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Characterization of promoter elements required for expression and induction by sucrose of the Arabidopsis *COX5b-1* nuclear gene, encoding the zinc-binding subunit of cytochrome *c* oxidase

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Abstract Arabidopsis *COX5b-1* encodes an isoform of the zinc binding subunit 5b of mitochondrial cytochrome *c* oxidase. A promoter region required for expression and induction by sucrose of this gene was analyzed using plants stably transformed with mutagenized promoter fragments fused to the *gus* reporter gene. Promoter dependent expression is absolutely dependent on a G-box present at −228 from the translation start site. This element interacts in vitro and in vivo with transcription factors from the bZip family, preferentially with the abscisic acid-responsive element binding factor AREB2/ABF4. A region located upstream of the G-box (−333/−259) contains elements with the core sequence ATCATT and distalB-like sequences (CCACTTG) that are required for expression in vegetative tissues. These sequences bind different sets of proteins present in plant nuclear extracts and participate in induction by sucrose (ATCATT) and abscisic acid (distalB) of the *COX5b-1* promoter. We propose that the *COX5b-1* promoter has acquired novel regulatory mechanisms during evolution after gene duplication. These novel mechanisms have allowed the diversification of expression patterns, but also the conservation of some responses that, as induction by sucrose, are shared by *COX5b-1* and other genes encoding components of the mitochondrial respiratory chain. Conservation of these responses may be a pre-requisite for the successful incorporation of new regulatory elements in this class of genes.

Keywords *Arabidopsis thaliana* · bZIP transcription factor · Cytochrome *c* oxidase · Promoter analysis · Sucrose responsive element

Abbreviations

COX	Cytochrome *c* oxidase
EMSA	Electrophoretic mobility shift assay
GUS	*β*-Glucuronidase
MUG	4-Methylumbelliferyl *β*-D-glucuronide
X-gluc	5-Bromo-4-chloro-3-indolyl-*β*-D-glucuronic acid

Introduction

Cytochrome *c* oxidase (COX), the terminal enzyme of the mitochondrial respiratory chain, is composed of at least 10 different polypeptides encoded either in the mitochondrial genome or the nuclear genome (Barrientos et al. 2002; Richter and Ludwig 2003). The three subunits encoded in the organelle in most plant species (COX1, COX2, and COX3) constitute the catalytic core of the enzyme and have relatives in prokaryotes, while the nuclear encoded subunits are most likely acquisitions that occurred in eukaryotes after endosymbiosis (Capaldi 1990; Richter and Ludwig 2003). The most conserved nuclear-encoded subunit is COX5b (COX4 in yeast), that is attached to the matrix side of the inner membrane and contains the binding site for a Zn(II) ion (Rizzuto et al. 1991; Grossman and Lomax 1997; Barrientos et al. 2002; Richter and Ludwig 2003).

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It is logical to assume that correct COX assembly requires the coordinated expression of the genes that encode its different subunits, or at least most of them. In animals, for example, it has been recently shown that the expression of all 10 nuclear-encoded COX subunits in neurons is regulated by transcription factor NRF-1, that also regulates the expression of transcription factors involved in mitochondrial gene expression (Dhar et al. 2008). In plants, expression of the mitochondrial genome does not seem to be coordinated with that of nuclear genes encoding respiratory chain components (Giegé et al. 2005). Accordingly, the expression of nuclear genes can be regarded as an important control point to regulate the amount of each respiratory complex within the cell. Current evidence indicates that the expression of several nuclear genes encoding respiratory chain components is coordinated at the level of transcription and that conserved regulatory elements, known as site II, present in a majority of the respective promoters are candidate target sites for transcription factors involved in regulation (Welchen and Gonzalez 2005, 2006; Gonzalez et al. 2007).

Particularly for *COX5b-1*, one of the two genes encoding subunit 5b in Arabidopsis, it was previously determined that it is induced by incubation of plants in the presence of carbohydrates (Welchen et al. 2002), an effect that was also observed for genes encoding cytochrome *c* and other COX subunits (Welchen et al. 2002; Curi et al. 2003). This raises the idea that expression of *COX5b-1*, the gene that is expressed in most Arabidopsis tissues, must be coordinated with that of other nuclear genes encoding respiratory chain components. Studies on the promoter region of *COX5b-1* determined that the regulatory elements involved in induction by carbohydrates are present in a fragment spanning nucleotides −387 to −196 respective to the translation start site that is also essential for expression (Welchen et al. 2004). Notably, this fragment does not contain site II elements as those present in other genes, raising the question that coordination may operate through non-conserved mechanisms for this gene.

In the present study, we have undertaken a detailed characterization of the *COX5b-1* promoter regions involved in expression. We have determined that a G-box present at −228, that interacts with the bZIP transcription factor AREB2/ABF4 (Choi et al. 2000; Uno et al. 2000), is essential for expression of the gene and that induction by carbohydrates requires the presence of motifs with an ATCATT core located upstream of the G-box. Accordingly, the *COX5b-1* gene seems to be a particular case that has acquired coordinated responses through the incorporation of novel regulatory mechanisms.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0) was purchased from Lehle Seeds (Tucson, AZ). Plants were grown on soil in a growth chamber at 22–24°C under long-day photoperiods (16 h of illumination by a mixture of cool-white and GroLux fluorescent lamps) at an intensity of approximately 200 μE m^{-2} s^{-1}. Plants used for the different treatments were grown in Petri dishes containing Murashige and Skoog (MS) medium, 0.8% agar and different additions, as indicated in the respective figure legends.

Reporter gene construct and plant transformation

Upstream deletions of the fragment spanning nucleotides −387 to −1 with respect to the ATG initiation codon of the *COX5b-1* gene (At3g15640) were obtained by PCR amplification of the respective clone using primers pCOX5b1-333 or pCOX5b1-259, together with pCOX5b1-R (Supplemental Table 1). The resulting fragments, containing *Hin*dIII and *Sal*I sites, were cloned in the binary vector pBI101.3. Complementary primers were used to generate an internal deletion of nucleotides −333/−259, scanning mutations along this portion of the promoter region, and specific mutations of putative regulatory elements (Supplemental Table 1). These primers were used with pCOX5b1-387 and pCOX5b1-R to amplify hybridizing downstream and upstream sequences. The resulting products were mixed in buffer containing 50 mM Tris–HCl (pH 7.2), 10 mM $MgSO_4$, and 0.1 mM DTT, incubated at 95°C during 5 min, and annealed by allowing the solution to cool to 24°C in approximately 1 h. After this, 0.5 mM of each dNTP and 5 units of the Klenow fragment of *E. coli* DNA polymerase I were added, and incubation was followed for 1 h at 37°C. A portion of this reaction was directly used to amplify the chimeric fragments using primers pCOX5b1-387 and pCOX5b1-R. Fragments were cloned into pBI101.3. All constructs were checked by DNA sequencing.

The respective constructs were introduced into *Agrobacterium tumefaciens* strain LB4404, and transformed bacteria were used to obtain transgenic Arabidopsis plants by the floral dip procedure (Clough and Bent 1998). Transformed plants were selected on the basis of kanamycin resistance and positive PCR carried out on genomic DNA with gene specific primers and the *gus* primer 5′-TTGGGGTTTCTACAGGAC-3′. Approximately 30 primary transformants for each construct were initially analyzed for GUS expression by histochemistry. From these, 10 independent lines with single insertions (as

deduced by kanamycin resistance segregation) and with representative expression patterns (those common to a majority of transformants) were further reproduced and homozygous T3 and T4 plants were used for detailed analysis of GUS expression. Plants transformed with pBI101.3 were obtained in a similar way.

β-Glucuronidase assays

β-Glucuronidase (GUS) activity of transgenic plants was analyzed by histochemical staining using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) as described by Hull and Devic (1995). Whole plants or separated organs were immersed in a 1 mM X-gluc solution in 100 mM sodium phosphate, pH 7.0, and 0.1% Triton X-100 and, after applying vacuum for 5 min, they were incubated at 37°C for specified times (see figure legends for details). Tissues were cleared by immersing them in 70% ethanol.

Specific GUS activity in protein extracts was measured using the fluorogenic substrate 4-methylumbelliferyl β-D-glucuronide (MUG) essentially as described by Jefferson et al. (1987). Total protein extracts were prepared by grinding the tissues in extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol) containing 0.1% (w/v) SDS and 1% Triton X-100, followed by centrifugation at 13000*g* for 10 min. GUS activity in supernatants was measured in extraction buffer containing 1 mM MUG and 20% methanol. Reactions were stopped at different times with 0.2 M Na_2CO_3 and the amount of 4-methylumbelliferone was calculated by relating relative fluorescence units with those of a standard of known concentration. Controls were made to ensure that activity measurements were within the linear range as a function of time and protein concentration. The protein concentration of extracts was determined as described by Sedmak and Grossberg (1977).

DNA binding assays

A fragment encoding amino acids 331–410, comprising the bZip domain of AREB2/ABF4, was amplified from the respective clone in pDEST22 and inserted in frame into the *Bam*HI and *Eco*RI sites of the expression vector pGEX-3X (Smith and Johnson 1988). For expression, *E. coli* cells bearing the corresponding plasmid were grown and induced as described previously (Palena et al. 1998). Purification of the recombinant protein was performed as indicated by Smith and Johnson (1988), with modifications described by Palena et al. (1998). Nuclear extracts were prepared from cauliflower buds (obtained from a local market) as described by Maliga et al. (1995).

For electrophoretic mobility shift assays (EMSAs), aliquots of extracts (10 μg) or purified recombinant protein (100 ng) were incubated with double stranded DNA (10,000 c.p.m.) obtained by amplification of the corresponding fragments with specific primers, followed by restriction enzyme cleavage and labeling with [α-^{32}P]dATP by filling-in the 3′-ends using the Klenow fragment of DNA polymerase. Binding reactions (20 μl) contained, in addition to labeled DNA, 20 mM HEPES (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 1.0 mM dithiothreitol, 0.5% Triton X-100, 10% glycerol, and 1.5 μg poly(dI-dC). Reactions were incubated for 20 min on ice, supplemented with 2.5% Ficoll and immediately loaded onto a running gel (5% acrylamide, 0.08% bis-acrylamide in 0.5× TBE plus 2.5% glycerol; 1× TBE is 90 mM Tris–borate, pH 8.3, 2 mM EDTA). The gel was run in 0.5× TBE at 30 mA and 4°C for 1.5 h and dried prior to autoradiography.

One-hybrid analysis in yeast

To obtain a yeast strain carrying the *COX5b-1* promoter region comprising nucleotides −259 to −196 in front of the *lacZ* reporter gene, three tandem copies of the fragment were cloned in plasmid pLacZi (Clontech). Plasmid linearized in its *Nco*I site was introduced into the *URA3* locus of the yeast aW303 strain. Alternatively, the *COX5b-1* promoter fragment was placed in front of the *HIS3* reporter gene contained in vector pHIS3NX (Meijer et al. 1998). The entire cassette was then transferred to the *Not*I–*Xba*I sites of the integrative yeast plasmid pINT1, which confers resistance to the antibiotic G418. An *Nco*I–*Sac*I fragment of the clone in pINT1 was introduced into the *PDC6* locus of the yeast strain Y187 as described (Meijer et al. 1998). The presence of the fragments of interest in the genome of yeast cells was analyzed by PCR with specific oligonucleotides.

Plasmids carrying the different transcription factor full-length coding regions fused to the GAL4 activation domain were obtained in pDEST22 (Invitrogen). The transcription factors used and the respective gene codes are: GBF-like (At1g32150), GBF1 (At4g36730; Schindler et al. 1992), GBF3 (At2g46270; Schindler et al. 1992), AREB2/ABF4 (At3g19290; Uno et al. 2000; Kang et al. 2002), bHLH080 (At1g35460; Heim et al. 2003; Toledo-Ortiz et al. 2003), and bHLH081 (At4g09180; Heim et al. 2003; Toledo-Ortiz et al. 2003). Plasmid DNA was introduced into yeast using the standard lithium acetate transformation method (Gietz et al. 1992).

Real-time RT-PCR measurements

RNA for real-time RT-PCR was prepared with Trizol reagent (Invitrogen) according to the manufacturer's

instructions. RNA (1 μg) was used for reverse transcription reactions using M-MLV reverse transcriptase (Promega Corp.). Quantitative PCR was carried out using an MJ Research Chromo4 apparatus in 25 μl final volume containing 1 μl SYBR Green, 10 pmol each of primers 5b1realtime-F and 5b1realtime-R, 3 mM $MgCl_2$, 5 μl of the reverse transcription reaction, and 0.2 μl platinum *Taq* polymerase (Invitrogen). Fluorescence was measured at 82°C during 40 cycles. Relative *COX5b-1* transcript levels of treated versus untreated samples were calculated by a comparative C_t method. Expression values were normalized using *ACT2* and *ACT8* transcript levels as standard (Charrier et al. 2002).

Results

Two different regions of the *COX5b-1* gene are required for expression

It was previously determined that a fragment spanning nucleotides −387 to −196 from the translation start site contains elements essential for *COX5b-1* expression (Welchen et al. 2004). To further delineate the presence of regulatory elements, we have performed two nested deletions of the −387 promoter fragment down to nucleotides −333 and −259 (Fig. 1a). The respective fragments, fused to *gus*, were stably introduced into Arabidopsis and GUS activity was determined in several independent lines carrying each construct, as described in "Materials and methods". Histochemical analysis showed GUS expression in cotyledons and hypocotyl (mainly vascular strands) and the root and shoot apical meristems in seedlings transformed with the −387 construct (Fig. 1b). Deletion down to −333 produced a moderate decrease in expression, while only expression in the shoot apical meristem was observed for the −259 construct (Fig. 1b). Analysis of adult plants revealed expression in roots, leaves, and flowers (mainly in anthers, stigma, and the junction of flowers with pedicels) for the two larger constructs, while only flowers were stained in plants transformed with the −259 construct (Fig. 1b). As previously described (Welchen et al. 2004), plants carrying the −387 promoter fragment have higher GUS activity, specially in leaves, when compared with plants carrying larger promoter fragments (Fig. 1b–e). Seedlings of plants transformed with the first deletion construct (−333) showed GUS activity levels that were about 50% of those of plants transformed with the −387 fragment (Fig. 1c). Deletion of nucleotides down to −259 originated a further decrease in GUS expression, of about 70–80% respective to the −333 fragment. Expression in flowers and leaves is also significantly reduced in plants carrying the different deletions (Fig. 1d, e; see Supplemental Fig. 1 for the values of individual lines). Deletion of the portion located upstream of −333 produced a more drastic decrease in expression in leaves respective to other organs (Fig. 1e). The results indicate the presence of positive regulatory elements in both promoter regions that were removed in the analysis. In addition, deletion of the region comprised between −333 and −259 within the context of the −387 promoter region (Fig. 1a) originated plants with similar expression levels and patterns as those of plants transformed with the −259 construct (Fig. 1b–e), suggesting that elements located upstream of −333 need the presence of the deleted portion of the promoter for activity. The lack of staining in vegetative parts of the plant observed when the fragment located between −333 and −259 was removed is most likely the consequence of a general decrease in expression that produces detectable activity only in flowers, where expression is higher. The results also indicate that additional element(s) essential for expression are present in the region located between −259 and −196, since removal of this fragment originates plants with a complete loss of GUS activity, as shown previously (Welchen et al. 2004).

A G-box present at −228 is essential for *COX5b-1* expression

We concentrated our study in the regions comprising nucleotides −259/−196 and −333/−259, whose deletion produces either a complete or a considerable decrease in expression, respectively. Informatic analysis of the more proximal of these regions indicated the presence of elements that could be involved in *COX5b-1* expression, like a G-box (CACGTG; −228; Salinas et al. 1992; Menkens et al. 1995), an ACGT motif (−204), and a sequence matching the core of a *telo* box (ACCCTA; −241; Manevski et al. 2000). Particularly, a G-box and an ACGT motif were shown to be required for expression of the *Cytc-2* gene (Welchen et al., unpublished), while a *telo* box is involved in expression of the *Cytc-1* gene (Welchen and Gonzalez 2005), both genes encoding cytochrome *c* from Arabidopsis. We then mutagenized these elements separately within the context of the −387 promoter region and obtained plants carrying the mutant promoters fused to *gus*. Histochemical analysis of these plants indicated that mutation of the G-box (CACGTG to CAATGG) completely abolishes GUS expression in all organs (Fig. 1b). Accordingly, GUS activity levels in protein extracts prepared from plants or isolated organs carrying the promoter fragment with a mutagenized G-box were close to background levels and similar to those obtained with plants transformed with a promoterless *gus* gene (Fig. 1c–e). Mutation of the other elements, in turn, does not produce changes in expression patterns (Supplemental Fig. 2). We conclude that the lack of activity

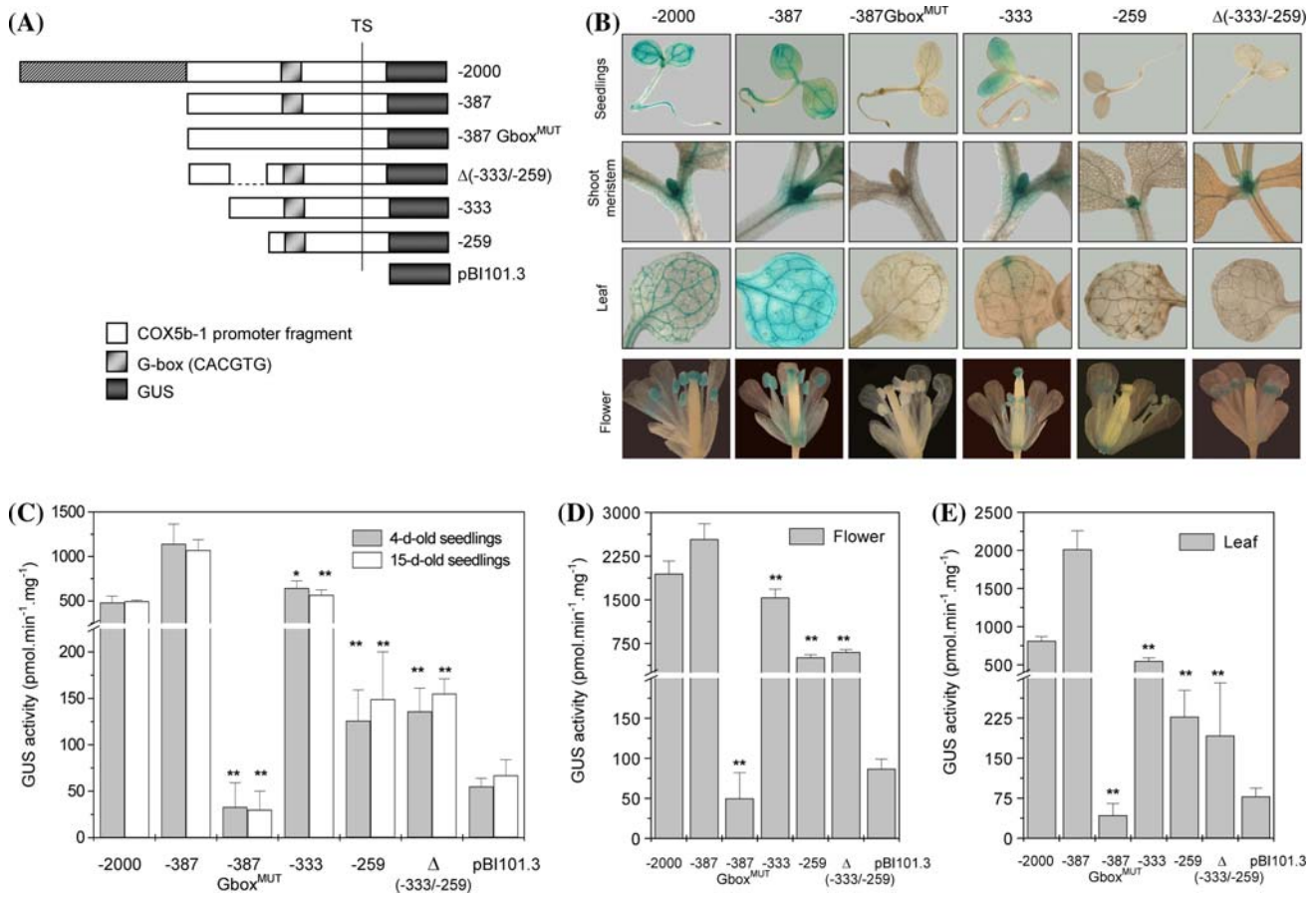

Fig. 1 Analysis of GUS activity driven by deleted and mutagenized forms of the *COX5b-1* promoter. **a** Schematic representation of different constructs used to transform Arabidopsis plants. The numbers indicate the upstream end of the promoter fragment present in each construct respective to the translation start site; the downstream end was at −1 for all constructs. TS indicates the putative transcription start site according to the longest cDNA sequence available. Gbox^{MUT} indicates a construct in which the G-box was mutagenized. **b** Histochemical localization of GUS activity in Arabidopsis plants transformed with the different constructs, as indicated. The images are representative from 10 lines analyzed for each deletion or mutagenized promoter construct. Incubation time in the staining solution was 12 h for plants of all stages and organs. **c**–**e** GUS expression levels measured in total protein extracts from 4- and 15-days-old seedlings (**c**), flowers (**d**), or leaves (**e**) of plants transformed with the different constructs. Plants transformed with the promoterless *gus* gene (pBI101) were also used. Significance of changes produced after each deletion or mutation was assessed using Student's *t* tests (*$P < 0.05$, **$P < 0.01$). The results indicate the mean (±SD) of five independent lines for each construct. Similar results were obtained in three other experiments in which different line combinations from a total of 10 were used

observed upon deletion of the fragment spanning nucleotides −259 to −196 is due to the removal of the G-box located at −228. This element is essential for expression and cannot be replaced by upstream regions.

The presence of nuclear proteins able to bind to the *COX5b-1* promoter fragment essential for expression (−259 to −196) was analyzed using nuclear extracts prepared from cauliflower inflorescence, used as a rich source of proteins from a species closely related to Arabidopsis. As shown in Fig. 2a, at least three shifted bands were observed in an EMSA in the presence of nuclear extract and labeled DNA. All shifted bands disappeared or were considerably reduced when a similar DNA fragment with a mutated G-box was used (Fig. 2a). Accordingly, it can be concluded that binding of nuclear proteins to the *COX5b-1* promoter fragment analyzed requires the integrity of the G-box. We have also used a competition approach to analyze the specificity of binding to the G-box. Figure 2b shows that a 25-fold molar excess of unlabeled fragment spanning nucleotides −259 to −196 efficiently competed the formation of the two complexes with lowest mobility, but competition was abolished upon mutation of the G-box. A 50-fold molar excess of wild-type fragment, in turn, competed the formation of the three complexes (Fig. 2c). Formation of the low mobility complexes was restored when a similar amount of a fragment with a mutated G-box was used. Competition was only marginally affected by mutation of the ACGT motif (Fig. 2c). It can be concluded that proteins present in the nuclear extract recognize specifically the G-box present in the *COX5b-1* promoter. This

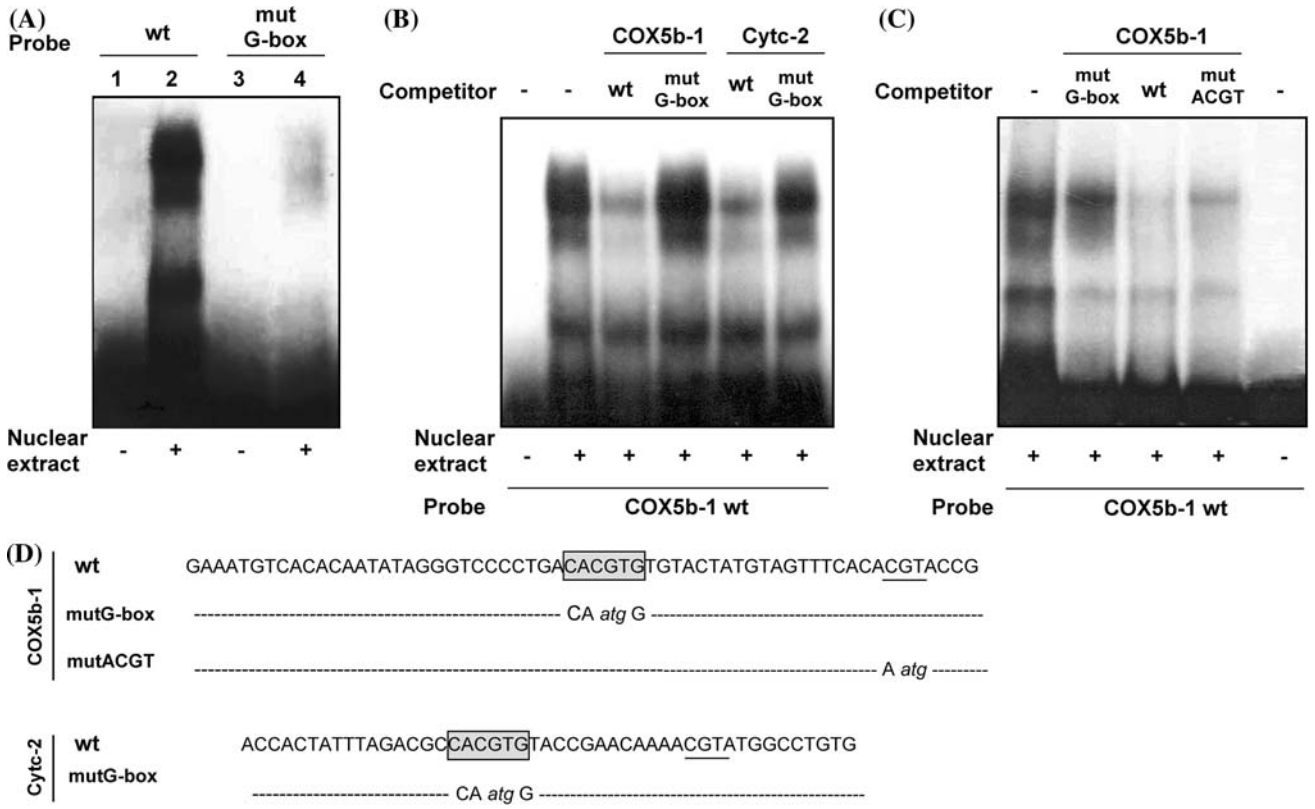

Fig. 2 Nuclear proteins bind specifically to the *COX5b-1* G-box. **a** Nuclear extracts (10 μg) from cauliflower inflorescences were analyzed by an EMSA for the presence of proteins that bind to labeled DNA spanning nucleotides −259 to −196 from the *COX5b-1* promoter. Binding was analyzed using either a labeled wild-type fragment (wt: *lanes 1 and 2*) or a similar fragment in which the G-box was mutagenized (mut G-box: *lanes 3 and 4*). **b** Binding of nuclear proteins to the non-mutagenized fragment used in (**a**) was analyzed with or without the addition of a 25-fold molar excess of unlabeled wild-type or mutated forms of the same fragment, or a fragment spanning nucleotides −189 to −139 from the *Cytc-2* promoter, that also contains a G-box, as competitors. **c** A competition experiment using a 50-fold molar excess of either wild-type or mutant forms of the *COX5b-1* promoter with modifications in the G-box or the ACGT motif as competitors. The sequences of the corresponding fragments are shown in (**d**). The respective G-boxes are boxed. ACGT motifs present in both promoter fragments are underlined. Nucleotides changed by mutagenesis are shown in lowercase letters

specific binding results in the formation of the two low mobility complexes. The complex of high mobility seems to arise from less specific interactions. In addition, a *Cytc-2* promoter fragment containing a G-box that is required for expression of this gene (Welchen et al., unpublished) also showed efficient competition that was abolished when the G-box was mutated (Fig. 2b). The *COX5b-1* and *Cytc-2* promoters show then the capacity to interact with similar sets of proteins.

The *COX5b-1* G-box region interacts with transcription factors from the bZIP family

The presence of a G-box and a nearby ACGT motif has been recently documented in the promoter region of the *Cytc-2* gene (Welchen et al., unpublished). Unlike the *COX5b-1* case, mutation of each of these elements produces a significant decrease in expression of this gene. One-hybrid screenings allowed the identification of six transcription factors from the bZIP and bHLH families that interact with the *Cytc-2* promoter, mainly through the G-box. The similarity of both promoter regions and the observed competition for binding to nuclear proteins led us to test the interaction of the transcription factors identified for the *Cytc-2* promoter with the region spanning nucleotides −259 to −196, essential for *COX5b-1* expression. For this purpose, three tandem copies of this fragment were cloned in front of the *LacZ* gene carrying a minimal promoter and introduced into yeast. As shown in Fig. 3a, expression of transcription factor AREB2/ABF4 fused to the GAL4 activation domain in this strain produced a 20-fold activation respective to the control strain. For GBF1 and the GBF-like protein, activation was about 4-fold. The other proteins assayed produced activity values that were similar to those of the control strain (Fig. 3a). A similar result was observed when the *HIS3* gene was used instead of *LacZ* as a reporter (not shown). We conclude that proteins from the bZIP family, and specially AREB2/ABF4, are able to interact

with the *COX5b-1* promoter region containing the G-box essential for expression of the gene.

Since we were unable to express the entire protein in recombinant form in *E. coli*, binding of AREB2/ABF4 to the *COX5b-1* promoter was studied in vitro using a protein region (amino acids 331–410) containing the bZip domain. EMSAs using this protein and labeled DNA comprising nucleotides −259 to −196 indicated the presence of a shifted band that was abolished after mutation of the G-box (Fig. 3b, right panel). On the contrary, mutation of the ACGT motif did not affect the interaction, suggesting that AREB2/ABF4 is unable to bind to this region of the promoter, even if it contains a sequence closely similar to the G-box/ABRE (TACGTGTG vs. CACGTGTC). Thus, the binding requirements of AREB2/ABF4 correlate with the effect of the respective mutations on *COX5b-1* promoter-dependent expression.

The region located upstream of −259 contains elements involved in expression in vegetative tissues

The presence of regulatory elements in the region located upstream of −259 was assayed by analyzing expression from a set of six −387 promoter constructs carrying site-directed mutations in 11–12 bp adjacent regions of the fragment spanning nucleotides −333 to −259 (Fig. 4a).

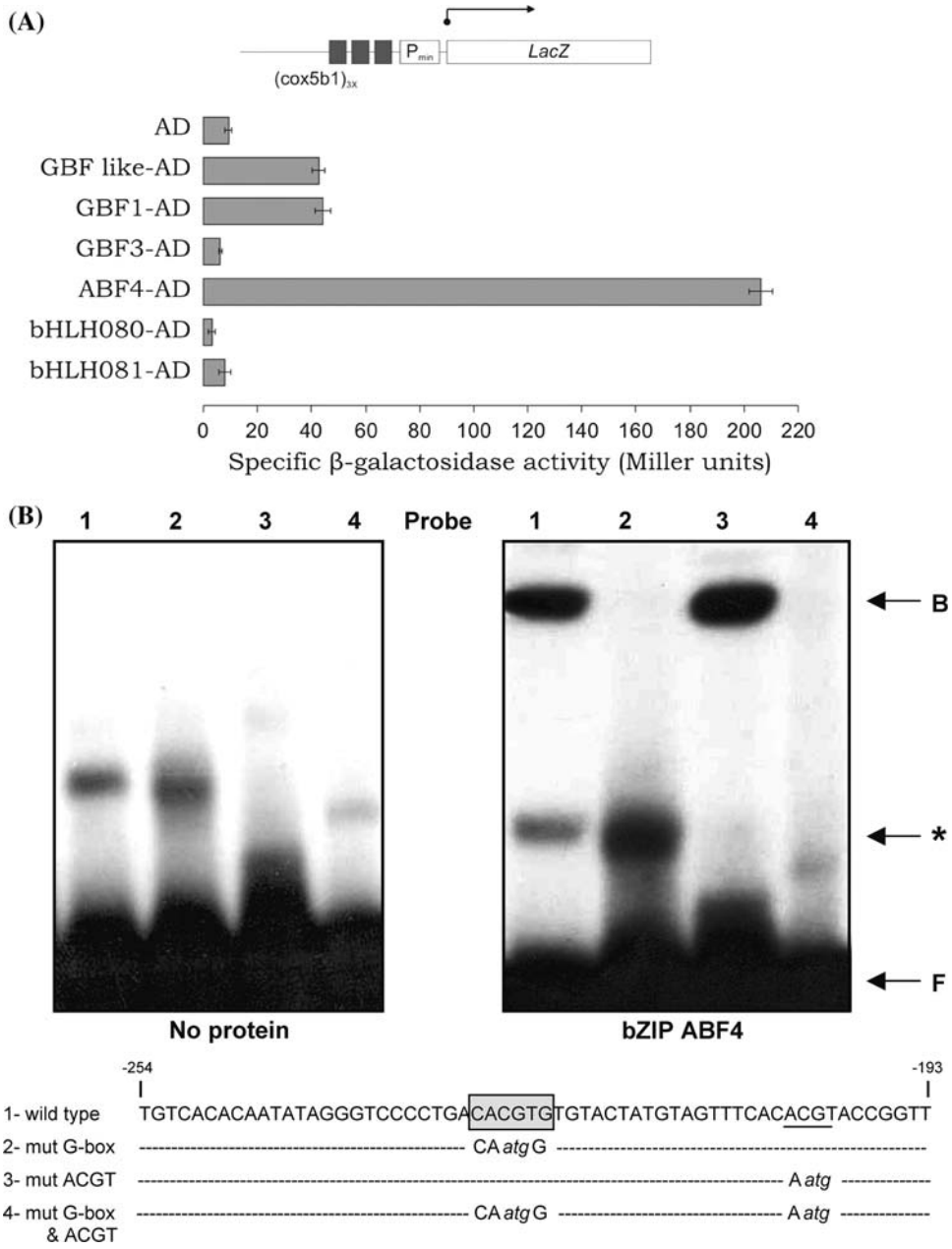

Fig. 3 bZIP transcription factors interact with the *COX5b-1* promoter. **a** *β*-galactosidase activity measurements in yeast cells containing the *lacZ* reporter gene fused to the *COX5b-1* promoter fragment spanning nucleotides −259 to −196 and transformed with the clones expressing different transcription factors fused to the GAL4 activation domain (AD). The bars represent mean *β*-galactosidase specific activity (±SD). AD represents a yeast strain transformed with a construct expressing only the GAL4 activation domain. **b** Binding of AREB2/ABF4 to the *COX5b-1* promoter in vitro. One hundred nanograms of recombinant protein were incubated with *COX5b-1* promoter fragments spanning nucleotides −259 to −196 (either wild-type or mutagenized in the G-box, the ACGT motif, or both) and analyzed in an EMSA. Sequences of the fragments used as probes are shown below. Free and bound DNA are indicated with F and B, respectively. Bands labeled with an asterisk correspond to species already present in the free probes as deduced from lanes in which no protein was added (*left panel*)

Histochemical analysis of the respective plants carrying fusions of the mutagenized promoter fragments fused to *gus* revealed that mutation M3 (−299/−289) produces a complete loss of activity in vegetative tissues, except for the apical meristem, in a similar way as it was observed upon deletion of the entire fragment (Fig. 4b). In addition, mutations in other regions (M1, M4, M5, M6) produce a moderate decrease in expression with no alteration in expression patterns (Fig. 4b). Notably, mutation M2 (−310/−300) produces a significant increase in expression, specially in roots (Fig. 4c), suggesting that this region contains a negative regulatory element. Fluorometric measurements of GUS activity in seedlings were in agreement with the observations made in the histochemical assay and indicated that mutation M3 produces a decrease of GUS activity levels to those observed upon deletion of the entire fragment comprising nucleotides −333 to −259 (Fig. 4d). Expression in flowers, in turn, was more profoundly affected by the deletion of the entire fragment than by mutation M3 (Fig. 4e). All other mutations, except M2, produced a partial but statistically significant decrease in expression, while mutation M2 originated a 6- to 10-fold increase in GUS activity levels (Fig. 4d, e; see Supplemental Fig. 1 for the values of individual lines).

Analysis of the M2 region evidenced the presence of an element (GTATATGC) described as a binding site of the PHR1 transcription factor, involved in responses to Pi starvation (Rubio et al. 2001). However, we did not detect any effect of incubation of plants under Pi deprivation conditions on *COX5b-1* gene expression (not shown). Regarding the other mutations, the fact that many of them affect GUS activity levels suggests that the analyzed fragment contains several positive elements involved in expression. Inspection of the sequence of the fragment revealed the presence of repetitive regions that may account for this behavior. We observed two repetitions of the sequences CATCATNNTNTCATT and TCANTANTTCCACTTG comprised in regions M1, M3 and M4, or M4 to M6, respectively (Fig. 4a). One of the repetitions of the first sequence is severely affected in mutation M3 (11 out of 15 nucleotides) that abolishes expression in vegetative tissues. We also noticed that the sequence mutated in M3 contains two tandem repetitions of the partially overlapping sequence TG(C/T)ATCATT(G/A)T (3-nt overlap). Hence, we constructed a mutant promoter where both TCA of the repetitive sequence were mutated to ATC (mutant M3a in Fig. 4a). Histochemical analysis of plants transformed with *gus* fusions of the mutant promoter showed expression only in flowers, as observed with mutant M3 (Fig. 4b). We also constructed a mutant in which three nucleotides located between both TCA were mutagenized (mutant M3b in Fig. 4a). Plants carrying this construct still showed activity in vegetative tissues, although lower than the one observed with the non-mutagenized construct (Fig. 4b). We conclude that sequences with an ATCATT core present in the fragment spanning nucleotides −333 to −259 are involved in enhancing expression from the *COX5b-1* promoter.

Fluorometric assays of GUS activity in seedling extracts indicated that mutations within both ATCATT sequences produce a significant decrease in expression, similar to that observed upon deletion of the fragment spanning nucleotides −333 to −259 (Fig. 4d). In addition, the reduced expression levels observed in mutant M1 (Fig. 4d) may be caused by mutation of elements with similarity to the ATCATT sequence (Fig. 4a). In flowers, mutation of ATCATT also produced a decrease in expression, but the effect was less pronounced (Fig. 4e), suggesting that other elements present in the −333/−259 fragment can also sustain expression in reproductive tissues.

The second of the repetitive sequences described above contains CCACTTG, the core of the distalB element (GCCACTTGTC) that has been implicated in ABA-dependent expression of the *napA* promoter (Ezcurra et al. 1999). We then constructed a mutant promoter in which nucleotides CAC from both distalB-like sequences were mutagenized (mutant M7 in Fig. 4a). Adult plants transformed with the mutagenized promoter fragment fused to *gus* showed activity only in flowers (Fig. 4b). It is noteworthy that mutations M4, M5 and M6, that affect only one of the distalB-like sequences, do not originate a complete loss of expression in vegetative tissues (Fig. 4b). It seems then that both distalB-like sequences have an additive effect on *COX5b-1* promoter-dependent expression. In addition, mutation of both ATCATT (as in M3 and the respective site-directed mutant) or both distalB-like sequences produces a loss of expression in vegetative tissues and seedlings (except for the apical meristem), while mutation of only one of each of these sequences (as in M4) does not. This may indicate that the interaction of proteins with both types of sequences is required for expression in vegetative tissues and seedlings. Accordingly, fluorometric assays of GUS activity in seedling extracts showed that mutation of both distalB-like sequences produces a similar effect as mutation of the ATCATT sequences or deletion of the entire −333/−259 fragment (Fig. 4d). In flowers, the different mutations have milder effects than deletion of the entire fragment (Fig. 4e). This may indicate that ATCATT sequences and distalB-like sequences have an additive effect on expression in flowers.

The interaction of proteins with the fragment of interest was analyzed by an EMSA using the nuclear extract mentioned above. A labeled fragment spanning nucleotides −333/−237 produced three complexes of different mobilities, named A–C in Fig. 5, in the presence of the extract and an excess of unspecific competitor. To delineate the regions of the fragment required for the formation of the respective complexes, a competition approach using

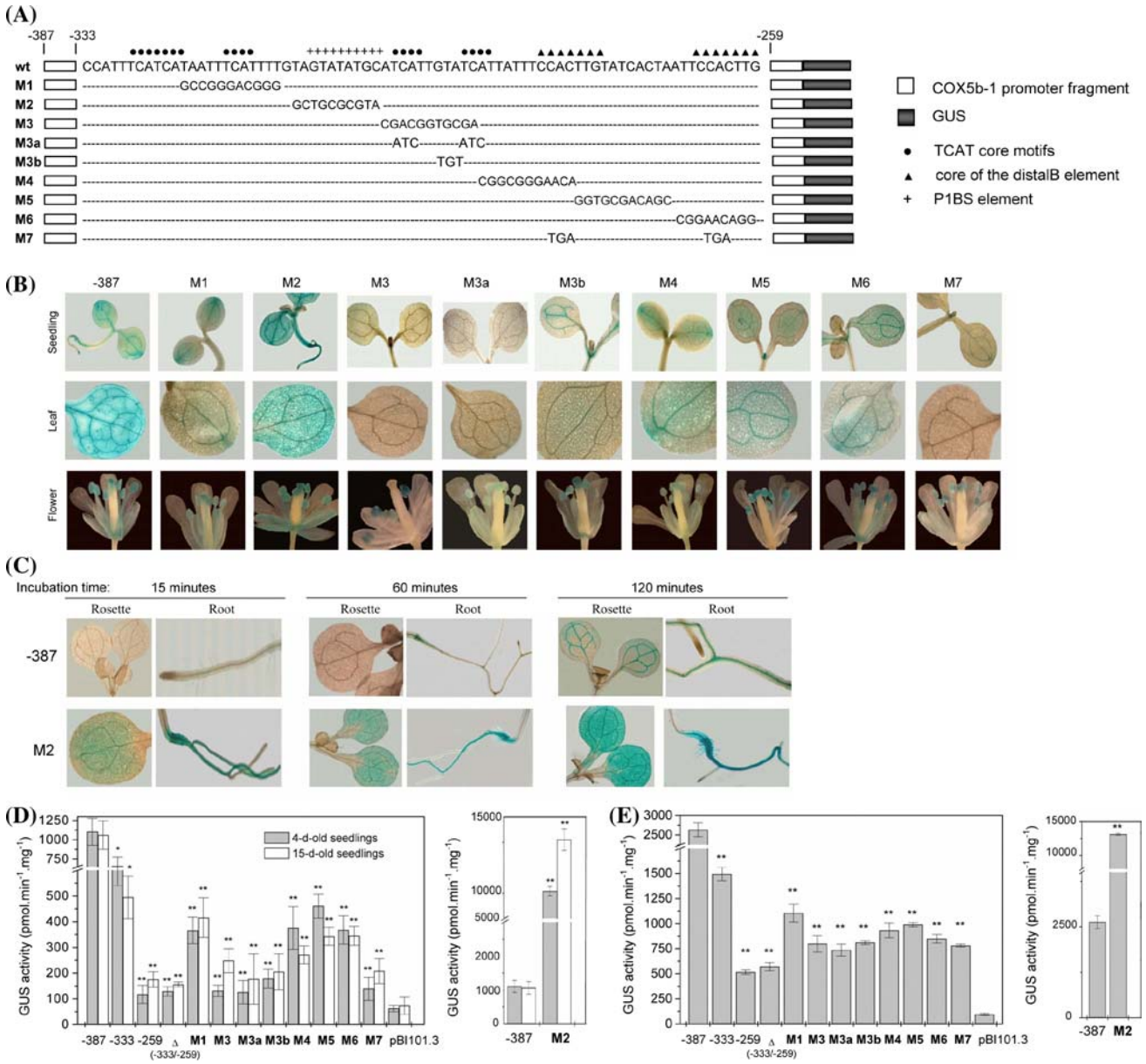

Fig. 4 Analysis of reporter gene expression in Arabidopsis plants transformed with *COX5b-1* promoter fragments mutagenized in the region spanning nucleotides −333 to −259 fused to *gus*. **a** Sequence of the analyzed fragment (wt) showing nucleotides that were mutagenized in each construct (M1–M7, only modified nucleotides are indicated). The location of motifs containing a TCAT core (*circles*), distalB-like elements (*triangles*), and the P1BS element (*crosses*) is indicated above the wild-type sequence. **b** Histochemical analysis of GUS activity in plants transformed with the different constructs. Incubation time in the staining solution was 12 h for plants of all stages and organs. The images are representative of 10 different lines from each construct. **c** Histochemical analysis of plants transformed with mutagenized fragment M2 fused to *gus*. GUS expression levels were compared in plants transformed with mutagenized fragment M2 or the respective non-mutagenized fragment (−387). Plants were incubated in the staining solution for different times, as indicated. **d**, **e** GUS expression levels measured in total protein extracts from 4- and 15-days-old seedlings (**d**) or flowers (**e**) of plants transformed with the different mutagenized fragments. Activity was also measured in extracts from plants transformed with a promoterless *gus* gene (pBI101). Bars indicate the mean activity values (±SD) obtained with five independent transformants for each construct. Significance of changes produced after each deletion or mutation respective to the non-mutagenized fragment was assessed using Student's *t* tests (*$P < 0.05$, **$P < 0.01$). Similar results were obtained in three other experiments in which different line combinations, from a total of 10, were used

different unlabeled DNA fragments was undertaken. The formation of all complexes was efficiently competed by a 25-fold molar excess of the same unlabeled fragment (Fig. 5). When either fragments containing 11-bp adjacent mutations in the regions spanning nucleotides −322 to −289 (mutants M1–M3) or a fragment spanning nucleotides

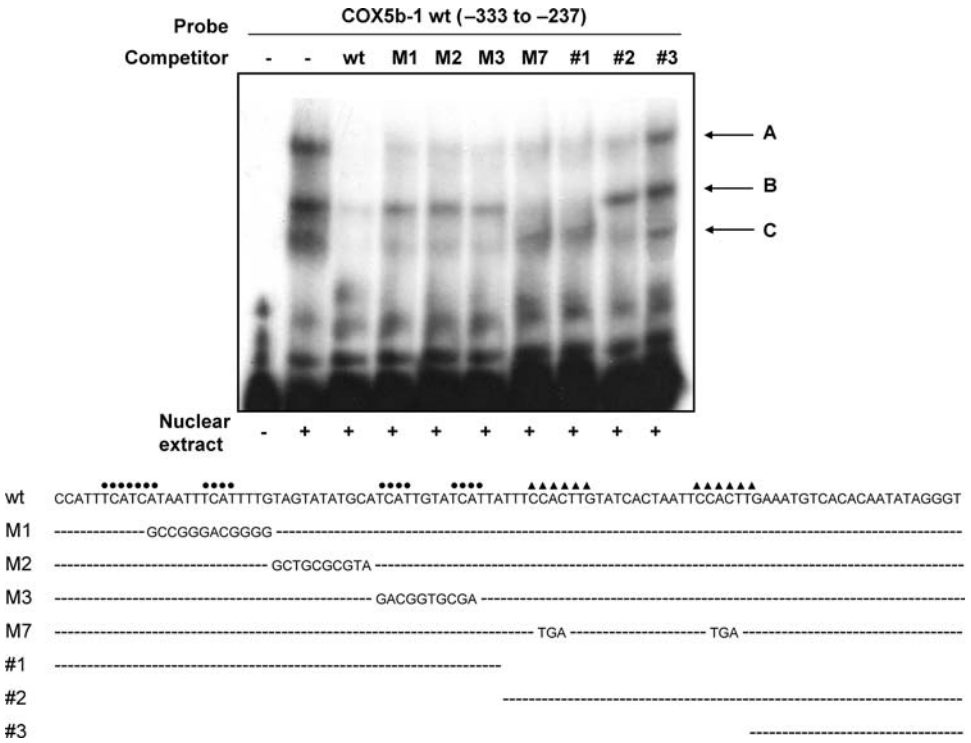

Fig. 5 Binding of nuclear proteins to the *COX5b-1* promoter fragment spanning nucleotides −333 to −237. Nuclear extracts (10 μg) from cauliflower inflorescences were analyzed by an EMSA for the presence of proteins able to bind to labeled DNA spanning nucleotides −333 to −237. A, B and C indicate the migration of three different complexes. Binding was analyzed with or without the addition of a 25-fold molar excess of different forms of unlabeled wild-type or mutated fragments of the *COX5b-1* promoter as competitors. Sequences of the fragments used for competition are shown below. Only nucleotide changes respective to the wild-type fragment are indicated. The location of motifs with a TCAT core (*circles*) and of distalB-like elements (*triangles*) is indicated above the wild-type sequence

−284 to −237 (named #2 in Fig. 5) were used as competitors, decreased competition, mainly of the complex with intermediate mobility (complex B), was observed (Fig. 5). In turn, when either a fragment with mutagenized distalB-like sequences (mutant M7) or a fragment spanning nucleotides −333 to −285 (named #1 in Fig. 5), that does not contain these sequences, were used, decreased competition of the high mobility complex (complex C) was observed (Fig. 5). This result suggests that complex C is formed by protein(s) that interact with the distalB-like sequences. Complex B may be formed by protein(s) that interact with the ATCATT sequences, since competition decreased when fragments with mutations (M1, M3) or lacking (−284/−237) these sequences were used (Fig. 5). Mutation M2 also produced a decrease in competition even if ATCATT sequences are not affected. Disturbance of binding to a couple of nearby ATCATT sequences may account for this behavior. The low mobility complex A may be formed by the interaction of proteins with both, the distalB and the ATCATT sequences. This is suggested by the fact that competition was similarly affected by the different mutations or deletions. In addition, a fragment spanning nucleotides −260/−237 (named #3 in Fig. 5), located outside the region that contains the ATCATT and the distalB-like sequences, competed less effectively the formation of the three complexes. The results evidence the presence of different sets of nuclear proteins that are able to interact with the *COX5b-1* promoter elements involved in expression located in the fragment spanning nucleotides −333 to −259.

The *COX5b-1* gene is induced by sucrose, ABA and other factors

It was previously shown that the *COX5b-1* promoter supports induction of a reporter gene by sucrose and cytokinin and that the sequences required for induction are located downstream of −387 (Welchen et al. 2004). In the present study, we have analyzed the effect of incubation of plants with different compounds in GUS expression levels driven by the *COX5b-1* promoter (−2000/−1). As shown in Fig. 6a, apart from sucrose and the cytokinin BAP, we observed that the *COX5b-1* promoter is also induced by treatment of plants with the hormones ABA and GA and the ethylene precursor ACC. Significant induction was also observed with Pi and H_2O_2 (Fig. 6a), suggesting that the

COX5b-1 promoter is able to respond to several factors. Among the compounds tested, carbohydrates were previously shown to produce an increase in the endogenous levels of the *COX5b-1* transcript (Welchen et al. 2002). Apart from sucrose, we also observed induction of the *COX5b-1* promoter in the presence of glucose and fructose, but not with mannitol (Supplemental Fig. 3), in close agreement with the results obtained by northern analysis of the endogenous transcript (Welchen et al. 2002). To determine if the other effectors of the *COX5b-1* promoter were also acting on the expression of the endogenous gene, we determined relative *COX5b-1* transcript levels in wild-type non-transformed plants after incubation with effectors using real-time PCR. It was observed that all treatments that induced GUS expression driven by the *COX5b-1* promoter also produced an increase in *COX5b-1* transcript levels (Fig. 6b), suggesting that, indeed, these compounds are effectors of the endogenous gene.

We then analyzed the minimal promoter fragments required for induction. Deletion of sequences located upstream of −609 abolished induction by GA and Pi, while the effect of ACC was eliminated by a further deletion down to −387 (Supplemental Fig. 4). Induction by H_2O_2, in turn, was conserved even in the fragment with a deletion upstream of −259, the shorter fragment that supports detectable GUS activity. For the cytokinin BAP, induction was observed in the −387 construct but not in the following deletion down to −333 (Supplemental Fig. 4). The element involved in cytokinin responses is then located in the 58-bp fragment that also produces a moderate decrease in basal expression when deleted. Interestingly, induction was also observed when the fragment spanning nucleotides −333 to −259 was removed, indicating that this downstream portion of the promoter is not required for cytokinin action. We did not study further the responses to the factors mentioned above.

In the case of sucrose, induction was observed for the construct containing nucleotides up to −333, but not when nucleotides −333 to −259 were removed, even in the presence of an upstream portion of the promoter (Fig. 7, left panel; see Supplemental Fig. 5 for the values of individual lines), suggesting that the putative sucrose-responsive elements of the *COX5b-1* promoter are present in this region. We then analyzed induction by sucrose in plants carrying the different mutations in the fragment mentioned above. No induction was observed in mutant M3, carrying mutations in the regions −299/−289 (Fig. 7, central panel). This region contains the ATCATT sequences that were shown above to be important for expression. In addition, point mutations in both TCA sequences present in the region covered by mutation M3 also abolish the induction by sucrose (mutant M3a in Fig. 7). We conclude, then, that the ATCATT sequences present in the *COX5b-1* promoter behave as part of a sucrose-responsive element. It is noteworthy that, although significant induction was still observed, the effect of sucrose was reduced in mutants M1 and M3b (Fig. 7, central panel). These mutations affect sequences with similarity to ATCATT (M1) or nucleotides adjacent to these sequences (M3b).

Although induction by ABA is present in all constructs that produce GUS activity, deletion of nucleotides −333 to −259 originates a decrease in induction levels (Supplemental Fig. 6). A similar decrease is observed in plants carrying point mutations in the distalB-like sequences (mutant M7 in Supplemental Fig. 6). This may reflect a role of the distalB-like sequences present in the *COX5b-1* promoter in maximal induction by ABA, perhaps in cooperation with the G-box/ABRE located downstream.

Discussion

Expression of an important set of nuclear genes encoding components of the mitochondrial respiratory chain is coordinated under certain conditions in plants (Gonzalez et al. 2007). Most of the genes that develop coordinated responses show the presence of elements named site II in their proximal promoter regions. However, part of the genes that participate in coordination do not possess recognizable site II elements, suggesting that they may have adopted different expression mechanisms to achieve similar responses. One of the genes showing these characteristics is *COX5b-1*, that is coordinately regulated by carbohydrates together with other respiratory chain component genes (Welchen et al. 2002).

With the idea that the knowledge of the elements and factors involved in *COX5b-1* expression and responses may have interesting mechanistic and evolutionary implications, we have performed a detailed characterization of the *COX5b-1* promoter. We have observed that a G-box located at −228 is essential for expression, and that a region located upstream of the G-box increases *COX5b-1* gene expression, mainly in vegetative tissues. The G-box in *COX5b-1* is recognized by bZip proteins from the GBF (G-box binding factor) subfamily (Schindler et al. 1992; Menkens et al. 1995; Sibéril et al. 2001), and by AREB2/ABF4, a bZip protein that has been isolated as an ABA responsive element (ABRE) binding factor (Choi et al. 2000; Uno et al. 2000; Kang et al. 2002). The ABRE consensus (C/T)ACGTGGC is similar to the sequence of the *COX5b-1* G-box and surrounding nucleotides in the complementary strand (CACGTGTC). These proteins also bind to a G-box/ABRE present at −172 in the *Cytc-2* gene, encoding cytochrome *c* (Welchen et al., unpublished), in agreement with the fact that both promoter regions compete for the binding to proteins present in nuclear extracts. Further studies *in planta* will be required to assess if these proteins, or other related members

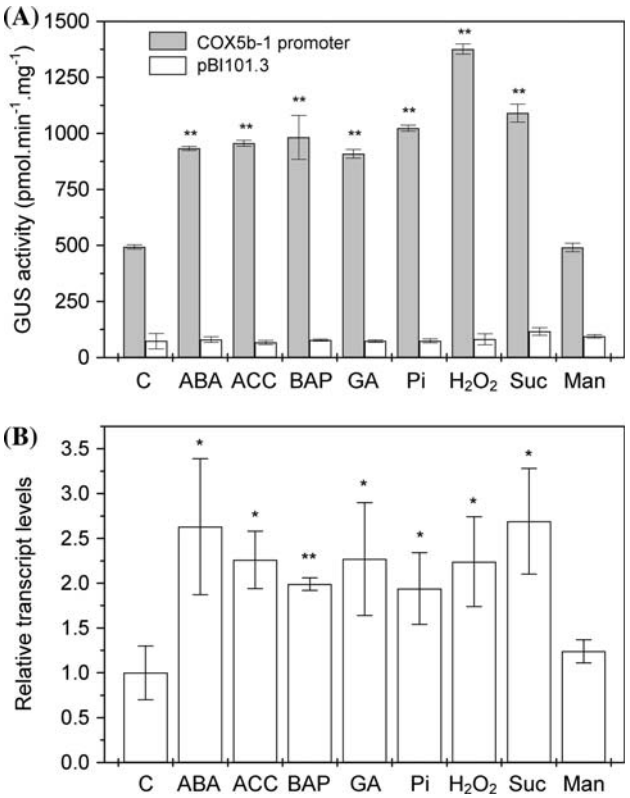

Fig. 6 The *COX5b-1* promoter responds to different compounds. **a** GUS activity was measured using the fluorogenic substrate MUG and protein extracts prepared from 21-days-old plants carrying a 2-kbp *COX5b-1* promoter fragment located upstream of the translation start site. Before the analysis, plants were grown in MS medium alone (C) or supplemented with either 0.1 mM abscisic acid (ABA), 0.1 mM aminocyclopropane-1-carboxylic acid (ACC), 0.1 mM 6-benzylaminopurine (BAP), 0.1 mM gibberellic acid (GA), 10 mM NaPi, 10 μM H_2O_2, 3% sucrose (Suc), or 3% mannitol (Man), as indicated. Plants transformed with the promoterless *gus* gene (pBI101.3) were used as controls. Bars indicate the mean activity values (±SD) obtained with five independent transformants for each construct. **b** Relative *COX5b-1* transcript levels in non-transformed plants after treatment with different compounds. Total RNA was prepared from 21-days-old plants treated as described in (**a**). Changes in *COX5b-1* transcript levels respective to control samples were analyzed by quantitative real-time RT-PCR, using *ACT2* and *ACT8* expression for normalization. The results shown indicate the mean (±SD) values of three independent measurements. In (**a** and **b**), significance of changes produced after each treatment respective to the non-treated sample was assessed using Student's *t* tests (*$P < 0.05$, **$P < 0.01$)

of the extensive bZip family, act as regulatory factors of the endogenous *COX5b-1* gene.

We have also observed that the *COX5b-1* promoter is induced by several compounds. Sucrose and other carbohydrates may act as signals to promote the synthesis of new respiratory chain components, which are involved in their utilization, as discussed previously (Felitti and Gonzalez 1998; Welchen et al. 2002). In fact, a coordinated regulation of nuclear genes encoding respiratory chain components seems to be exerted by carbohydrates in several systems analyzed (Giegé et al. 2005; Gonzalez et al. 2007). A cross-talk between sugar and ABA signalling pathways has been observed for the regulation of a variety of genes, and ABA and carbohydrates are both repressors of photosynthetic gene expression (Rook et al. 2006). It has been speculated that repression of photosynthetic genes by ABA is related with the reduced photosynthetic capacity of plants exposed to drought, that also accumulate carbohydrates (Wingler and Roitsch 2008). Expression of *COX5b-1* seems to be antagonistic with respect to that of photosynthetic genes in the presence of sugars and ABA, perhaps reflecting the need for enhanced respiratory energy production when photosynthesis ir reduced. Unlike induction by sugars, response to ABA has not been reported as a general characteristic of respiratory chain component genes. Induction by cytokinins has been observed for genes encoding other respiratory chain component, namely cytochrome *c* (Welchen and Gonzalez 2005; Welchen et al., unpublished results). In this case, induction may be related with an increase in cell proliferation promoted by the hormone. Increased cell proliferation is associated with the synthesis of new cellular components (Conlon and Raff 1999) and mitochondrial biogenesis may be required to supply energy for this process and to maintain the number of mitochondria in rapidly dividing cells. Our results do not indicate if expression of the gene is responding directly to the presence of the hormone or to other factors associated with its effects on cell proliferation or metabolism. Induction by H_2O_2 treatment may be related with a requirement for the synthesis of new subunits to replace those damaged by oxidative stress. COX5b contains bound Zn and this may convert it in a preferred target of ROS action. Several genes encoding proteins involved in COX biogenesis are also induced by oxidative stress (Attallah et al. 2007a, b). The physiological meaning of induction by other compounds reported here is less clear. The multiplicity of responses observed for *COX5b-1* suggests that it may constitute a preferential regulatory target of COX biogenesis, as also suggested by the fact that it is a target of a miRNA involved in the regulation of copper homeostasis, even if it does not encode any of the copper binding subunits of the complex (Yamasaki et al. 2007).

The region located upstream of the G-box is required for the response to sucrose and ABA. This region contains elements with the sequence ATCATT and distalB-like sequences. The distalB element has been previously described in the *napA* promoter, where it drives ABA-dependent seed-specific expression in conjunction with the proxB element (A/TCNAACAC; Ezcurra et al. 1999). It has been suggested that the proxB element constitutes a coupling element that restricts expression from the distalB element to the seed. The fact that a proxB element does not seem to be present in the *COX5b-1* promoter may explain the

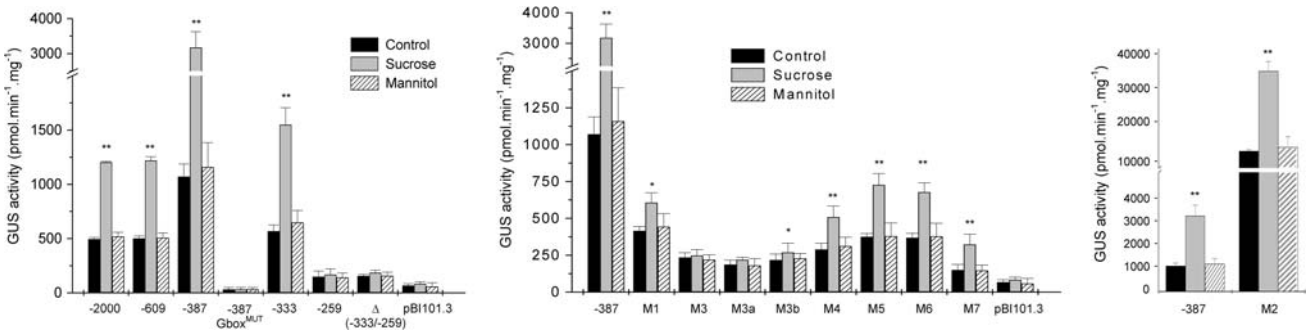

Fig. 7 Identification of promoter regions involved in the response of the *COX5b-1* gene to sucrose. Arabidopsis plants transformed with different wild-type or mutagenized fragments of the *COX5b-1* promoter were grown in MS medium with the addition of 3% sucrose or mannitol, as indicated. GUS activity in protein extracts was measured using the fluorogenic substrate MUG. Activity was measured in extracts from five independent lines for each construct and the bars indicate the mean (±SD) of these measurements. Significance of changes produced after each treatment respective to control was assessed using Student's *t* tests (*$P < 0.05$, **$P < 0.01$). Similar results were obtained in three other experiments in which different line combinations, from a total of 10, were used. The names under the bars correspond to the mutagenized segments shown in Fig. 4a. Numbers indicate the upstream end of the promoter fragment present in each construct respective to the translation start site; the downstream end was at −1 for all constructs

observation that the distalB-like sequences are functional in vegetative tissues. The *napA* promoter also contains a G-box located downstream of distalB (Ezcurra et al. 1999). This supports the notion that, as in *napA*, the G-box and distalB-like sequences may act in combination to drive expression of the *COX5b-1* gene.

The elements containing the sequence ATCATT present in *COX5b-1* seem to be arranged in pairs separated by four nucleotides. We did not find any evidence in the literature of similar elements known to be involved in the expression of other genes. We have only found that similar sequences (TTCATT and ATCATC) present in the *Phaseolus vulgaris* β-phaseolin promoter are strongly protected by tobacco proteins from immature seeds during in vivo footprinting studies (Li and Hall 1999). In the *COX5b-1* promoter, these sequences participate in determining basal levels of expression and induction by sucrose.

The Arabidopsis genome contains a second gene encoding COX5b (*COX5b-2*; At1g80230). The process of duplication that originated both Arabidopsis *COX5b* genes seems to be relatively recent (Welchen et al. 2002). The *COX5b-2* promoter contains functional site II elements, directs expression specifically in root and shoot meristems and in pollen, and is induced by sucrose (unpublished results). Thus, it shares some expression characteristics with *COX5b-1*, but this last one has a broader expression pattern. We speculate that the ancestral form of the *COX5b* gene contained site II elements and that, after duplication, incorporation of new transcriptionally active elements may have allowed the progressive elimination of site II elements from the resulting *COX5b-1* gene promoter. For this to occur, these new elements must have conferred expression characteristics that were required for coordination with other respiratory chain component genes. The fact that some expression characteristics are conserved in both genes produced after duplication may indicate that the existence of more than one gene encoding certain respiratory chain components may be used by the plant not only for the diversification of protein function or expression characteristics, but also to increase the robustness of the genetic system towards changes that may hinder the correct biogenesis of the mitochondrial machinery involved in energy production.

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