A Nuclear Gene for the Iron–Sulfur Subunit of Mitochondrial Complex II is Specifically Expressed During Arabidopsis Seed Development and Germination

Alvaro Elorza 1,2,3, Hanneliz Roschütztardtz 1, Isabel Gómez 1, Armand Mouras 2, Loreto Holugue 1, Alejandro Araya 2 and Xavier Jordana 1,*

1 Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, P. Universidad Católica de Chile, Casilla 114-D, Santiago, Chile
2 UMR 0619-Physiologie et Biotechnologie Végétales, Institut National de la Recherche Agronomique et Université Victor Segalen-Bordeaux II, IBVM CR-INRA Bordeaux, BP 81, 33883 Villenave d’Ornon Cedex, France
3 Laboratoire de Réplication et Expression des Gènes Eucaryotes et Rétroviraux, UMR 5097, CNRS et Université Victor Segalen-Bordeaux II, 33076 Bordeaux-Cedex, France

Three nuclear genes, SDH2-1, SDH2-2 and SDH2-3, encode the essential iron–sulfur subunit of mitochondrial complex II in Arabidopsis thaliana. SDH2-1 and SDH2-2 probably arose via a recent duplication event and we reported that both are expressed in all organs from adult plants. In contrast, transcripts from SDH2-3 were not detected. Here we present data demonstrating that SDH2-3 is specifically expressed during seed development. SDH2-3 transcripts appear during seed maturation, persist through desiccation, are abundant in dry seeds and markedly decline during germination. Analysis of transgenic Arabidopsis plants carrying the SDH2-3 promoter fused to the β-glucuronidase reporter gene shows that the SDH2-3 promoter is activated in the embryo during maturation, from the bent-cotyledon stage. β-Glucuronidase expression correlates with the appearance of endogenous SDH2-3 transcripts, suggesting that control of this nuclear gene is achieved through transcriptional regulation. Furthermore, progressive deletions of this promoter identified a 159 bp region (–223 to –65) important for SDH2-3 transcriptional activation in seeds. Interestingly, the SDH2-3 promoter remains active in embryonic tissues during germination and post-germinative growth, and is turned off in vegetative tissues (true leaves). In contrast to SDH2-3 transcripts, SDH2-1 and SDH2-2 transcripts are barely detected in dry seeds and increase during germination and post-germinative growth. The opposite expression patterns of SDH2 nuclear genes strongly suggest that during germination the embryo-specific SDH2-3 is replaced by SDH2-1 or SDH2-2 in mitochondrial complex II.

Keywords: Arabidopsis thaliana – Iron–sulfur subunit — Plant mitochondria — Seed-specific expression — Succinate dehydrogenase — Transcriptional regulation.

Abbreviations: ABRE, abscisic acid-responsive element; DAF, days after flowering; dpi, days post-inhibition; GUS, β-glucuronidase; LEA, late embryogenesis abundant; MUG, 4-methylumbelliferyl-β-D-glucuronide; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR; SDH, succinate dehydrogenase; UTR, untranslated region

Introduction

Succinate:ubiquinone oxidoreductase [succinate dehydrogenase (SDH), EC 1.3.5.1], commonly referred to as complex II, catalyzes the oxidation of succinate to fumarate and the reduction of ubiquinone to ubiquinol. This mitochondrial membrane-associated complex is a functional part of both the citric acid cycle and the aerobic respiratory chain, and has been well characterized in bacteria and heterotrophic eukaryotes (Lemire and Oyedotun 2002, Yankovskaya et al. 2003). In these organisms, complex II contains only four polypeptides: two peripheral membrane proteins, a flavoprotein (SDH1) and an iron–sulfur protein (SDH2), and two small integral membrane proteins (SDH3 and SDH4). The succinate-binding site is formed by the SDH1 polypeptide, which is covalently linked to a FAD molecule acting as acceptor of a hydride ion at an early step of succinate oxidation. This flavoprotein interacts with the SDH2 subunit, which transfers the electrons to the membrane through its three non-heme iron–sulfur centers. SDH3 and SDH4 anchor the SDH1–SDH2 subcomplex to the matrix side of the inner membrane and contain a b-type heme and the ubiquinone-binding site (Yankovskaya et al. 2003). Interestingly, it has been shown recently that plant complex II may contain additional subunits of unknown function (Eubel et al. 2003, Millar et al. 2004).

Complex II subunits are all encoded in the nuclear genome in Arabidopsis thaliana, just like complex II from heterotrophic eukaryotes (Scheffler 1998, Figueroa et al. 2001, Figueroa et al. 2002, Millar et al. 2004). Moreover, several of the complex II subunits are encoded by more than one gene in Arabidopsis. For instance, we have described that three nuclear genes, designated SDH2-1 (At3g27380), SDH2-2 (At5g40650)
and SDH2-3 (At5g65165), encode the iron–sulfur subunit in *A. thaliana* (Figueroa et al. 2001). All three proteins are probably functional as complex II subunits since they are highly conserved compared with their homologs in other organisms. Moreover, they contain the cysteine motifs involved in binding the three iron–sulfur clusters essential for electron transport and they are actively imported into isolated plant mitochondria (Figueroa et al. 2001).

The presence of three SDH2 genes in Arabidopsis is intriguing and raises interesting questions about their origin and function. It is worth mentioning that Roos and Tielens (1994) described two differentially expressed SDH2 genes in the larval and adult stages of the sheep nematode *Haemonchus contortus*, a fact that may be related to a switch in energy metabolism during development. To our knowledge, this is the only report describing more than one SDH2 gene in a eukaryotic organism. Several observations have suggested that Arabidopsis SDH2-1 and SDH2-2 genes arose via a relatively recent duplication event, and that separation with SDH2-3 would be more ancient (Figueroa et al. 2001). First, SDH2-1 and SDH2-2 have similar exon–intron structures and encode nearly identical proteins (96% identity), whereas SDH2-3 has a completely different exon–intron structure and encodes a protein only 67% similar to SDH2-1 and SDH2-2. Secondly, whereas the N-terminal mitochondrial targeting sequences of SDH2-1 and SDH2-2 are similar, a completely different one is attached to SDH2-3.

To gain insight into the regulation of nuclear genes encoding mitochondrial proteins and the role of multiple SDH2 genes in complex II biogenesis, we undertook a detailed analysis of SDH2 expression during plant development. Recently we found that SDH2-1 and SDH2-2 are expressed in all organs from adult plants, with the highest mRNA levels in reproductive organs (Elorza et al. 2004). In contrast, we were unable to detect SDH2-3 expression, raising questions about its role.

Seed germination and post-germinative growth are crucial for seedling establishment. Germination begins with imbibition, terminates with radicle emergence and is followed shortly after by the mobilization of seed nutrient reserves which provide essential energy and carbon skeletons to support growth until the seedling becomes photoautotrophic (Bewley 1997, Bewley et al. 2000). Several studies have implicated mitochondrial activity and mitochondrial biogenesis in these processes (Ehrenshaft and Brambl 1990, Logan et al. 2001 and references therein). Furthermore, a specific role for the non-decarboxylative portion of the citric acid cycle, comprising the steps catalyzed by SDH and fumarase, has been proposed in oilseeds for the pathway converting storage lipids into soluble carbohydrates (Falk et al. 1998, Oliver et al. 1998, Eastmond and Graham 2001). These data led us to analyze SDH2 gene expression during germination and post-germinative growth. Unexpectedly, we found high levels of SDH2-3 transcripts in dry seeds, prompting us to characterize SDH2-3 expression during seed development. Here, we show that SDH2-3 is specifically expressed in the embryo during the maturation phase of seed development. SDH2-3 transcripts are abundant in dry seeds and decline during germination. Furthermore, transgenic Arabidopsis plants carrying SDH2-3 promoter fusions to the GUS reporter gene allowed us to show that SDH2-3 expression is regulated at the transcriptional level during seed development, and to define a promoter region involved in this tissue-specific expression.

**Results**

**Expression of SDH2 genes during germination**

In Arabidopsis, germination (radicle emergence) takes place within 24 h of imbibition under optimal conditions. This is followed by a period of 2 d of heterotrophic growth, which is supported by seed storage reserves. By the end of this period, the transition to photoautotrophic growth has occurred (Eastmond and Graham 2001). Since mitochondria are expected to play a central role during reserve mobilization, we studied SDH2 expression during germination and post-germinative growth in the dark, to avoid carbon and energy sources for seedling growth being switched to mainly photosynthetic reactions. We also analyzed the expression of the fumarase gene (At2g47510, accession No. AF020303), since both SDH and fumarase activities are essential for carbohydrate synthesis from lipid (Eastmond and Graham 2001).

RNA was isolated from Arabidopsis and *Brassica napus* seeds and seedlings at different times after imbibition, and the expression of SDH2 and fumarase genes was examined using Northern analysis. Hybridization to Arabidopsis SDH2-1, SDH2-2 and SDH2-3 mRNAs was performed with genespecific probes generated from the 3′ untranslated regions (UTRs) (Fig. 1A). Since Brassica SDH2 genes have not been characterized, blots were hybridized with probes derived from Arabidopsis SDH2-2 and SDH2-3 coding regions (Fig. 1B). Fumarase transcripts were detected with probes from the coding region of either the Arabidopsis (Fig. 1A) or the Brassica (Fig. 1B) genes.

In both species, the steady-state abundance of fumarase, SDH2-1 and SDH2-2 transcripts was very low or undetectable in seeds and increased significantly during germination and post-germinative growth. Unexpectedly, SDH2-3 transcript levels were high in seeds and markedly declined during germination and post-germinative growth. It is worth mentioning that it was the first time we were able to detect significant levels of SDH2-3 expression. In previous studies, SDH2-3 mRNA had been revealed only in reverse transcription–PCR (RT–PCR) and rapid amplification of 3′ cDNA ends (3′ RACE) experiments (Figueroa et al. 2001, Elorza et al. 2004), and data on SDH2-3 expression are very scarce in the existing large expression databases. Either SDH2-3 probes are absent, for instance in the Affymetrix chips (see at http://www.genevestigator.ethz.ch, Zimmermann et al. 2004), or seeds have not been analyzed and expression is barely detected (e.g. by massively parallel
Mitochondrial complex II gene expression in seeds

These results suggested that SDH2-3 is specifically expressed during seed development and prompted us to analyze its expression during this developmental phase.

Expression of the SDH2-3 gene during silique development

Seed development is a key process in the life cycle of higher plants. In Arabidopsis, it can be roughly divided into three stages (Baud et al. 2002). Morphogenesis begins with fertilization, and terminates with a bent-cotyledon embryo having acquired the basic architecture of the plant. During maturation, the second phase of development, embryo cells go through a period of cellular expansion and accumulate storage proteins, lipids and carbohydrates in their cotyledons. In the last stage (late maturation), an acute loss of water takes place in the seed and the embryo becomes metabolically quiescent (dormant) and tolerant to desiccation.

SDH2-3 transcripts could not be detected in flowers, confirming previous results (Figueroa et al. 2001, Elorza et al. 2004). Furthermore, no significant SDH2-3 expression was observed in siliques at early developmental stages, corresponding to the morphogenesis phase of embryo development (Fig. 2). Expression occurs in long green siliques, where most embryos have completed morphogenesis and entered the maturation phase (Fig. 2, lanes 4 and 5). Transcript levels persist through desiccation (lanes 5 and 6), and dry seeds contain abundant SDH2-3 mRNA (lane 7), confirming the results shown in Fig. 1. The signal obtained for RNA extracted from dry seeds is stronger than that for RNA from whole siliques, suggesting that transcripts detected in siliques were in fact derived from seeds.

The SDH2-3 promoter is activated in the embryo during maturation

We have previously fused 1,603 bp of the SDH2-3 promoter to the β-glucuronidase (GUS) reporter gene (construct P5), and found that GUS is not expressed in flowers, leaves, stems and roots of adult plants (Elorza et al. 2004). In view of the results described in the previous sections, we used this con-
Mitochondrial complex II gene expression in seeds

struct to unveil the mechanism of regulation of SDH2-3 expression during silique development. GUS activity was initially quantified in protein extracts from transgenic T2 seeds, and was found to be high in eight out of 12 independent transformed lines (results not shown). Then homozygous plants were obtained for several transgenic lines. Protein extracts were prepared in triplicate from samples equivalent to those employed in Fig. 2, i.e. recently opened flowers and siliques at different developmental stages. GUS activity was assayed in these extracts, and a representative result is shown in Fig. 3A.

GUS activity was low in protein extracts from flowers and siliques at early developmental stages, corresponding to the embryo morphogenesis phase (Fig. 3A, samples 1–3). GUS expression was induced concomitantly with embryo maturation (samples 4 and 5), and was maintained through desiccation (samples 5 and 6). This activity profile mimics the expression pattern of the endogenous SDH2-3 transcript (Fig. 2).

Results of GUS staining in transgenic flowers and siliques carrying the P5 SDH2-3 construct are shown in Fig. 3B. No GUS staining was observed in flowers (sample 1), as previously described (Elorza et al. 2004). Moreover, young siliques also lack GUS activity (samples 2 and 3). In agreement with quantitative GUS assays, GUS expression was detected in sample 4 and increased as siliques enlarged and became yellow. In addition, this histochemical analysis clearly showed that GUS is specifically expressed in seeds. Finally, embryos were dissected out from developing seeds and then stained for GUS activity. GUS expression was not detected in torpedo embryos (not shown), was induced at the bent-cotyledon stage, during the maturation phase of seed development, and increased as maturation proceeded (Fig. 3C). Staining appeared first in embryo cotyledons and then extended to the other embryo tissues.

Analysis of the SDH2-3 promoter

Arabidopsis transgenic plants carrying progressive deletions of the SDH2-3 promoter fused to the GUS reporter gene have been obtained (Elorza et al. 2004). In this previous work, however, only adult plant tissues and 13-day-old seedlings were stained for GUS activity. To investigate regulatory control regions involved in seed SDH2-3 expression, here we determined GUS activity in mature dry seeds from these transgenic plants. For each construct, GUS activity in seeds was quantified in duplicate seed extracts from 7–9 independent transformed lines. Results are shown in Fig. 4A.

Deletion of the promoter to –223 (P3 construct) did not significantly affect GUS activity. Interestingly, removal of the region between –223 and –65 (P2 construct) drastically reduced GUS expression, revealing the presence of important cis-elements involved in SDH2-3 transcriptional activation in seeds.

The sequence from –230 to the SDH2-3 ATG initiation codon is presented in Fig. 4B. Identification of transcription initiation sites was performed on seed RNA by 5’ RACE, and the major 5’ end was found to map 47–49 nucleotides upstream.
Mitochondrial complex II gene expression in seeds

The 159 bp promoter region (–223/–65) defined as important for expression in seeds contain motifs putatively involved in this organ-specific expression. In silico analysis of this sequence revealed the presence of two potential abscisic acid-responsive elements (ABREs, underlined in Fig. 4B), characterized by the consensus sequence YACGTGGC and the core ACGT (Busk and Pagès 1998, Leung and Giraudat 1998). Moreover, these putative elements are separated by a short 6 bp sequence and may act in concert. An additional putative ABRE motif is present in the P2 construct (at –62, underlined in Fig. 4B), and an RY-like enhancer element, which might be also involved in seed gene expression, is located eight nucleotides downstream (double underlined in Fig. 4B).

The SDH2-3 promoter is inactivated in vegetative tissues

To determine whether SDH2-3 expression is also regulated at the transcriptional level during germination, seeds from
three homozygous transgenic lines harboring the P5 construct were germinated in the dark for 4 d. Protein extracts were prepared and GUS activity was measured. Similar results were obtained for the three lines, and those for one of them are shown in Fig. 5A. GUS activity remained fairly constant between 0 and 4 d after imbibition, indicating that the SDH2-3 promoter is still active in embryonic tissues during germination and post-germinative growth.

Since the SDH2-3 promoter is not active in vegetative tissues and flowers from adult plants (Elorza et al. 2004 and Fig. 3B), we investigated when it is inactivated. Four- and 8-day-old seedlings were analyzed via histochemical GUS staining (Fig. 5B). In 4-day-old seedlings, GUS activity appeared strong in cotyledons and hypocotyl, confirming the quantitative data. In 8-day-old seedlings, decreased GUS activity was seen in cotyledons and hypocotyl, and, most importantly, no GUS staining was observed in first true new leaves. This result clearly showed that the SDH2-3 promoter is turned off in vegetative tissues.

**Discussion**

Considering that in most organisms there is a single SDH2 gene, as expected for a supposedly housekeeping gene with a well-defined function, the presence of three genes in Arabidopsis raises questions about their roles during plant development and led us to undertake a detailed analysis of their expression. We recently reported that SDH2-1 and SDH2-2 genes, which have similar structures and encode nearly identical proteins, have similar expression patterns (Elorza et al. 2004). These results are consistent with the fact that SDH2-1 and SDH2-2 arose via a relatively recent duplication event and are probably redundant (Elorza et al. 2004).

Here we have found a highly tissue-specific expression pattern for the SDH2-3 gene. This gene, which is structurally different and would have diverged for a long time from SDH2-1 and SDH2-2, is highly expressed in the embryo during seed development. Altogether, our results suggest that, after its emergence from a common ancestor, SDH2-3 differentiated to acquire a specific role in seed maturation and/or early germination.

**SDH2-3 is expressed in the embryo during seed development**

SDH2-3 transcripts accumulate during the maturation phase of seed development and remain high during the desiccation phase and in dry seeds (Fig. 2). Parcy et al. (1994) monitored the expression pattern of multiple marker mRNAs during Arabidopsis seed development and identified classes of coordinately expressed mRNAs. The seed-specific expression of SDH2-3 described here overlaps with that of genes encoding abundant seed storage proteins (e.g. A2S3, CRC and PAP85) and late-embryogenesis abundant (LEA) proteins. The expression of these proteins is under tight developmental control, since they are not expressed in vegetative tissues and their expression is restricted to the maturation and/or desiccation phases of embryogenesis, as is the case for SDH2-3.

To gain insight into the mechanism of SDH2-3 gene regulation during silique development, its promoter was fused to the GUS reporter gene and analyzed in transgenic plants (Fig. 3). The correlation between SDH2-3 mRNA appearance (Fig. 2) and GUS expression (Fig. 3) during embryo maturation suggests that control of this gene is achieved through transcriptional regulation. Furthermore, histochemical GUS staining revealed that SDH2-3 is specifically expressed in the embryo from the bent-cotyledon to dry seed stages. Progressive deletions of the promoter defined a promoter region (–223 to –65) important for high expression in seeds (Fig. 4). Two potential ABRE elements, separated by 6 bp and which may act in concert, are present in this region and might represent seed-specific elements (Fig. 4B).

ABRE elements have been implicated in seed storage and LEA protein gene regulation (Busk and Pagès 1998). ABRE functions effectively when two copies are located in tandem, or when it is associated with a coupling or enhancer element (Nambara and Marion-Poll 2003). One kind of element is the RY motif, which acts as an enhancer for seed-specific transcription. Consistently, genome-wide profiling of Arabidopsis seed transcripts revealed that ABREs are over-represented in the promoters of highly expressed genes (Nakabayashi et al. 2005). Moreover, multiple copies of ABRE or the presence of the RY motif in combination with ABRE were associated with particularly high expression. In this context, it has to be pointed out that an additional putative ABRE element and an RY-like motif are located downstream from –64 (P2 construct, Fig. 4B). Our results clearly demonstrate that these two elements present in P2 are not sufficient for high SDH2-3 expression in seeds, but they do not exclude the possibility that they are necessary. Further work will be needed to ascertain the functional role of these or other sequences.

**SDH2-3 expression decline during germination**

The steady-state abundance of SDH2-3 transcripts is high in seeds and markedly declines during germination and post-germinative growth (Fig. 1A). In contrast, SDH2-1, SDH2-2 and fumarase transcript levels are barely detected in dry seeds and then increase. These opposite expression patterns suggest that during germination and post-germinative growth a SDH2-3-containing complex II is replaced by a complex II containing SDH2-1 or SDH2-2. It is likely that transcript level decreases observed for SDH2-1, SDH2-2 and fumarase at 2 d post-imbibition were due to the fact that transition to photo-autotrophic growth does not occur in the dark.

Similar results were obtained in *B. napus*, leading to the important conclusion that the presence of two types of iron–sulfur subunit (SDH2-1/2-2 and SDH2-3 types) is not specific to Arabidopsis (Fig. 1B). It is worth mentioning that a SDH2-1/2-2-like gene has been described previously in maize and rice (Figueroa et al. 1999, Kubo et al. 1999), and that database
searches have allowed us to identify a SDH2-3 homolog in several monocots (unpublished results). Thus, the presence of two types of SDH2 genes appears to be conserved among angiosperms.

The expression profiles of SDH2-1, SDH2-2 and fumarase genes mirror those of the genes involved in fatty acid β-oxidation or the glyoxylate cycle (Ryllot et al. 2001). These results suggest that the increased expression of SDH2-1 and SDH2-2 is part of an overall metabolic program that enables the mobilization of storage reserves, and argue against a role for SDH-2-3 in lipid mobilization and conversion to soluble carbohydrates during the post-germinative period of seedling growth.

The imbibing seed resumes metabolic activity within minutes of water entering its cells, and rapid increases in respiration rate accompany the earliest stages of germination (Bewley and Black 1994). For instance, Logan et al. (2001) studied mitochondrial biogenesis during maize embryo germination, and showed that succinate-dependent O2 consumption and citric acid cycle activity were low in embryos from dry seeds, but increased rapidly during imbibition. Resumption of protein synthesis is another early event during imbibition, and utilizes mRNAs conserved in the dry seed (Rajjou et al. 2004). Therefore, it is tempting to speculate that SDH2-3 transcripts accumulate in the dry seed to allow for their rapid translation at early germination, before SDH2-1 and SDH2-2 are expressed.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) plants were grown hydroponically at 22°C, under a 16 h light/8 h dark cycle (Gibeaut et al. 1997). Staging of developing siliques (Fig. 2, 3) was performed according to their morphology. Besides recently opened flowers and dry siliques, five samples were collected: short siliques (<5 mm long, 1–3 d after flowering (DAF)), thin green siliques (5–10 mm long, 4–5 DAF), long and thick green siliques (10–14 mm long, ~2 mm thick, 6–9 DAF), siliques with yellow tips (10–11 DAF) and yellow siliques (12–14 DAF).

For germination experiments, seeds were surface sterilized with a solution of 10% (v/v) commercial bleach for 10 min, rinsed thoroughly and plated on sterile distilled water-soaked filter papers. Germination was scored 10 mm long, 4–5 DAF), long and thick green siliques (10–14 mm long, ~2 mm thick, 6–9 DAF), siliques with yellow tips (10–11 DAF) and yellow siliques (12–14 DAF).

For germination experiments, seeds were surface sterilized with a solution of 10% (v/v) commercial bleach for 10 min, rinsed thoroughly and plated on sterile distilled water-soaked filter papers. Germination and seedling growth were carried out in the dark. Brassica napus seeds were incubated at 22–24°C, and Arabidopsis seeds were first cold treated at 4°C for 48 h and then incubated at 22–24°C.

RNA preparation and analysis

Tissues were harvested into liquid nitrogen. Total RNA was isolated by the method of Vicient and Delensy (1999) and further purified using the RNeasy Plant mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s protocol.

Northern analyses were performed by standard procedures, with 32P-labeled probes (Ausubel et al. 1994). To obtain gene-specific probes to analyze Arabidopsis transcripts (Fig. 1A), DNA fragments from the 3′ UTRs were PCR amplified using the following primer pairs: for SDH2-1, 5′ AACAGATTCACTACAAGAAGC 3′ and 5′ TAGTTTAAATTTACTCAAGTTT 3′; for SDH2-2, 5′ AACAGATCACACACATCAAGC 3′ and 5′ AGCAGAAGTTTTATGCTTTGTGTTGTT 3′; and for SDH2-3, 5′ GAGAGGCTACAGGCAATAACTGAG 3′ and 5′ AGTGGGATGATTGACCTTTG 3′. To analyze Brassica SDH2 transcripts (Fig. 1B), probes were derived from Arabidopsis coding sequences. The following primer pairs were used: for Arabidopsis SDH2-2 coding region, 5′ CTAGATGCTTGATTAAAGATG 3′ and 5′ AAATCATAGTAACTGCTT 3′; and for Arabidopsis SDH2-3 coding region, 5′ AGTGCAGCTGCTTTGCCCTGTGTTT 3′ and 5′ AGACACCTTCTTGTTCTCAACTACGGA 3′. The fumarase probes were obtained by RT–PCR, using total RNA from either Arabidopsis flowers or B. napus seedlings, and the primers 5′ AAGCCTTGCTGCTGACACAG 3′ and 5′ ATCAATCGGGGACCACATC 3′. The PCR products were cloned into a pGem-T plasmid (Promega, Madison, WI, USA).

RT–PCR multiplex experiments were carried out as previously described (Erolza et al. 2004). 5′ RACE experiments were performed according to the supplier’s protocol (Invitrogen, Carlsbad, CA, USA). Three nested oligonucleotides were used: 5′ TTTCCTTTGCTTGATGAATGCTTGGGATT 3′, 5′ TTGGAAAGCTTCTGACCGCT 3′ and 5′ CGTTTAGATCCATATAAGGATTTGA 3′. PCR products were cloned into the pGem-T Easy plasmid and several clones were sequenced to identify transcription initiation sites.

GUS assays

Soluble extracts of plant tissues were assayed for GUS activity by fluorometric measurements, using 4-methylumbelliferyl-β-D-glucuronide (MUG, Sigma, St. Louis, MO, USA) as substrate (Jefferson 1987). Protein concentrations were determined by the Bradford method (Bradford 1976), and GUS activities were calculated as nmol MU h−1 mg−1 of protein.

Histochimical GUS staining was performed as described (Erolza et al. 2004). Samples were incubated at 37°C overnight into GUS buffer [100 mM sodium phosphate buffer, pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium lauryl sarcosine] plus 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc, BD Biosciences Clontech, Palo Alto, CA, USA) (Jefferson 1987).

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