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RESEARCH PAPER

The promoter of the *Arabidopsis* nuclear gene *COX5b-1*, encoding subunit 5b of the mitochondrial cytochrome *c* oxidase, directs tissue-specific expression by a combination of positive and negative regulatory elements

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Abstract

In the present work, the promoter of the *Arabidopsis thaliana* nuclear gene *COX5b-1*, encoding subunit 5b of the mitochondrial cytochrome *c* oxidase, has been analysed. For this purpose, plants, stably transformed with different promoter fragments fused to the β-glucuronidase reporter gene, have been obtained. Histochemical staining indicated that the *COX5b-1* promoter directs expression in meristems and in vascular tissues of cotyledons, roots, and hypocotyls, as well as in anthers and pollen and the central leaf vein. Quantitative measurements in extracts prepared from different organs suggested that expression is higher in roots. The analysis of progressive upstream deletions of the promoter suggested the presence of negative regulatory elements, preferentially active in leaves, between nucleotides −609 and −387 from the translation start site. A further deletion down to nucleotide −195 completely abolished expression. The inclusion of sucrose or the cytokinin 6-benzylaminopurine in the culture medium induced *COX5b-1* promoter-dependent β-glucuronidase expression. This induction was observed with all constructs that produced β-glucuronidase activity. Putative regulatory elements involved in the regulation of other genes were detected in the promoter fragment required for expression. A detailed analysis of these elements will help to elucidate the molecular mechanisms that participate in the expression of this and, possibly, other components of the cytochrome *c*-dependent respiratory pathway.

Key words: Cytochrome *c* oxidase, gene expression, mitochondrion, promoter analysis, regulatory elements.

Introduction

The plant mitochondrial respiratory chain ends up in two different pathways that transfer electrons from ubiquinone to O_2 (Brownleader *et al.*, 1997). These pathways differ in their sensitivity to cyanide and their involvement in ATP production. The cyanide-resistant or alternative pathway is composed of a single oxidase encoded by a small number of homologous nuclear genes (Wagner and Krab, 1995; Vanlerberghe and McIntosh, 1997). The cyanide-sensitive or cytochrome *c*-dependent pathway is similar to that found in other organisms and involves cytochrome *c* reductase, cytochrome *c*, and cytochrome *c* oxidase. Cytochrome *c* oxidase is a multimeric complex composed of several different subunits (Capaldi, 1990; Jänsch *et al.*, 1996), two or three of them (depending on the plant species) encoded by the mitochondrial genome (Unseld *et al.*, 1997) and the rest encoded in the nucleus. Up to now, four different nuclear-encoded subunits, namely COX5b, COX5c, COX6a, and COX6b, have been identified in plants (Nakagawa *et al.*, 1990; Kadowaki *et al.*, 1996; Ohtsu *et al.*, 2001; Curi *et al.*, 2003). It is assumed that the biogenesis of this enzyme requires the co-ordinated expression of the different genes present in both genomes.

Most mitochondrial components show enhanced expression in flowers (Huang *et al.*, 1994; Landschütze *et al.*, 1995; Felitti *et al.*, 1997; Heiser *et al.*, 1997; Zabaleta *et al.*, 1998). This correlates with the fact that the number of

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mitochondria per cell increases considerably during flower development (Lee and Warmke, 1979). *In situ* hybridization studies have shown that the expression in flowers is cell-specific, and that there is a good correlation in the expression of several mitochondrial genes (Smart *et al.*, 1994) and the nuclear gene encoding cytochrome *c* (Ribichich *et al.*, 2001), suggesting the existence of co-ordinated expression mechanisms. Besides this organ- or cell-type specific expression, it has been observed that the levels of transcripts encoding cytochrome *c* and the subunits 5b, 6a, and 6b of cytochrome *c* oxidase are modified by the incubation of plants in solutions containing metabolizable sugars (Felitti and Gonzalez, 1998; Welchen *et al.*, 2002; Curi *et al.*, 2003). To gain an insight into the molecular mechanisms involved in the expression of genes encoding cytochrome *c* oxidase components, the promoter region of the *Arabidopsis thaliana* gene *COX5b-1*, one of two genes encoding cytochrome *c* oxidase subunit 5b (COX5b), the most conserved nuclear-encoded cytochrome *c* oxidase subunit (Rizzuto *et al.*, 1991; Grossman and Lomax, 1997) has been analysed. The results indicate that promoter regions located between −387 and −193 of the translation start site are essential for the transcriptional activity of the *COX5b-1* gene and contain regulatory elements involved in tissue-specific expression and induction by carbohydrates and cytokinins. Negative elements required for preferential repression in leaves are present upstream of this region.

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 either 200 mM mannitol, 200 mM sucrose or 50 μM 6-benzylaminopurine (BAP), as indicated. The dishes were kept at 4 °C for 2 d and then transferred to growth chamber conditions and kept under complete darkness for 7 d.

Isolation of genomic clones

A *COX5b-1* EST clone encoding exons two to six (clone 234B12T7; accession no. N65183) was obtained from ABRC. For the isolation of clones containing the *COX5b-1* gene, the cDNA probe was used to screen 1×10^5 pfu from an *Arabidopsis* genomic library (Voytas *et al.*, 1990). Phage DNA was transferred to Hybond-N and, after overnight hybridization, filters were washed and exposed to X-ray films. Positive clones were purified through successive rounds of plating and hybridization. Purified clones were used to isolate phage DNA. A 4 kbp *Hin*dIII fragment from one of the isolated clones, which contains the *COX5b-1* transcribed region down to the fifth intron and upstream sequences, was subcloned in vector pBluescript SK^-. This clone was checked by partial sequencing and named VBATH1.

Reporter gene construct and plant transformation

A 2 kbp *Hin*dIII/*Sal*I fragment from the *COX5b-1* gene, comprising sequences upstream of the ATG initiation codon, was amplified from clone VBATH1 using oligonucleotide COXB12: 5′-GGCGTCGAC-GATGATGATGAGTCAAAG-3′ (*Sal*I site underlined) and universal primer −40 and cloned in the same sites of plasmid pBI101.3 in front of the *gus* coding region. Constructs containing upstream deletions were obtained by PCR amplification with oligonucleotide COXB12 and either COXB13 (5′-CGGAAGCTTATTGTGTACGTCTT-TATC-3′), COXB14 (5′-GGGAAGCTTCTTGCTTGTGCCTGTG-TG-3′), COXB15 (5′-GCCAAGCTTAATATCGAGATTCCAAGT-3′), or COXB16 (5′-CGCAAGCTTTTTGGGGAGTTTCTTCGC-3′), followed by cloning in pBI101.3. The resulting constructs were introduced into *Agrobacterium tumefaciens* strain GV2260, 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 primers specific for *COX5b-1* and the *gus*-specific primer 5′-TTGGGGTTTCTACAG-GAC-3′. Ten independent lines for each construct (five for the construct including the −609/−1 promoter fragment) were further reproduced and homozygous T_3 and T_4 plants were used to analyse *gus* expression. Plants transformed with pBI101.3 or pBI121 were obtained in a similar way.

β-glucuronidase assays

β-glucuronidase (GUS) activity of transgenic plants was analysed 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), 0.1% Triton X-100 and, after applying vacuum for 5 min, they were incubated at 37 °C until satisfactory staining was observed. Tissues were cleared by immersing them in 70% ethanol and images were taken with a Nikon Coolpix 995 digital camera connected to a light microscope or a stereomicroscope.

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 13 000 *g* for 10 min. GUS activity in supernatants was measured in extraction buffer containing 1 mM MUG and 20% methanol. Reactions were stopped 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. The protein concentration of extracts was determined as described by Sedmak and Grossberg (1977).

Results

The COX5b-1 *promoter directs tissue-specific GUS expression*

An *Arabidopis thaliana* genomic region covering 2002 nt upstream of the initiation codon of the *COX5b-1* gene was isolated by direct screening of a genomic library using EST 234B12T7 as probe, followed by subcloning into pBluescript SK^-. To characterize the expression patterns conferred by this promoter region, the entire 2 kbp fragment was cloned in front of the *gus* gene coding region contained in the binary vector pBI101.3 and this clone was used to obtain *Arabidopsis* transgenic plants by *Agrobacterium*-mediated transformation. Five independent transgenic lines were analysed by histochemical staining of GUS activity

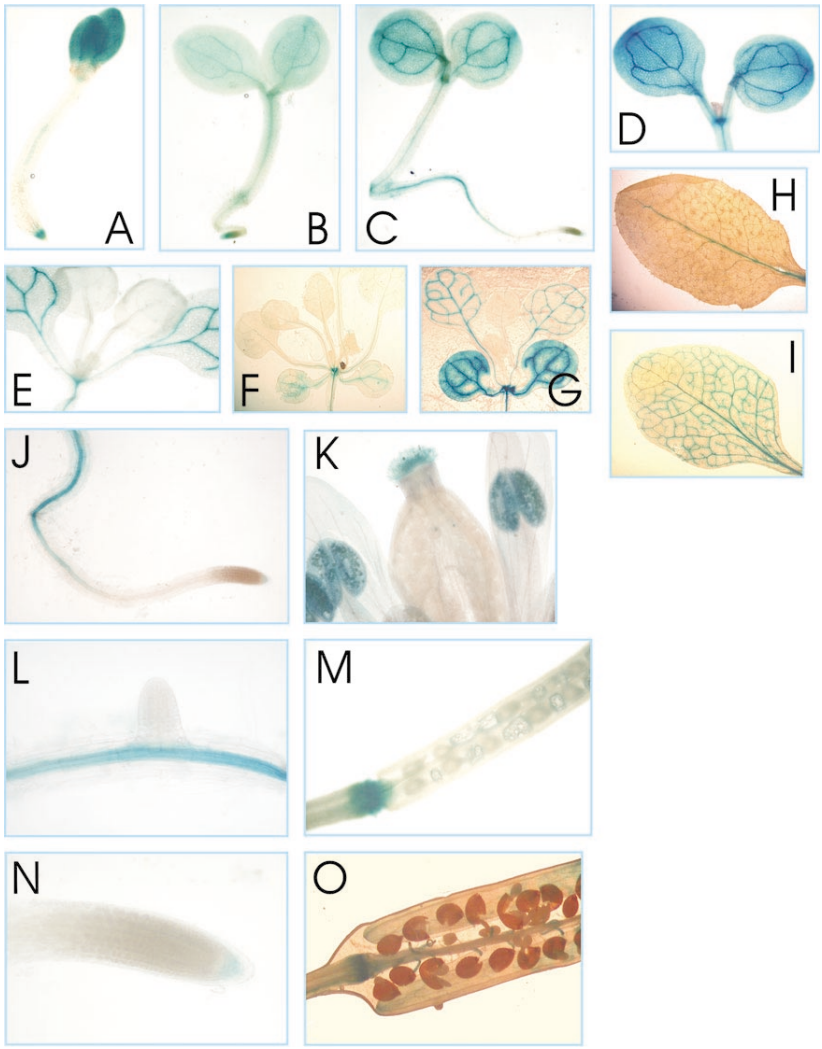

Fig. 1. Histochemical localization of GUS activity in *Arabidopsis* plants transformed with the *COX5b-1* promoter fused to the *gus* reporter gene. Except where indicated, plants transformed with the 2 kbp *COX5b-1* promoter fragment are shown. (A–D) 2-, 3-, 5-, and 6-d-old seedlings, respectively. (E, F) 10- d-old and 15-d-old plants. (G) 15-d-old plant carrying the 387 bp proximal promoter fragment. (H, I) Mature leaves from plants carrying the 2 kbp or the 387 bp promoter fragments, respectively. (J, L, N) Primary roots from mature plants. (K) Mature flowers. (M, O) Developing and mature siliques, respectively.

using X-gluc as substrate. At early stages of development, GUS activity was detected mainly in cotyledons and the root meristem (Fig. 1A). Upon seedling growth, staining was progressively localized to the vascular cylinder of the root and hypocotyl and the vascular tissues of the cotyledons (Fig. 1B, C). GUS activity was also detected in the root and shoot meristems (Fig. 1B–D), but not in leaf primordia (Fig. 1D, E). Young leaves were not stained, but older leaves displayed GUS activity mainly in the central vein (Fig. 1E, F, H). In adult plants, expression was detected preferentially in the vascular cylinder of primary and secondary roots (Fig. 1J, L) and in anthers, more specifically in pollen grains (Fig. 1K). Expression was also evident in the root meristem (Fig. 1N), in the central vein of leaves (Fig. 1H), in the stigma (Fig. 1K), and in pedicels at the junction with siliques (Fig. 1M, O).

The amount of GUS activity present in different organs was quantitatively assessed by a fluorometric GUS assay using protein extracts prepared from transgenic plants. GUS activity, related to the total amount of protein, was significantly higher in roots (1520 ± 270 pmol min^{-1} mg^{-1}) than in all other organs tested (leaves, stems, flowers, and siliques). The activity in leaves showed an intermediate level (450 ± 120 pmol min^{-1} mg^{-1}). The results indicate that the promoter region of *COX5b-1* directs organ- and cell-type specific expression. The patterns of GUS histochemical staining suggest that *COX5b-1* is predominantly expressed in proliferating tissues, perhaps in relation with

cell division or metabolic activity, and in vascular tissues throughout development.

Induction of the COX5b-1 *promoter by sucrose and BAP*

It has previously been shown that *COX5b-1* transcript levels are regulated by the incubation of plants in solutions containing metabolizable carbohydrates (Welchen *et al.*, 2002). To investigate if this regulation takes place at the transcriptional level, plants were grown in Petri dishes containing MS medium supplemented with either mannitol, as a control of the effect of osmotic pressure, or sucrose. The GUS-specific activity of extracts prepared from independent transgenic lines was 2–3-fold higher when plants were grown in the presence of sucrose (Fig. 2A), suggesting the existence of transcriptional regulation by carbohydrates.

The effect of hormones on the expression of the *COX5b-1* gene was also tested. In northern blot experiments, it was observed that the treatment of plants with the cytokinin BAP produces an increase in *COX5b-1* transcript levels (not shown). Accordingly, GUS activity was also measured after BAP treatment in plants carrying the *COX5b-1* promoter–*gus* fusion. As shown in Fig. 2B, this treatment produced a significant increase in GUS specific activity with respect to untreated plants in several independent lines. Induction by BAP seems to be slightly higher than that observed with sucrose.

The combined effect of sucrose and BAP was also tested. Fluorometric GUS activities of extracts prepared from plants treated with both compounds did not differ significantly from activities exhibited by extracts from BAP-treated plants. This suggests that BAP and sucrose may exert their effects through common components or regulatory elements.

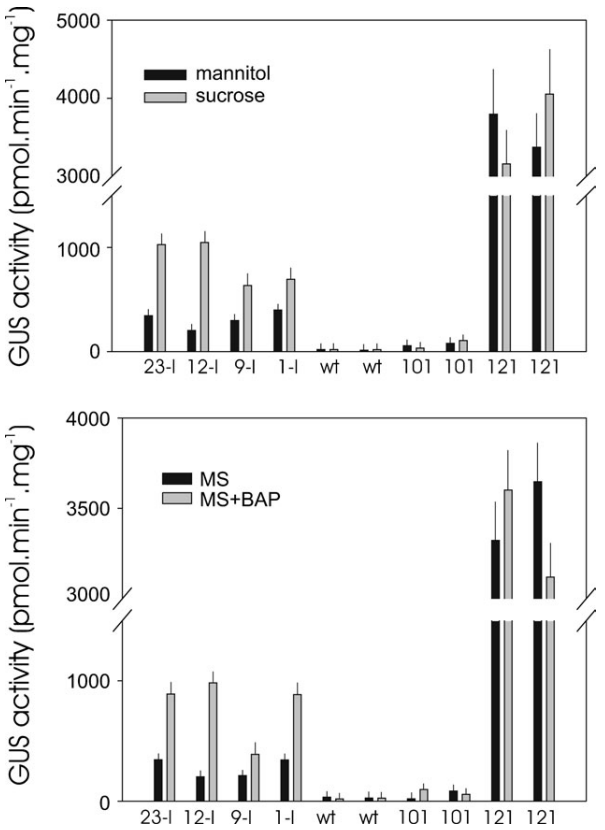

Fig. 2. Induction of the *COX5b-1* gene by sucrose and cytokinins. GUS activity was measured using the fluorogenic substrate MUG and protein extracts prepared from 7-d-old seedlings grown in MS medium alone or supplemented with either 200 mM mannitol, 200 mM sucrose or 50 μM BAP, as indicated. The plants used for the analysis were four independent lines carrying the 2 kbp *COX5b-1* promoter–*gus* fusion (23-I, 12-I, 9-I, and 1-I), and two batches of plants carrying either the promoterless *gus* gene (101) or the *gus* gene fused to the 35SCaMV promoter (121). Activity was also measured in extracts from non-transformed plants (wt). Bars indicate mean specific activity (±SD) of three independent measurements.

The COX5b-1 *promoter contains positive and negative regulatory elements*

To define the minimal promoter region required for the observed responses, a series of nested deletions from the upstream portion of the 2 kbp promoter fragment was performed (Fig. 3A). The different constructs were introduced into *Arabidopsis* and GUS activity was analysed in transformed plants. Fluorometric assays indicated that a fragment located between −387 and −195 of the start codon is required for *COX5b-1* transcription, since no GUS activity could be detected in plants carrying the proximal 195 bp fragment fused to *gus* (Fig. 3B). In accordance, histochemical analysis of these plants using X-gluc did not show GUS expression in any tissue or cell type. A similar result was obtained with plants transformed with the −96 bp construct (not shown). Interestingly, the −387 construct produced plants with considerably higher GUS activity than plants carrying larger promoter fragments, suggesting that a negative regulator of transcription is located between −609 and −387 (Fig. 3B). Although differences in transcriptional activity may arise from the fact that the transgenes are inserted in different parts of the genome, higher transcription rates were consistently observed in several lines containing the −387 construct. In addition, while plants transformed with larger constructs displayed higher GUS activities in roots with respect to leaves, those carrying the −387 construct showed the opposite behaviour (Fig. 4). This difference was mainly due to an increase in the specific GUS activity of leaves transformed with the −387 construct (Fig. 4), suggesting that the repression effect of the upstream portion operates more effectively in leaves. This fact was also evident in histochemical GUS assays of plants carrying the 387 bp promoter fragment. These plants showed strong staining in leaves of different ages, mainly in vascular tissues, that were not stained (except for the central vein) in plants carrying larger promoter fragments (Fig. 1F–I). In addition, a positive regulatory element seems to be present upstream from −609, since plants carrying only the 609 bp

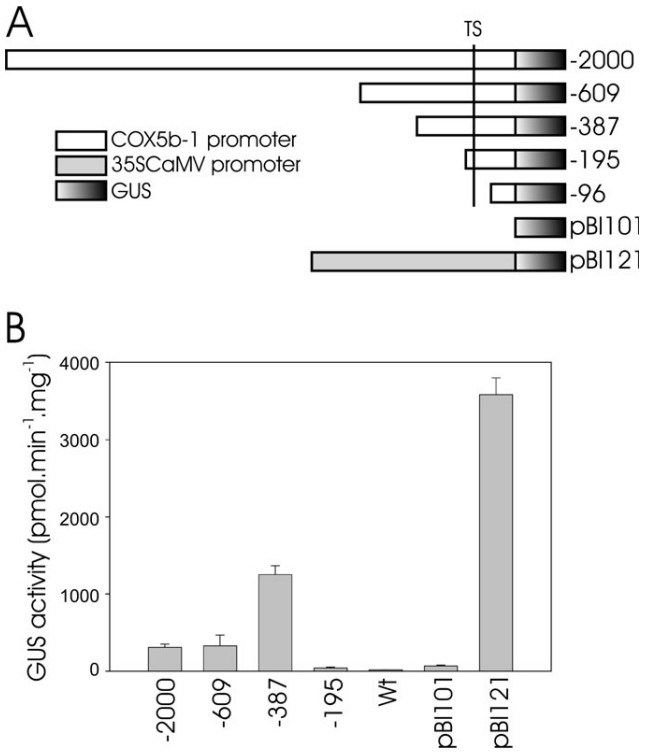

Fig. 3. Analysis of GUS activity driven by truncated forms of the *COX5b-1* promoter. (A) Constructs used for the analysis of the *COX5b-1* promoter. Numbers indicate the upstream end of the promoter fragment present in each construct, respective to the translation start site; the downstream end was at −1 for all constructs. The putative transcription start site is indicated (TS). Plants were also transformed with the promoterless *gus* gene (pBI101) or with the *gus* gene under the control of the 35SCaMV promoter (pBI121). (B) GUS expression levels in plants carrying the different constructs. Specific GUS activity was determined using protein extracts prepared from seedlings transformed with the constructs indicated in (A). The results indicate the mean (±SE) of five independent lines measured in two different experiments. Numbers below the bars indicate the respective construct. GUS activity was also measured in extracts from non-transformed plants (wt).

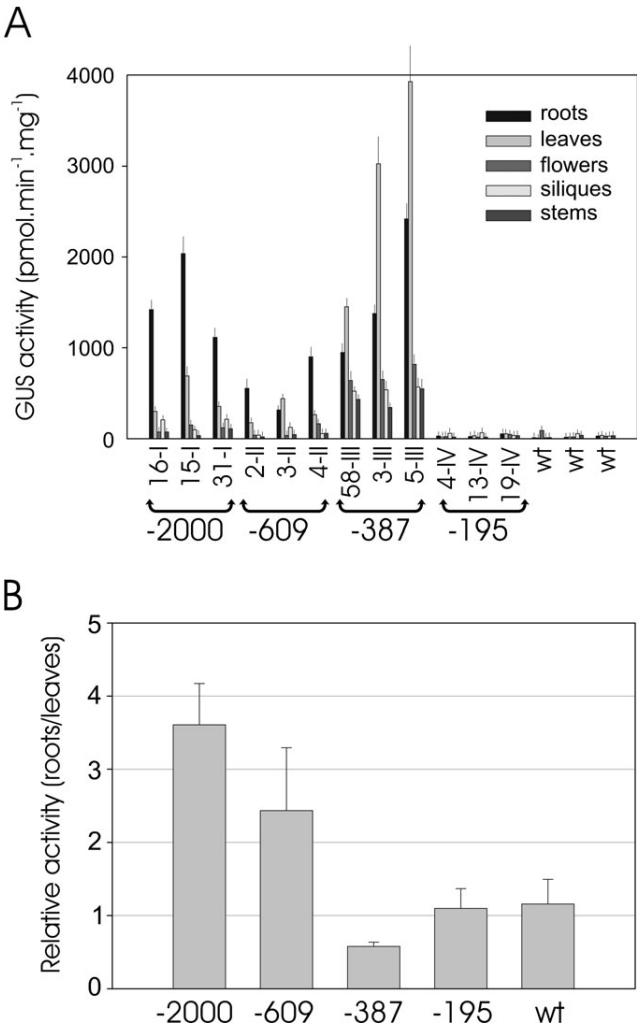

Fig. 4. Analysis of GUS activity in protein extracts from different organs of transformed plants. (A) Specific GUS activity (mean ±SD of three independent measurements) was determined using protein extracts prepared from different organs (as indicated) of plants carrying upstream deletions of the *COX5b-1* promoter fused to *gus*. The plants used for the analysis were 12 independent lines carrying either the 2 kbp *COX5b-1* promoter–*gus* fusion (16-I, 15-I, and 31-I), or truncated forms up to −609 (2-II, 3-II, and 4-II), −387 (58-III, 3-III, and 5-III), or −195 (4-IV, 13-IV, and 19-IV). Activity was also measured in extracts from non-transformed plants (wt). The relative activity in root extracts versus that in leaves is shown in (B). Error bars in (B) represent SE.

proximal promoter fragment displayed reduced levels of GUS activity in both fluorometric (Fig. 3B) and histochemical assays (not shown).

Regarding the transcriptional regulation by carbohydrates and cytokinins, all constructs that produced GUS expression also showed induction by these compounds at similar levels than those observed with the larger construct (Fig. 5). The putative elements involved in induction seem then to be located in the same −387 to −195 region required for basal expression. It cannot be ruled out that the same elements are involved in basal expression and induction by these compounds. The presence of elements necessary, but not sufficient, for expression and induction within the −195 region cannot be ruled out either.

Putative regulatory elements present in the COX5b-1 *promoter*

The region from −387 to −1 was analysed for the presence of known plant regulatory elements that could be related to the expression patterns observed in transgenic plants using the PLACE database (Higo *et al.*, 1999). A canonical TATA-box near the putative transcription start site, deduced from the longest cDNA available in data banks, was not found. TATA-less promoters are frequent in nuclear genes encoding mammalian COX subunits (Grossman and Lomax, 1997). A total number of ten motifs involved in pollen-specific expression (Bate and Twell, 1998; Rogers *et al.*, 2001) and a region exactly matching the pollen-quantitative element of the maize *ZM13* gene (AGGTCA in the reverse orientation; Hamilton *et al.*, 1998) were

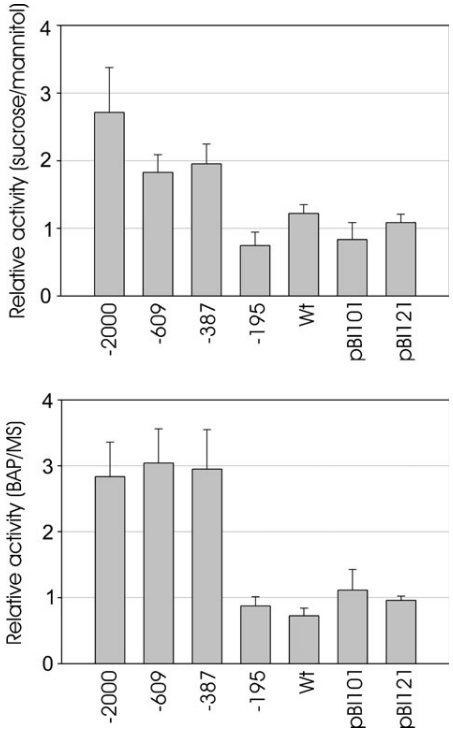

Fig. 5. Induction of GUS activity by sucrose and BAP in plants carrying different *COX5b-1* promoter fragments. Specific GUS activity was determined using protein extracts prepared from transgenic seedlings carrying different upstream deletions of the *COX5b-1* promoter fused to *gus* grown in MS medium alone or supplemented with either 200 mM mannitol, 200 mM sucrose or 50 μM BAP. The bars indicate the relative GUS activity (mean ±SE) in the presence of sucrose versus mannitol (upper panel) or BAP versus MS (lower panel). Five independent lines were analysed for each construct in two different experiments. The numbers below the bars (−2000, −609, −387, and −195) indicate the upstream end of the promoter fragment present in each construct, with respect to the translation start site; the downstream end was at −1 for all constructs. GUS activity was also measured in extracts from non-transformed plants (wt) or from plants carrying the promoterless *gus* gene (pBI101) or the *gus* gene under the control of the 35SCaMV promoter (pBI121).

observed. Among the others, it is worth mentioning two close ACGT boxes located between −200 and −230. The first of these motifs matches the G-box (CACGTG) involved in binding transcription factors of the b-Zip family related to different regulatory signals (Menkens *et al.*, 1995). The sequence surrounding the G-box (ACGTGTC) contains the ABRE consensus found in genes regulated by abscisic acid (Hattori *et al.*, 2002). The second ACGT motif (GTACGTG) is similar to the one involved in the expression of the glutelin *GluB-1* gene in rice endosperm (Washida *et al.*, 1999; Wu *et al.*, 2000). Notably, a GCN4 motif (TGAGTCA) is present at −15. This element is also involved in the expression of the rice *GluB-1* gene together with the ACGT box and an AACA box (Washida *et al.*, 1999; Wu *et al.*, 2000). Adjacent to the second ACGT box, a GT-1 element is also present (Villain *et al.*, 1996). Within the region that, when removed, produces an increase in the expression in leaves (−609 to −388), the presence of two copies of the AACCAA motif, named REalpha by Degenhardt and Tobin (1996), was observed. This element, present in the *Lhcb2-1* gene of *Lemna gibba*, binds nuclear proteins which are more abundant in etiolated leaves, suggesting that they may act as repressors. It is speculated that similar interactions may be responsible for the down-regulation of the *COX5b-1* gene in leaves.

Discussion

Accumulating evidence suggests that several genes encoding mitochondrial respiratory chain components are co-ordinately regulated. Transcript levels for a set of these genes are considerably higher in flowers and, specifically, in anther tissues (Smart *et al.*, 1994; Zabaleta *et al.*, 1998; Ribichich *et al.*, 2001). It has also been shown that a group of genes for components of the cytochrome *c*-dependent pathway are similarly regulated by carbon and nitrogen sources (Welchen *et al.*, 2002; Curi *et al.*, 2003). However, little is known about the elements that govern the transcriptional regulation of these genes. Zabaleta *et al.* (1998) have studied the promoter regions involved in pollen/anther expression of three genes that encode components of the NADH dehydrogenase (Complex I). Within these regions, they have identified conserved GT-rich elements similar to those found in other genes expressed in pollen. They have postulated that these motifs are involved in the co-ordinated expression of these three genes.

In this work, the promoter region of the *COX5b-1* gene from *Arabidopsis*, one of two genes encoding cytochrome *c* oxidase subunit 5b has been analysed. Using northern experiments, it has previously been shown that both *COX5b* genes show similar expression characteristics (Welchen *et al.*, 2002). While the *COX5b-1* gene, as those encoding Complex I components mentioned above, shows expression in anthers and pollen, it is also expressed at different levels in vegetative tissues, notably roots and leaves. It seems then that the expression patterns of genes encoding components of two different respiratory chain complexes seem only partially to overlap. It would be interesting to know if genes for other cytochrome *c* oxidase subunits show a similar behaviour. It has previously been shown that transcript levels of genes encoding subunits 5b, 6a, and 6b show similar responses to carbon and nitrogen compounds (Welchen *et al.*, 2002; Curi *et al.*, 2003). An *in silico* analysis of the promoter regions of the *Arabidopsis* nuclear genes encoding these subunits indicates the presence of common putative regulatory elements in some of them (Table 1). Further analysis of these promoters will indicate whether they direct similar expression patterns.

It has been found that sequences located between −387 and −195 are required for expression in all organs. Since

Table 1. *Known regulatory motifs shared by COX5b-1 and other COX gene promoters*

A 1 kbp region located upstream of the translation start site of the *COX5b*, *COX6a* and *COX6b* genes was screened for the presence of known regulatory motifs. Only those motifs with a complexity of six or more nucleotides are indicated. *COX6b-4* may be a pseudogene.

Motif	Location in *COX5b-1*	Other *COX* genes (location)	Name and function	Reference
ACGTGKC	−223	*COX5b-2* (−628), *COX6b-2* (−402), *COX6b-4* (−88; −109)	ABRE, involved in ABA responsiveness	Hattori *et al.*, 2002
CACGTG	−223	*COX5b-2* (−628), *COX6b-4* (−111)	G-box, involved in transcriptional regulation in several genes	Menkens *et al.*, 1995
CAANNNNATC	−34; −467	*COX5b-2* (−315), *COX6b-1* (−264; −579), *COX6b-2* (−979), *COX6b-3* (−102; −777)	Circadian expression of tomato Lhc gene	Piechulla *et al.*, 1998
TGAGTCA	−11	*COX5b-2* (−500), *COX6b-3* (−394)	GCN4 motif, involved in endosperm expression	Washida *et al.*, 1999
AGGTCA	−56	*COX5b-2* (−711), *COX6a* (−722), *COX6b-2* (−260)	Q-element, involved in pollen expression	Hamilton *et al.*, 1998
GGTTAA	−191	*COX6b-3* (−15; −71; −153; −168)	GT1 motif, involved in transcriptional regulation of several genes	Villain *et al.*, 1996
TAACTG	−361	*COX6a* (−632), *COX6b-3* (−173)	At-Myb2 binding site, involved in water-stress responses	Urao *et al.*, 1993
AACCAA	−474; −401	*COX6b-1* (−333; −478), *COX6b-3* (−99; −922), *COX6b-4* (−23; −708; −937)	REalpha, involved in phytochrome regulation	Degenhardt and Tobin, 1996

the putative transcriptional start site is present at −163 from the initial ATG, this means that most relevant promoter elements are proximal to this site. This is also true for the elements involved in regulation by sucrose and cytokinins. Motifs involved in the partial repression of the *COX5b-1* gene in leaves must be present further upstream (from −609 to −387).

Cytokinin treatment induced the expression of the *COX5b-1* gene. Since cytokinins are known to induce cell proliferation (D'Agostino and Kieber, 1999), it is logical to assume that the expression of genes involved in mitochondrial metabolism may be up-regulated in response to cell proliferation, perhaps related to the existence of higher energetic demands. It is interesting to note that the expression patterns observed in histochemical GUS assays also indicate that *COX5b-1* is preferentially expressed in proliferating tissues. Accordingly, the tissue-specific patterns of *COX5b-1* expression may be the consequence of responses to cell-specific factors or to metabolic changes associated with cell proliferation. The observed regulation by sucrose may be a consequence of the same type of response. This may explain why the same promoter region seems to determine expression in organs and inducibility by sucrose and cytokinins.

In summary, it has been established that the promoter of the *COX5b-1* gene directs expression in specific organs and tissues by a combination of negative and positive elements. Induction by carbohydrates and cytokinins has also been observed. A detailed analysis of the promoter elements that confer the observed responses will be helpful to elucidate the molecular mechanisms involved in the expression of this and other genes encoding components of the cytochrome *c*-dependent mitochondrial respiratory pathway.

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