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Identification of regulatory elements involved in expression and induction by sucrose and UV-B light of the *Arabidopsis thaliana COX5b-2* gene, encoding an isoform of cytochrome *c* oxidase subunit 5b

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The promoter sequences required for expression of the *Arabidopsis thaliana COX5b-2* gene, encoding an isoform of cytochrome *c* oxidase subunit 5b, were analyzed using plants transformed with deleted and mutagenized forms of the promoter fused to *gus*. A 1000-bp promoter fragment produces expression in root and shoot meristems, leaf and cotyledon tips, and anthers. Deletion analysis indicated the presence of positive and negative regulatory elements. A regulatory element located between −660 and −620 from the translation start site was identified as a G-box by mutagenic analysis. Mutation of the G-box, that is present within the coding region of the preceding gene in the genome, increases expression of *COX5b-2* in cotyledon and leaf lamina and abolishes induction by ultraviolet-B (UV-B) light, which presumably acts through the removal of an inhibitory factor. Identified positive regulatory elements include a site II element (TGGGCC), a related element with the sequence TGGGTC and four initiator elements (YTCANTYY) that completely abolish expression when mutated in combination. Site II elements are also involved in the response to sucrose. The results imply that the *COX5b-2* gene has retained expression characteristics presented by most respiratory chain component genes, but its expression mechanisms have diverged from those employed by *COX5b-1*, the other gene encoding cytochrome *c* oxidase subunit 5b in Arabidopsis.

Introduction

Mitochondrial biogenesis requires the expression of genes located in the nucleus and within the organelle. An example of this requirement is the enzyme cytochrome *c* oxidase (COX), or complex IV, that is composed of three subunits encoded in the organelle in most eukaryotes and a variable number of subunits encoded in the nuclear genome (Barrientos et al. 2002). Plant COX contains six to seven nuclear-encoded subunits, some of them homologous to polypeptides present in COX from other organisms and others that seem to be specific from plants (Jänsch et al. 1996, Millar et al. 2004). One of the questions regarding the biogenesis of this enzyme is how the expression of the many different genes that encode its polypeptidic components is coordinated to achieve an efficient assembly of the complex. Conditions that produce an increase in mitochondrial biogenesis,

Abbreviations – COX, cytochrome *c* oxidase; EMSA, electrophoretic mobility shift assay; GUS, β-glucuronidase; MS, Murashige and Skoog; MUG, 4-methylumbelliferyl β-D-glucuronide; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; UV-B, ultraviolet-B; X-gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid.

such as the development of anthers and pollen (Huang et al. 1994, Lee and Warmke 1979, Smart et al. 1994) and the re-addition of sucrose to starved cells in culture (Giegé et al. 2005), are expected to require the active biosynthesis of respiratory complexes in a coordinated manner. Using the cell culture system, Giegé et al. (2005) showed that the synthesis of subunits encoded in the organelle is not coordinated with the synthesis of those encoded in the nucleus and proposed that the amount of nuclear-encoded subunits is the limiting factor for complex assembly. Accordingly, several nuclear genes that encode respiratory chain components show increased expression in anthers and are induced by sucrose or other carbohydrates (Elorza et al. 2004, Gonzalez et al. 2007, Mufarrege et al. 2009, Welchen and Gonzalez 2005, Welchen et al. 2004, Zabaleta et al. 1998).

Specifically for COX, it was observed that transcript levels of nuclear genes encoding subunits 5b, 6a and 6b are higher when plants are incubated in the presence of carbohydrates, but this is not observed for the organellar gene *COX2* (Curi et al. 2003, Welchen et al. 2002). Induction by carbohydrates has also been observed for the two *Arabidopsis* genes encoding cytochrome *c*, whose activity is closely related to COX function (Welchen et al. 2002). Studies of the promoter regions of the different genes mentioned above have uncovered the existence of common and divergent regulatory mechanisms. An example of conservation is given by the presence of elements known as site II (TGGGCY) in the proximal upstream regions of the six genes that encode different isoforms of cytochrome *c* and COX subunits 6a and 6b (Welchen and Gonzalez 2006). While all of these elements were shown to be functional by mutagenic analysis, the effect of their mutation varies from a complete loss of expression to a marginal effect depending on the gene (Mufarrege et al. 2009, Welchen and Gonzalez 2005, Welchen et al. 2009). This may be explained by the presence of additional, gene-specific elements that differently influence the action of site II elements. Site II elements were also shown to be required for the response to sucrose of some of the genes, usually in combination with other elements too (Mufarrege et al. 2009, Welchen et al. 2009).

An interesting case is that of *COX5b* genes from Arabidopsis. Northern analysis indicated that these genes, namely *COX5b-1* (At3g15640) and *COX5b-2* (At1g80230), show different expression patterns, and that *COX5b-1* is expressed in most tissues. In addition, both genes are induced by sucrose. The *COX5b-1* promoter directs expression in anthers, meristems and vascular tissues of leaves, cotyledons, roots and hypocotyls (Welchen et al. 2004). It has been shown that an essential G-box and particular upstream elements are involved in the response of this gene to sucrose (Comelli et al. 2009). In the present study, we investigated in detail the structure of the promoter of the *COX5b-2* gene. We have found that the expression characteristics of this gene are determined by site II-like and initiator elements, together with an upstream G-box that has a negative effect on expression. In addition, site II-like elements and the G-box are involved in the responses of this gene to sucrose and ultraviolet-B (UV-B) light, respectively.

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 100 μmol 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

A fragment spanning 1000 bp upstream of the ATG initiation codon of *A. thaliana COX5b-2* (At1g80230) was obtained by polymerase chain reaction (PCR) amplification of Arabidopsis genomic DNA using primers listed in Table S1. The resulting fragment, containing *Bam*HI and *Hin*dIII sites, was cloned in the binary vector pBI101.3. Deletions of upstream portions of the promoter were constructed in a similar way using different upstream primers (Table S1). Complementary primers were used for the introduction of specific mutations in putative regulatory elements (Table S1), using overlap extension mutagenesis by PCR (Silver et al. 1995). 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 β-glucuronidase (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.

GUS assays

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 m*M* X-gluc solution in 100 m*M* 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 m*M* sodium phosphate, pH 7.0, 10 m*M* ethylenediaminetatraacetic acid (EDTA), 10 m*M* β-mercaptoethanol) containing 0.1% (w/v) sodium dodecyl sulfate (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 m*M* 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

For electrophoretic mobility shift assays (EMSAs), aliquots of nuclear extracts (10 µg) 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 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.5 m*M* EDTA, 1.0 m*M* dithiothreitol (DTT), 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 × Tris-borate-EDTA (TBE) plus 2.5% glycerol; 1 × TBE is 90 m*M* Tris-borate, pH 8.3, 2 m*M* EDTA). The gel was run in 0.5 × TBE at 30 mA and 4°C for 1.5 h and dried prior to autoradiography. Nuclear extracts were prepared from cauliflower buds (obtained from a local market) as described by Maliga et al. (1995).

Real-time RT-PCR measurements

RNA for real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (1 µg) was used for reverse transcription reactions using 200 units of M-MLV reverse transcriptase (Promega Corp. Madison, WI, USA) in a final volume of 30 µl. Quantitative PCR was carried out using an MJ Research Chromo4 apparatus in a 25 µl final volume containing 1 µl SYBR Green, 10 pmol each of primers 5b2realtime-F and 5b2realtime-R, 3 m*M* $MgCl_2$, 5 µl of the reverse transcription reaction and 0.25 units platinum *Taq* polymerase (Invitrogen). Fluorescence was measured at 82°C during 40 cycles. Relative *COX5b-2* transcript levels of treated vs 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

The *COX5b-2* promoter contains positive and negative regulatory elements

As a first step toward the elucidation of the mechanisms involved in expression of the *Arabidopsis COX5b-2* gene, we cloned different fragments containing sequences located upstream of the translation start site in front of the *uidA* (*gus*) reporter gene (Fig. 1A) and used these constructions to transform Arabidopsis plants. As shown in Fig. 1A, some of the constructs comprised transcribed regions from the upstream *At1g80240* gene. The larger promoter fragment used, of 1000 bp, directed expression specifically in root and shoot meristems, at the tip of cotyledons and leaves, and in anthers (Fig. 1B). Expression was also visible in the receptacle and stigma of flowers and siliques. GUS activity measurements in protein extracts indicated that expression is higher in flowers than in leaves (Fig. 1D, E). Interestingly, removal of a 380-bp upstream portion of the promoter originated the appearance of expression in leaf and cotyledon lamina and in the entire root (Fig. 1B, construct −620),

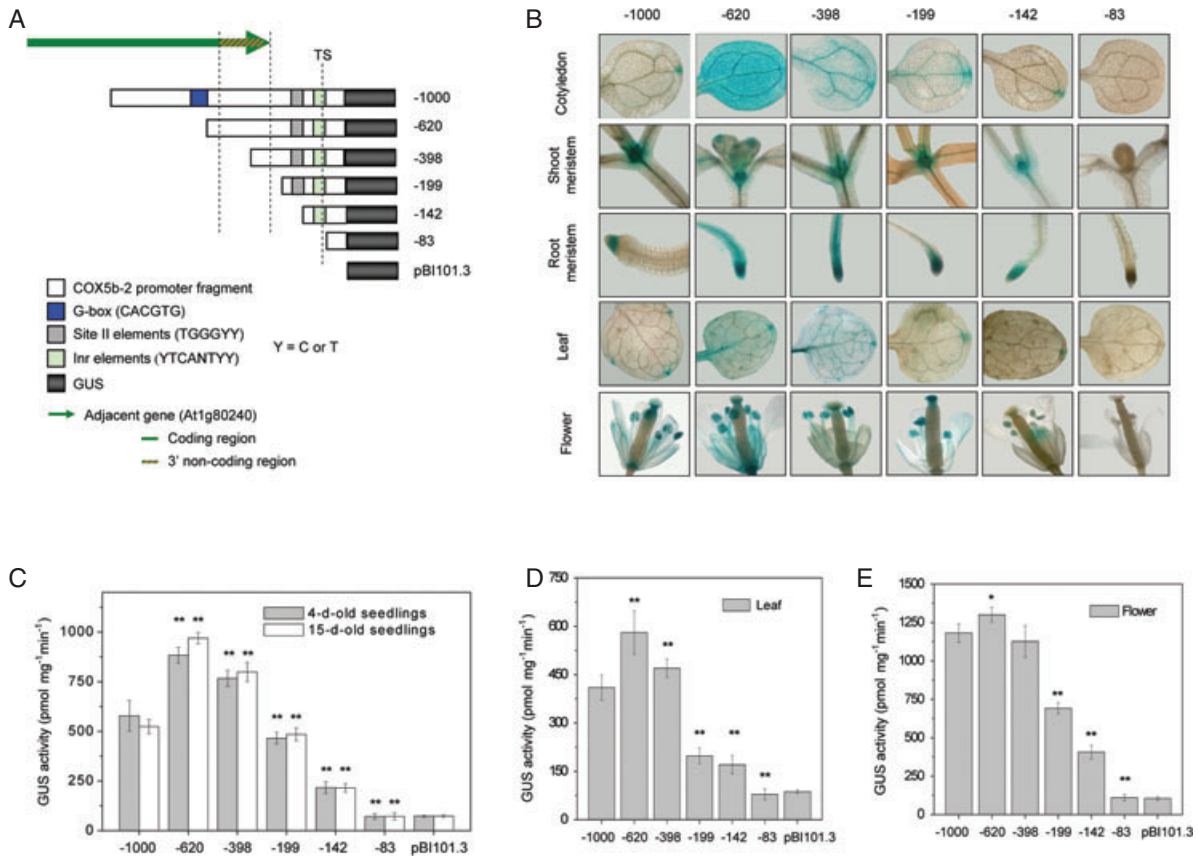


Fig. 1. Analysis of GUS activity driven by the *COX5b-2* promoter. (A) Schematic representation of different constructs used to transform Arabidopsis plants. The numbers indicate the upstream end of the promoter fragment present in each construct respective to the *COX5b-2* translation start site; the downstream end was at -1 for all constructs. TS indicates the putative transcription start site according to available cDNA sequences. The green arrow indicates the location of the adjacent *At1g80240* gene that is transcribed from the same strand as the *COX5b-2* gene. The location of elements analyzed in this work is indicated. (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 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-day-old seedlings (C), leaves (D) or flowers (E) of plants transformed with the different constructs. Plants transformed with the promoterless *gus* gene (pBI101.3) were also used. Significance of changes produced after each deletion 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.

suggesting that a negative regulatory element is present in this region. Indeed, an increase in GUS activity levels in protein extracts prepared from seedlings and leaves was evident upon deletion of the 380-bp segment (Fig. 1C, D). Further deletions produced a progressive decrease in GUS activity until its complete disappearance when only the downstream 83 bp were left (Fig. 1B-E). These results point to the existence of several positive elements located in different portions of the promoter.

A G-box is responsible for the repressive effect of the upstream portion of the *COX5b-2* promoter

Analysis of the 380-bp segment that produces an increase in expression when removed indicated the existence of a G-box (CACGTG), an element involved in the expression of a vast number of genes in plants (Ishige et al. 1999, Menkens et al. 1995, Salinas et al. 1992). To explore the possibility that this element participates in the repression of the *COX5b-2* gene, we constructed a promoter form with a slightly shorter deletion of its upstream portion (340 bp instead of 380 bp) so that the G-box and surrounding nucleotides become included (Fig. 2A). GUS expression levels and patterns produced by this construct were very similar to those produced by the 1000-bp fragment (Fig. 2A), suggesting that the negative element is present between −660 and −620 from the translation start site. We then assayed the effect of mutagenizing the G-box in the −1000 and the −660 constructs. Both

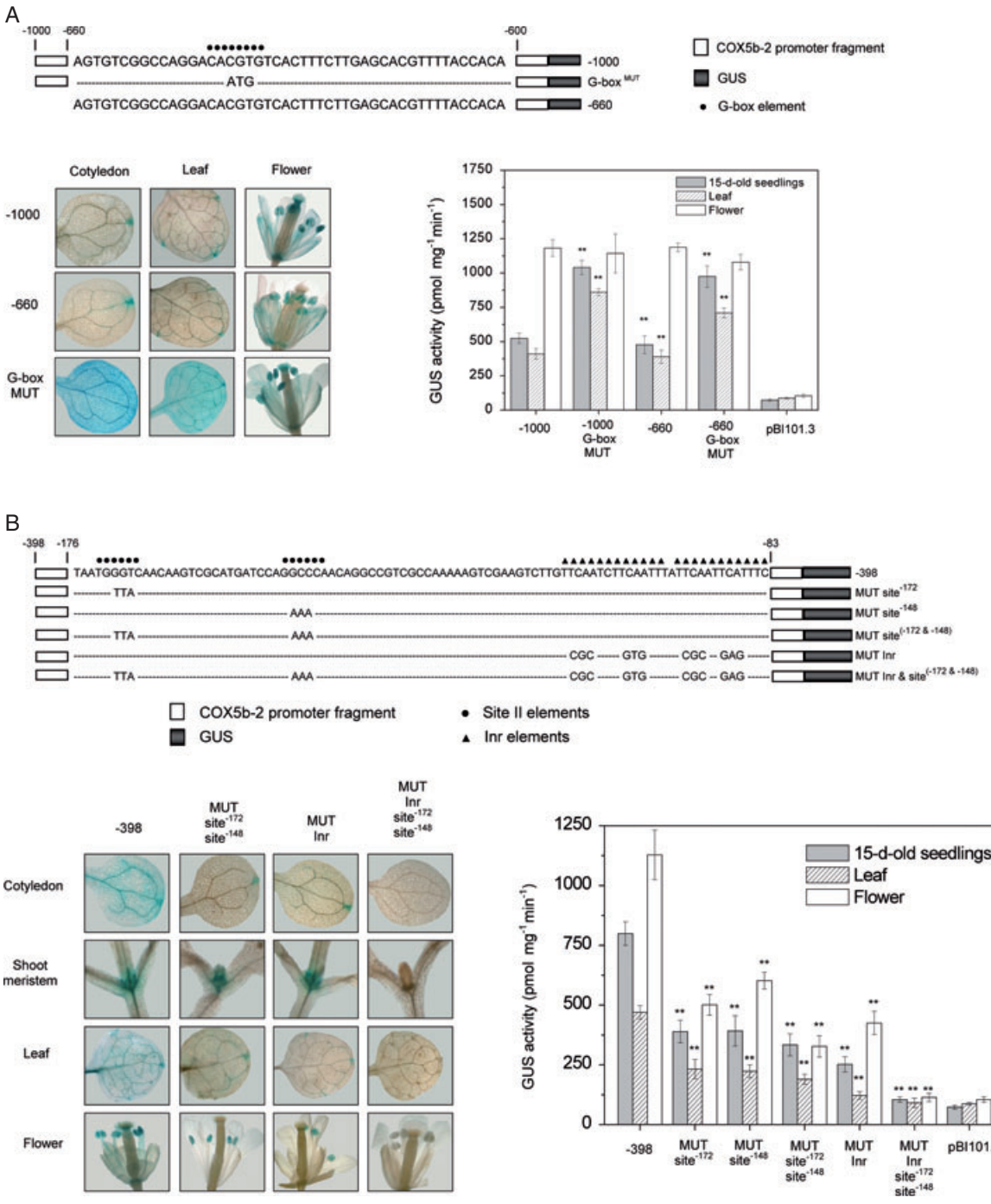


Fig. 2. Analysis of reporter gene expression in Arabidopsis plants transformed with mutagenized *COX5b-2* promoter fragments fused to *gus*. (A) Histochemical and fluorometric analysis of plants transformed with −1000 or −660 promoter fragments carrying mutations in the G-box compared with plants transformed with the respective non-mutagenized fragments. The sequence of the fragment spanning nucleotides −660 to −600 and the nucleotides that were modified are shown above. (B) Histochemical and fluorometric analysis of plants transformed with the −398 promoter fragment carrying mutations in the site II elements or the initiator elements compared with plants transformed with the non-mutagenized fragment. The sequence of the fragment spanning nucleotides −176 to −83 and the nucleotides that were modified in each construct are shown above. Activity was also measured in extracts from plants transformed with a promoterless *gus* gene (pBI101.3). 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 (**$P < 0.01$). Similar results were obtained in three other experiments in which different line combinations, from a total of 10, were used.

mutant promoter forms produced expression in cotyledon and leaf lamina and increased GUS activity levels in seedlings and leaves, but not in flowers, respective to the non-mutated forms (Fig. 2A). We conclude that the G-box located at -636 acts as a negative regulatory element for expression in vegetative parts of the plant.

Two site II-like elements and initiator elements participate in the establishment of basal expression levels of the *COX5b-2* gene

As mentioned in the Introduction section, site II elements are required for expression of several genes encoding components of the respiratory chain. A site II element (TGGGCC in the non-coding strand) is present in the *COX5b-2* promoter in the region, located between −199 and −142, that produces a decrease in expression when removed. Because site II elements usually occur in closely located pairs (Tremousaygue et al. 2003, Welchen and Gonzalez 2006), we also assayed the importance of the sequence TGGGTC, located upstream (Fig. 2B), for expression. Mutation of each of these sequences produced a significant decrease in expression from the −398 promoter form (Fig. 2B), indicating that both elements participate in expression of the *COX5b-2* gene. Interestingly, expression was not completely abolished by the mutations, and the simultaneous mutation of both elements did not produce a further decay in GUS activity (Fig. 2B). On one side, this indicates that additional elements present in the −398 promoter fragment are able to sustain expression in the absence of site II elements, in agreement with the fact that positive elements were detected upstream and downstream of site II elements by promoter deletion analysis (Fig. 1). On the other side, both site II elements seem to act in concert, because mutation of any of them produces a similar effect as mutation of both.

We also noticed the presence of four repetitions of the initiator element (YTCANTYY; Y = C or T; Nakamura et al. 2002), located near the putative transcription start site of the *COX5b-2* gene, as deduced from available cDNAs. As removal of the region that contains the initiators (together with upstream regions) produces a complete loss of expression, we tested the effect of mutating the four initiators on expression from the −398 promoter fragment (Fig. 2B). Mutation of the initiators produced a similar decrease in expression as mutation of site II elements, except for leaves, where a larger effect was observed (Fig. 2B). Combined mutation of site II elements and initiators, on the other hand, produced a complete loss of expression (Fig. 2B).

The promoter fragments that contain the site II elements and the initiator were used in EMSAs to evaluate the presence of nuclear proteins able to bind to these elements. As shown in Fig. 3A (left panel), two specific shifted bands were observed when the fragment spanning nucleotides -195 to -113, that contains both site II elements, was used, but not with a similar fragment with mutagenized site II elements. In addition, a 25-fold molar excess of unlabeled wild-type fragment efficiently competed binding, while a similar amount of the mutated fragment was ineffective (Fig. 3A, right panel). In a similar way, four different shifted bands were observed when the fragment containing the initiators (−120 to −37) was used in an EMSA and these bands disappeared when the initiators were mutagenized (Fig. 3B, left panel). Competition for binding was observed only with the fragment that contains intact initiator elements (Fig. 3B, right panel). The results show the presence of nuclear proteins that specifically bind to the promoter elements involved in expression of the *COX5b-2* gene. Crossed competition between the fragments that contain the site II elements (−195 to −113) and the initiators (−120 to −37) was not observed (Fig. S1), indicating that different proteins bind to these two regions of the promoter.

The G-box and site II elements are involved in the response of the *COX5b-2* gene to UV-B treatment and sucrose, respectively

Using plants transformed with the 1000-bp *COX5b-2* promoter fragment fused to GUS, we performed a search of conditions that may influence the activity of the promoter. For this purpose, we incubated plants in the presence of several compounds or placed them under different environmental conditions and then measured GUS activity levels in extracts from treated plants. In this way, we observed that the *COX5b-2* promoter becomes more active when plants are treated with the auxin indole-3-acetic acid (IAA), the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), H_2O_2, UV-B light, inorganic phosphate and sucrose (Fig. S2A, left panel). Determination of relative *COX5b-2* transcript levels in non-transformed plants after incubation with the different effectors using real-time PCR confirmed that these compounds induce the expression of the *COX5b-2* gene (Fig. S2A, right panel).

Analysis of plants transformed with deleted promoter fragments indicated that the responses to IAA and ACC require the presence of sequences located upstream of −660, while sequences located between −620 and −398 are important for the effects of H_2O_2 and phosphate (Fig. S2B). Interestingly, the putative element required for induction by UV-B is located between −660 and −620, in the same fragment that produces

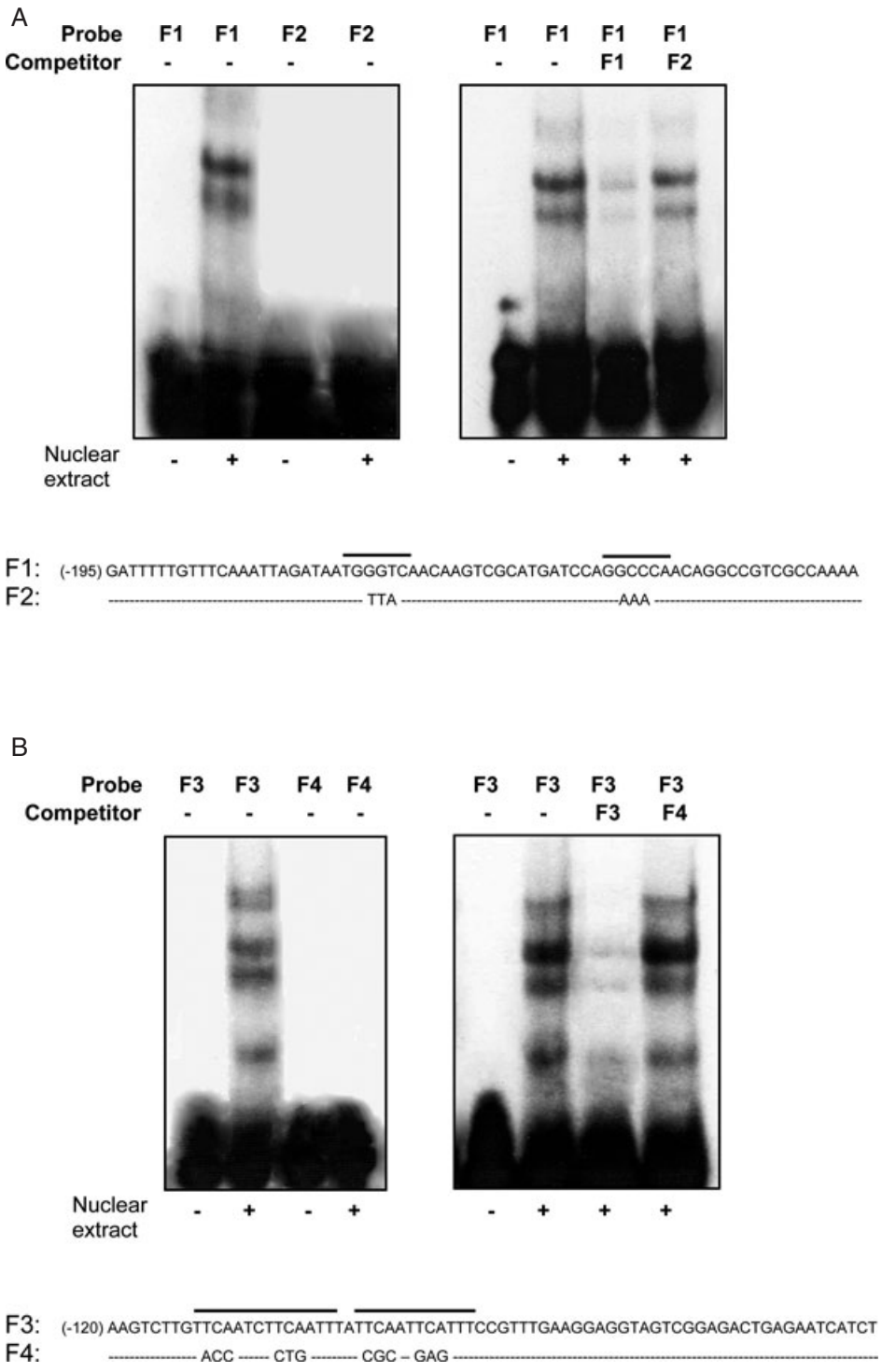
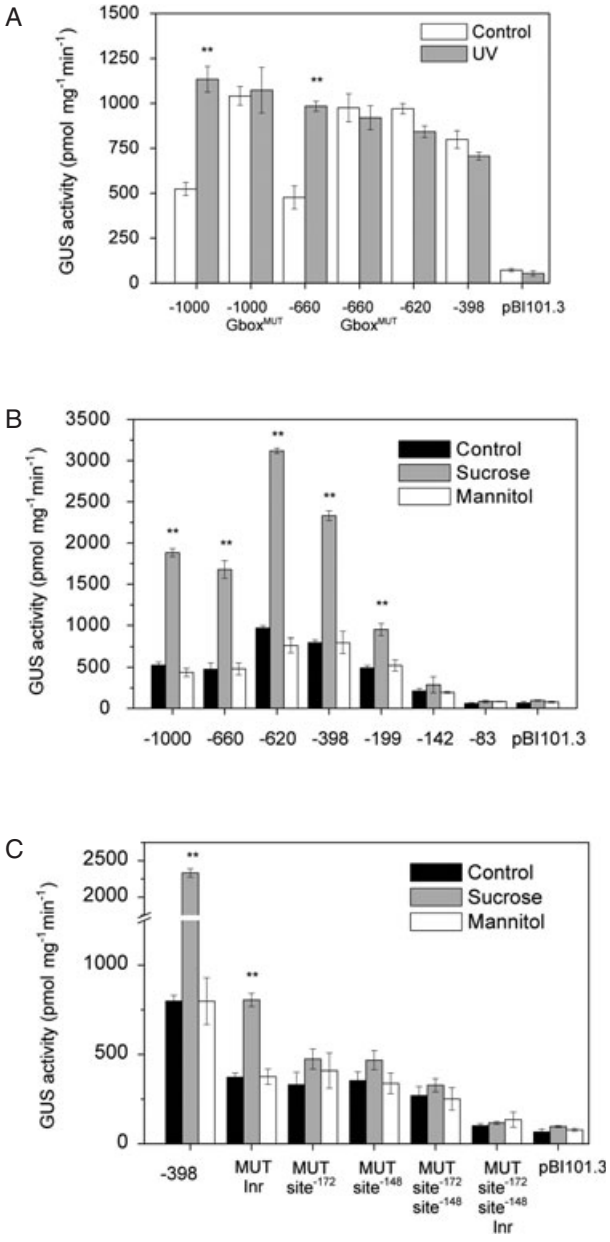


Fig. 3. Nuclear proteins bind specifically to the *COX5b-2* site II elements and initiators. (A) Nuclear extracts (10 μg) from cauliflower inflorescences were analyzed by an EMSA for the presence of proteins that bind to labeled DNA spanning nucleotides −195 to −113 of the *COX5b-2* promoter. Binding was analyzed using either a labeled wild-type fragment (F1) or a similar fragment in which the site II elements were mutagenized (F2). Where indicated, a 25-fold molar excess of unlabeled F1 or F2 fragment was included as competitor. Below, the sequence of the corresponding fragments is indicated. (B) Binding of nuclear proteins to labeled DNA spanning nucleotides −120 to −37 of the *COX5b-2* promoter. Binding was analyzed using either a labeled wild-type fragment (F3) or a similar fragment in which the initiator elements were mutagenized (F4). Where indicated, a 25-fold molar excess of unlabeled F3 or F4 fragment was included as competitor. Below, the sequence of the corresponding fragments is indicated.

an increase in expression when removed (Fig. 4A). As the repressive effect of this fragment is because of the presence of a G-box, we assayed induction by UV-B in plants transformed with promoter forms with a mutated G-box. In these plants, basal expression was higher but the response to UV-B was lost (Fig. 4A). The fact that UV-B treatment produces a similar effect as mutation of the G-box suggests that induction by UV-B is originated by the removal of a repressor bound to this element.

Induction by sucrose was lost when the fragment located between −199 and −142 was removed (Fig. 4B). A similar effect was observed when site II elements were either individually or collectively mutated in a larger promoter fragment (Fig. 4C). Mutation of initiators in the same fragment did not alter the sucrose responsiveness of the promoter.



Discussion

The *COX5b-2* promoter contains a mixture of positive and negative regulatory elements, as deduced from the effect of deletions of progressive portions of the promoter. The larger promoter fragment used in this study directs localized expression in root and shoot meristems, leaf and cotyledon tips, and anthers. Previous northern analysis of *COX5b-2* expression in different organs suggested that transcript levels are higher in leaves than in flowers (Welchen et al. 2002), while the opposite is evident from the GUS activity measurements described here. This apparent discrepancy can be explained because northern analysis was performed on total RNA extracted from the entire rosette or inflorescence, while only mature leaves and isolated flowers were used here for the GUS activity measurements. In addition, post-transcriptional mechanisms may operate to increase the stability of the corresponding mRNA in leaves respective to other tissues.

The *COX5b-2* promoter shows induction by several compounds. Induction by sucrose is a property of a majority of genes encoding components of the mitochondrial respiratory chain (Curi et al. 2003, Gonzalez et al. 2007, Welchen et al. 2002). As sugars are repressors of photosynthesis genes, it has been speculated that this may represent a mechanism to balance the processes involved in the synthesis and utilization of carbohydrates. The responses to H_2O_2 and

Fig. 4. Identification of promoter regions involved in the response of the *COX5b-2* gene to UV-B and sucrose. (A) Twenty-one-day-old Arabidopsis plants transformed with different *COX5b-2* promoter fragments, or the same fragments with mutations in the G-box, were treated with UV-B light (280-320 nm) during 60 min and analyzed for GUS activity using the fluorogenic substrate MUG. (B, C) Arabidopsis plants carrying different deletions (B) or mutagenized forms (C) of the *COX5b-2* promoter were grown in MS medium with or without the addition of either 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 (**$P < 0.01$). 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. 2. 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.

UV light may reflect a requirement for the synthesis of COX subunits to replace those damaged by oxidative stress generated under these conditions. As COX5b contains a Zn atom, it may constitute a preferential target of ROS action. It is noteworthy that *COX5b-1* is also induced by H_2O_2 and several genes encoding proteins involved in COX biogenesis are induced by oxidative stress conditions (Attallah et al. 2007a,b, Comelli et al. 2009). The physiological significance of the regulation by other compounds reported here is less evident, but it is noteworthy that ACC and inorganic phosphate also act as inducers of the *COX5b-1* promoter (Comelli et al. 2009).

A G-box located at −636 from the *COX5b-2* start codon acts as a repressive element in leaves and seedlings and participates in induction by UV-B that probably operates through the removal of a repressor. The fact that mutation of the *COX5b-2* G-box produces an increase in expression in leaves and seedlings, but not in flowers, suggests that higher promoter activity in flowers may be because of the absence of the repressor. G-boxes or related elements have been implicated in the induction by UV light of the chalcone synthase gene (Hartmann et al. 1998, 2005, Schulze-Lefert et al. 1989). The location of the *COX5b-2* G-box is unusual, as it lies within the coding region of the nearby *At1g80240* gene (Fig. 1A). Indeed, the 3′ non-coding region of this gene begins at −520 and ends at −357 from the *COX5b-2* translation start site. We have not found any evidence in the literature of the presence of a regulatory element of a gene within the coding region of another gene. According to public microarray expression data (http://bbc.botany.utoronto.ca/), At1g80240 is expressed in roots, mainly in the root cap, and is expressed at very low levels in other parts of the plant, implying that there is no or very little overlap between the expression patterns of At1g80240 and *COX5b-2* and that both genes are regulated independently. In addition, At1g80240 is not induced by UV light. It can be speculated that transcription of At1g80240 and regulation of *COX5b-2* expression by factors bound to the G-box region would operate independently and not interfere with one another. It is also noteworthy that there is an ACGT motif located downstream of the G-box, with a 14-bp spacing region. This arrangement, with 13- and 18-bp spacers, respectively, is present in the *Cytc-2* and *COX5b-1* promoters, but the G-boxes are positive regulators of expression in these genes (Comelli et al. 2009, Welchen et al. 2009). G-boxes can be recognized by transcription factors from the bZIP and bHLH families (Heim et al. 2003, Sibéril et al. 2001, Toledo-Ortiz et al. 2003), composed of 73 and 161 members in Arabidopsis, respectively (http://arabidopsis.med.ohio-state.edu/AtTFDB/). It is possible that nucleotides adjacent to the G-box determine the specificity of interaction with different transcription factors, as has been proposed previously (Ishige et al. 1999). Regarding the *COX5b-2* ACGT motif, it is unlikely that this element has an important role in transcription, because it is present within the fragment located between −620 and −399, that produces only minor changes when removed.

Basal expression of the *COX5b-2* gene depends on site II-like and initiator elements present in proximal promoter regions. Site II elements, defined as elements with the sequence TGGGCY, are present in proximal promoter regions in a majority of nuclear genes encoding respiratory chain components, usually in more than one copy (Welchen and Gonzalez 2006). Site II elements are recognized in vitro by members of the teosinte branched1, cycloidea and PCF (TCP) family of transcription factors (Tremousaygue et al. 2003, Welchen and Gonzalez 2005), but there is no definitive evidence that these proteins recognize these elements in vivo . The *COX5b-2* gene contains one site II element and a nearby element with the related sequence TGGGTC that probably acts as a site II element too. Mutational analysis indicated that these elements are also involved in the response of *COX5b-2* to sucrose. A similar observation has been made for the site II elements present in the *Cytc-2* and the three *COX6b* genes from Arabidopsis, encoding cytochrome *c* and COX subunit 6b, respectively (Mufarrege et al. 2009, Welchen et al. 2009). Considering these results, a picture is emerging that site II elements act as transducers of signals originated by an increase in sucrose concentration in plants.

Initiators are elements located around the transcription start site that are involved in the interaction with the basal transcription machinery, specially in TATA-less promoters as the *COX5b-2* promoter (Smale and Baltimore 1989). The *COX5b-2* promoter has four initiators located around the transcription start site, which is located within the second of these elements according to available cDNAs. Functional initiators have been identified in the genes encoding plastid ribosomal protein Rpl21 and the photosystem I component PsaD from spinach and tobacco, respectively (Achard et al. 2003, Nakamura et al. 2002). In the *psaD/b* gene, that contains three consecutive initiators, these elements are required for the response to light (Nakamura et al. 2002). Nothing is known about the proteins that interact with initiators in plants. It is noteworthy that mutation of the four initiators of the *COX5b-2* gene does not completely abolish transcription, apparently because site II elements are capable of promoting expression in their absence.

This is supported by the fact that the combined mutation of site II elements and the initiators produces a complete loss of expression. Accordingly, site II elements may also be viewed as components of the core promoter with a role in basal transcription. The fact that about 5-10% of *Arabidopsis* genes contain site II elements in their proximal promoter regions (Welchen and Gonzalez 2006) agrees with this view.

The elements that participate in expression of the two Arabidopsis *COX5b* genes are different, indicating that the respective promoters have diverged considerably after the duplication process that originated both gene forms. Interestingly, the promoters of the *COX5b-1*/*COX5b-2* gene pair share characteristics with those of the *Cytc-1*/*Cytc-2* pair, encoding cytochrome *c* isoforms. While *COX5b-2* and *Cytc-1* depend for their expression on site II elements, *COX5b-1* and *Cytc-2* depend mainly on the presence of G-boxes linked to ACGT motifs (Comelli et al. 2009, Welchen and Gonzalez 2005, Welchen et al. 2009). In this sense, the *COX5b-2* and *Cytc-1* promoters direct a more localized expression mainly in meristems and anthers, while the *COX5b-1* and *Cytc-2* promoters produce broader expression patterns. The four genes, however, are expressed in anthers and respond to sucrose, suggesting that the conservation of these characteristics is important for the function of all of them.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of oligonucleotides used.

Figure S1. Fragments that contain the site II elements and the initiators do not show crossed competition.

Figure S2. Response of the *COX5b-2* promoter to different treatments.

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