation of the Brassica napus napin promoter by ABI3 requires interaction of the conserved B2 and B3 domains of ABI3 with

different cis-elements: B2 mediates activation through an ABRE, whereas B3 interacts with an RY/G-box. *Plant J* **24**, 57-66.

Felsenstein J, 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783-91.

Figueroa P, Leon G, Elorza A, Holuigue L, Araya A, Jordana X, 2002. The four subunits of mitochondrial respiratory complex II are encoded by multiple nuclear genes and targeted to mitochondria in Arabidopsis thaliana. *Plant Mol Biol* **50**, 725-34.

Figueroa P, Leon G, Elorza A, Holuigue L, Jordana X, 2001. Three different genes encode the iron-sulfur subunit of succinate dehydrogenase in Arabidopsis thaliana. *Plant Mol Biol* **46**, 241-50.

Friml J, 2003. Auxin transport - shaping the plant. *Curr Opin Plant Biol* **6**, 7-12.

Fuentes D, 2011. *Caracterización de plantas deficientes en el complejo II mitocondrial en Arabidopsis thaliana*: Pontificia Universidad Católica de Chile, Tesis de Doctorado.

Gendreau E, Traas J, Desnos T, Grandjean O, Caboche M, Hofte H, 1997. Cellular basis of hypocotyl growth in Arabidopsis thaliana. *Plant Physiol* **114**, 295-305.

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM, 1992. Isolation of the Arabidopsis ABI3 gene by positional cloning. *Plant Cell* **4**, 1251-61.

Goffman FD, Alonso AP, Schwender J, Shachar-Hill Y, Ohlrogge JB, 2005. Light enables a very high efficiency of carbon storage in developing embryos of rapeseed. *Plant Physiol* **138**, 2269-79.

Goldberg RB, De Paiva G, Yadegari R, 1994. Plant Embryogenesis: Zygote to Seed. *Science* **266**, 605-14.

Hayashi M, Toriyama K, Kondo M, Nishimura M, 1998. 2,4-Dichlorophenoxybutyric acid-resistant mutants of Arabidopsis have defects in glyoxysomal fatty acid beta-oxidation. *Plant Cell* **10**, 183-95.

Higo K, Ugawa Y, Iwamoto M, Korenaga T, 1999. Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res* **27**, 297-300.

Hughes DW, Galau GA, 1989. Temporally modular gene expression during cotyledon development. *Genes Dev* **3**, 358-69.

Jefferson RA, Kavanagh TA, Bevan MW, 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* **6**, 3901-7.

Kirby J, Kavanagh TA, 2002. NAN fusions: a synthetic sialidase reporter gene as a sensitive and versatile partner for GUS. *Plant J* **32**, 391-400.

Kroemer K, Reski R, Frank W, 2004. Abiotic stress response in the moss Physcomitrella patens: evidence for an evolutionary alteration in signaling pathways in land plants. *Plant Cell Rep* **22**, 864-70.

Kroj T, Savino G, Valon C, Giraudat J, Parcy F, 2003. Regulation of storage protein gene expression in Arabidopsis. *Development* **130**, 6065-73.

Kubis SE, Pike MJ, Everett CJ, Hill LM, Rawsthorne S, 2004. The import of phosphoenolpyruvate by plastids from developing embryos of oilseed rape, Brassica napus (L.), and its potential as a substrate for fatty acid synthesis. *J Exp Bot* **55**, 1455-62.

Lara P, Onate-Sanchez L, Abraham Z*, et al.*, 2003. Synergistic activation of seed storage protein gene expression in Arabidopsis by ABI3 and two bZIPs related to OPAQUE2. *J Biol Chem* **278**, 21003-11.

Lemire BD, Oyedotun KS, 2002. The Saccharomyces cerevisiae mitochondrial succinate:ubiquinone oxidoreductase. *Biochim Biophys Acta* **1553**, 102-16.

Leon G, Holuigue L, Jordana X, 2007. Mitochondrial complex II Is essential for gametophyte development in Arabidopsis. *Plant Physiol* **143**, 1534-46.

Leung J, Giraudat J, 1998. Abscisic Acid Signal Transduction. *Annu Rev Plant Physiol Plant Mol Biol* **49**, 199-222.

Lin Y, Ulanov AV, Lozovaya V*, et al.*, 2006. Genetic and transgenic perturbations of carbon reserve production in Arabidopsis seeds reveal metabolic interactions of biochemical pathways. *Planta* **225**, 153-64.

Logan DC, Millar AH, Sweetlove LJ, Hill SA, Leaver CJ, 2001. Mitochondrial biogenesis during germination in maize embryos. *Plant Physiol* **125**, 662-72.

Luerssen H, Kirik V, Herrmann P, Misera S, 1998. FUSCA3 encodes a protein with a conserved VP1/AB13-like B3 domain which is of functional importance for the regulation of seed maturation in Arabidopsis thaliana. *Plant J* **15**, 755-64.

Marella HH, Sakata Y, Quatrano RS, 2006. Characterization and functional analysis of ABSCISIC ACID INSENSITIVE3-like genes from Physcomitrella patens. *Plant J* **46**, 1032-44.

Marzabal P, Gas E, Fontanet P, Vicente-Carbajosa J, Torrent M, Ludevid MD, 2008. The maize Dof protein PBF activates transcription of gamma-zein during maize seed development. *Plant Mol Biol* **67**, 441-54.

Mayer U, Torres-Ruiz RA, Berleth T, Miseera S, Juurgens G, 1991. Mutations affecting body organization in the Arabidopsis embryo. *Nature* **353**, 402-7.

Millar AH, Eubel H, Jansch L, Kruft V, Heazlewood JL, Braun HP, 2004. Mitochondrial cytochrome c oxidase and succinate dehydrogenase complexes contain plant specific subunits. *Plant Mol Biol* **56**, 77-90.

Molina C, Grotewold E, 2005. Genome wide analysis of Arabidopsis core promoters. *BMC Genomics* **6**, 25.

Nambara E, Marion-Poll A, 2003. ABA action and interactions in seeds. *Trends Plant Sci* **8**, 213-7.

Nap JP, Keizer LCP, Jansen RC, 1993. First-generation transgenic plants and statistics. *Plant Molecular Biology Reports* **11**, 156-64.

Nishiyama T, Hiwatashi Y, Sakakibara I, Kato M, Hasebe M, 2000. Tagged mutagenesis and gene-trap in the moss, Physcomitrella patens by shuttle mutagenesis. *DNA Res* **7**, 9-17.

O´Brien M, 1981. *The Study of Plant Structure: Principles and Selected Methods*. Termarcarphi Pty. Ltd., Melbourne Australia.

Onate-Sanchez L, Vicente-Carbajosa J, 2008. DNA-free RNA isolation protocols for Arabidopsis thaliana, including seeds and siliques. *BMC Res Notes* **1**, 93.

Parcy F, Valon C, Raynal M, Gaubier-Comella P, Delseny M, Giraudat J, 1994. Regulation of gene expression programs during Arabidopsis seed development: roles of the ABI3 locus and of endogenous abscisic acid. *Plant Cell* **6**, 1567-82.

Penfield S, Graham S, Graham IA, 2005. Storage reserve mobilization in germinating oilseeds: Arabidopsis as a model system. *Biochem Soc Trans* **33**, 380-3.

Prigge MJ, Bezanilla M, 2010. Evolutionary crossroads in developmental biology: Physcomitrella patens. *Development* **137**, 3535-43.

Raz V, Bergervoet JH, Koornneef M, 2001. Sequential steps for developmental arrest in Arabidopsis seeds. *Development* **128**, 243-52.

Rohde A, Kurup S, Holdswoth M, 2000. ABI3 emerges from the seed. *Trends Plant Sci* **5**, 418-9.

Roos MH, Tielens AG, 1994. Differential expression of two succinate dehydrogenase subunit-B genes and a transition in energy metabolism during the development of the parasitic nematode Haemonchus contortus. *Mol Biochem Parasitol* **66**, 273-81.

Roschzttardtz H, 2007. *Caracterización de la expresión del gen SDH2-3 en el desarrollo de semillas de Arabidopsis thaliana*: Pontificia Universidad Católica de Chile, Tesis de Doctorado.

Roschzttardtz H, Fuentes I, Vasquez M*, et al.*, 2009. A nuclear gene encoding the iron-sulfur subunit of mitochondrial complex II is regulated by B3 domain transcription factors during seed development in Arabidopsis. *Plant Physiol* **150**, 84-95.

Ruuska SA, Girke T, Benning C, Ohlrogge JB, 2002. Contrapuntal networks of gene expression during Arabidopsis seed filling. *Plant Cell* **14**, 1191-206.

Saitou N, Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406-25.

Santos-Mendoza M, Dubreucq B, Baud S, Parcy F, Caboche M, Lepiniec L, 2008. Deciphering gene regulatory networks that control seed development and maturation in Arabidopsis. *Plant J* **54**, 608-20.

Schaefer DG, Zryd JP, 2001. The moss Physcomitrella patens, now and then. *Plant Physiol* **127**, 1430-8.

Schmidt RJ, Ketudat M, Aukerman MJ, Hoschek G, 1992. Opaque-2 is a transcriptional activator that recognizes a specific target site in 22-kD zein genes. *Plant Cell* **4**, 689-700.

Schruff MC, Spielman M, Tiwari S, Adams S, Fenby N, Scott RJ, 2006. The AUXIN RESPONSE FACTOR 2 gene of Arabidopsis links auxin signalling, cell division, and the size of seeds and other organs. *Development* **133**, 251-61.

Schwartz SH, Leon-Kloosterziel KM, Koornneef M, Zeevaart JA, 1997. Biochemical characterization of the aba2 and aba3 mutants in Arabidopsis thaliana. *Plant Physiol* **114**, 161-6.

Schwender J, Ohlrogge JB, 2002. Probing in vivo metabolism by stable isotope labeling of storage lipids and proteins in developing Brassica napus embryos. *Plant Physiol* **130**, 347-61.

Schwender J, Shachar-Hill Y, Ohlrogge JB, 2006. Mitochondrial metabolism in developing embryos of Brassica napus. *J Biol Chem* **281**, 34040-7.

Shen Q, Zhang P, Ho TH, 1996. Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. *Plant Cell* **8**, 1107-19.

Shinozaki K, Yamaguchi-Shinozaki K, 2000. Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Curr Opin Plant Biol* **3**, 217-23.

Steinmetz LM, Scharfe C, Deutschbauer AM*, et al.*, 2002. Systematic screen for human disease genes in yeast. *Nat Genet* **31**, 400-4.

Stone SL, Kwong LW, Yee KM*, et al.*, 2001. LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development. *Proc Natl Acad Sci U S A* **98**, 11806-11.

Suzuki M, Kao CY, Mccarty DR, 1997. The conserved B3 domain of VIVIPAROUS1 has a cooperative DNA binding activity. *Plant Cell* **9**, 799-807.

Suzuki M, Mccarty DR, 2008. Functional symmetry of the B3 network controlling seed development. *Curr Opin Plant Biol* **11**, 548-53.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731-9.

Vandesompele J, De Preter K, Pattyn F*, et al.*, 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, RESEARCH0034.

Vicente-Carbajosa J, Carbonero P, 2005. Seed maturation: developing an intrusive phase to accomplish a quiescent state. *Int J Dev Biol* **49**, 645-51.

Weigelt K, Kuster H, Radchuk R*, et al.*, 2008. Increasing amino acid supply in pea embryos reveals specific interactions of N and C metabolism, and highlights the importance of mitochondrial metabolism. *Plant J* **55**, 909-26.

Weltmeier F, Ehlert A, Mayer CS*, et al.*, 2006. Combinatorial control of Arabidopsis proline dehydrogenase transcription by specific heterodimerisation of bZIP transcription factors. *EMBO J* **25**, 3133-43.

White JA, Todd J, Newman T*, et al.*, 2000. A new set of Arabidopsis expressed sequence tags from developing seeds. The metabolic pathway from carbohydrates to seed oil. *Plant Physiol* **124**, 1582-94.

Wolstenholme DR, Fauron CM, 1995. *Mitochondrial genome organization*. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Xiao W, Custard KD, Brown RC*, et al.*, 2006. DNA methylation is critical for Arabidopsis embryogenesis and seed viability. *Plant Cell* **18**, 805-14.

Yamamoto YY, Yoshitsugu T, Sakurai T, Seki M, Shinozaki K, Obokata J, 2009. Heterogeneity of Arabidopsis core promoters revealed by high-density TSS analysis. *Plant J* **60**, 350-62.

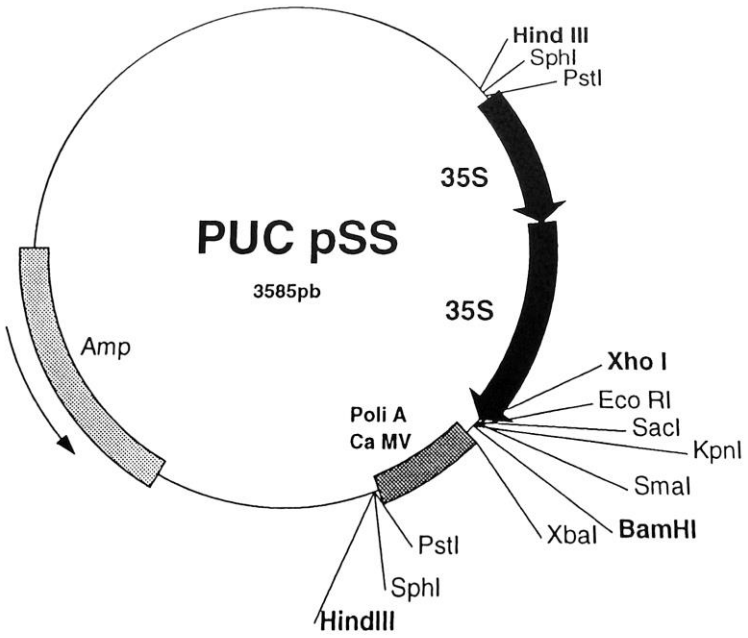
Yankovskaya V, Horsefield R, Tornroth S*, et al.*, 2003. Architecture of succinate dehydrogenase and reactive oxygen species generation. *Science* **299**, 700-4.

Yoo SD, Cho YH, Sheen J, 2007. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc* **2**, 1565-72.

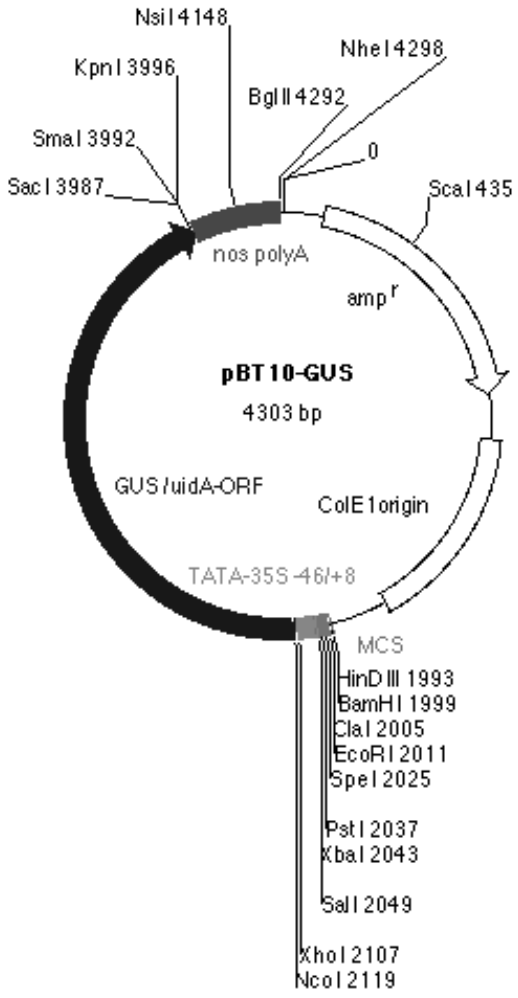
Zhang X, Yazaki J, Sundaresan A*, et al.*, 2006. Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis. *Cell* **126**, 1189-201.

Zuckerkandl E, Pauling L, 1965. Evolutionary divergence and convergence in proteins. In. *Evolving Genes and Proteins.* Academic Press, New York., 97-166. (Bryson V, Vogel HJ, eds.)

8. APPENDIX



Appendix 1. PUC pSS vector used for effector expression in transient assays (Figure 14).



Appendix 2. pBT10-GUS vector used for GUS expression under the control of the *SDH2-3* or *2S1* promoter for expression in transient assays (Figure 14).