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Common Sets of Promoter Elements Determine the Expression Characteristics of Three Arabidopsis Genes Encoding Isoforms of Mitochondrial Cytochrome *c* Oxidase Subunit 6b

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Short Communication

The promoters of the three Arabidopsis nuclear genes encoding mitochondrial cytochrome *c* oxidase subunit 6b (*AtCOX6b*) have similar expression patterns, with preferential expression in anthers and meristems, and are induced by sucrose and etiolation. Additionally, induction of *AtCOX6b-1* by GA_3 and *AtCOX6b-3* by 6-benzylaminopurine was observed. Site II elements (TGGGCC/T) present in the three promoters bind common nuclear proteins and are important for basal and induced expression. Induction by sucrose requires, in addition, the integrity of elements with the sequence TACTAA. The results imply the participation of common regulatory factors in the expression of the three Arabidopsis *COX6b* genes.

Keywords: *Arabidopsis thaliana* • *COX6b* gene • Gene expression • Promoter analysis • Site II element • Sucrose-responsive element.

Abbreviations: BAP, 6-benzylaminopurine; COX, cytochrome *c* oxidase; EMSA, electrophoretic mobility shift assay; GUS, β-glucuronidase.

Cytochrome *c* oxidase (COX), performs the final step of electron transport in the mitochondrial respiratory chain (Barrientos et al. 2002). The enzyme is composed of three subunits encoded in the organelle and a variable number of subunits encoded in the nucleus. Plant COX contains 6–7 nuclear-encoded subunits, some of them homologous to subunits from other eukaryotes and others specific to plants (Millar et al. 2004). It is assumed that the assembly of a functional enzyme requires the coordinated expression of its subunits. Recent studies have shown that expression of subunits encoded in the nucleus is not coordinated with the synthesis of those encoded in the organelle (Giegé et al. 2005). Since subunits encoded in the mitochondrion are synthesized in excess, assembly of functional complexes is dictated by the amount of nuclear-encoded subunits. Expression of nuclear-encoded subunits is, in turn, coordinated at the transcriptional level. In this sense, it has been shown that several genes encoding COX subunits are induced by incubation of plants with carbohydrates (Welchen et al. 2002, Curi et al. 2003, Giegé et al. 2005).

Coordination of genes involved in respiration becomes more complex if one considers that, apart from the numerous genes necessary to encode respiratory chain components, some subunits are encoded by more than one gene in certain plant species. An example of this is COX6b from Arabidopsis, for which three different nuclear genes have been described (Ohtsu et al. 2001).

Subunit 6b is present in COX from several eukaryotic organisms and plays an essential role in the assembly of the complex (Taanman et al. 1990, Carrero-Valenzuela et al. 1991). One of the genes present in Arabidopsis, *AtCOX6b-1*, encodes a protein that is twice as large as those from other eukaryotes, while *AtCOX6b-2* and *AtCOX6b-3* encode proteins of similar size (Ohtsu et al. 2001). The presence of a homolog of *AtCOX6b-1* in rice suggests that the duplication event that originated this isoform occurred before the separation of monocots and dicots. The divergence of *AtCOX6b-2* and *AtCOX6b-3* is comparatively more recent.

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Plant Cell Physiol. 50(7): 1393–1399 (2009) doi:10.1093/pcp/pcp080, available online at www.pcp.oxfordjournals.org

To gain insight into the mechanisms that govern the expression of *AtCOX6b* genes, we studied the respective promoter regions using reporter gene fusions. Constructs comprising about 1,200 bp regions located upstream of the translation start codons of *AtCOX6b-1* (At1g22450), *AtCOX6b-2* (At5g57815) and *AtCOX6b-3* (At4g28060) fused to the *uidA* (*gus*) reporter gene were introduced into Arabidopsis. (We use the gene nomenclature adopted by Ohtsu et al. 2001 to name the Arabidopsis *COX6b* genes.) Histochemical assays of β-glucuronidase (GUS) activity from independent lines carrying each construct showed that the three promoters produce similar expression patterns (**Fig. 1**, left panels). In seedlings, expression was detected in shoot and root apical meristems, in the root vascular cylinder and in cotyledon vascular strands. In adult plants, expression was evident in root vascular tissues, in leaf veins and lamina, and in pollen, stigma and the receptacle in flowers and siliques. Preferential expression in meristems and anthers has also been described for other genes encoding components of the mitochondrial respiratory chain (Zabaleta et al. 1998, Elorza et al. 2004, Welchen et al. 2004, Welchen and Gonzalez 2005) and agrees with the presence of an increased number of mitochondria in these parts of the plant (Lee and Warmke 1979, Kuroiwa et al. 1992).

The fact that all *AtCOX6b* genes display similar expression characteristics suggests that they may be the target of the same regulatory factors. Analysis of the respective promoters revealed the presence of conserved elements known as site II (TGGGCC/T) that have been described as important for expression in proliferating tissues (Tremousaygue et al. 2003), in similar locations in the three promoters (**Fig. 2A**). The role of site II elements in expression of *AtCOX6b* genes was analyzed with two types of constructs (**Fig. 2A**): the first type contained promoter regions comprising site II elements and downstream sequences, and the second type contained the entire regions used in the initial studies carrying point mutations (TGGGCC/T to TGTTCC/T) in site II elements.

Plants with the shorter promoter forms fused to *gus* displayed similar expression patterns and levels to those observed with the entire promoters (**Fig. 2B–D**), suggesting that all elements required for basal expression are present in the downstream portion of the promoters. Mutagenesis of site II elements produced a pronounced decrease in expression to levels observed in plants transformed with a promoterless *gus* gene in seedlings, leaves and roots (**Fig. 2B–D**). In flowers and siliques, residual GUS activity was observed. Indeed, GUS histochemical assays demonstrated the existence of activity in the shoot apical meristem, pollen, stigma

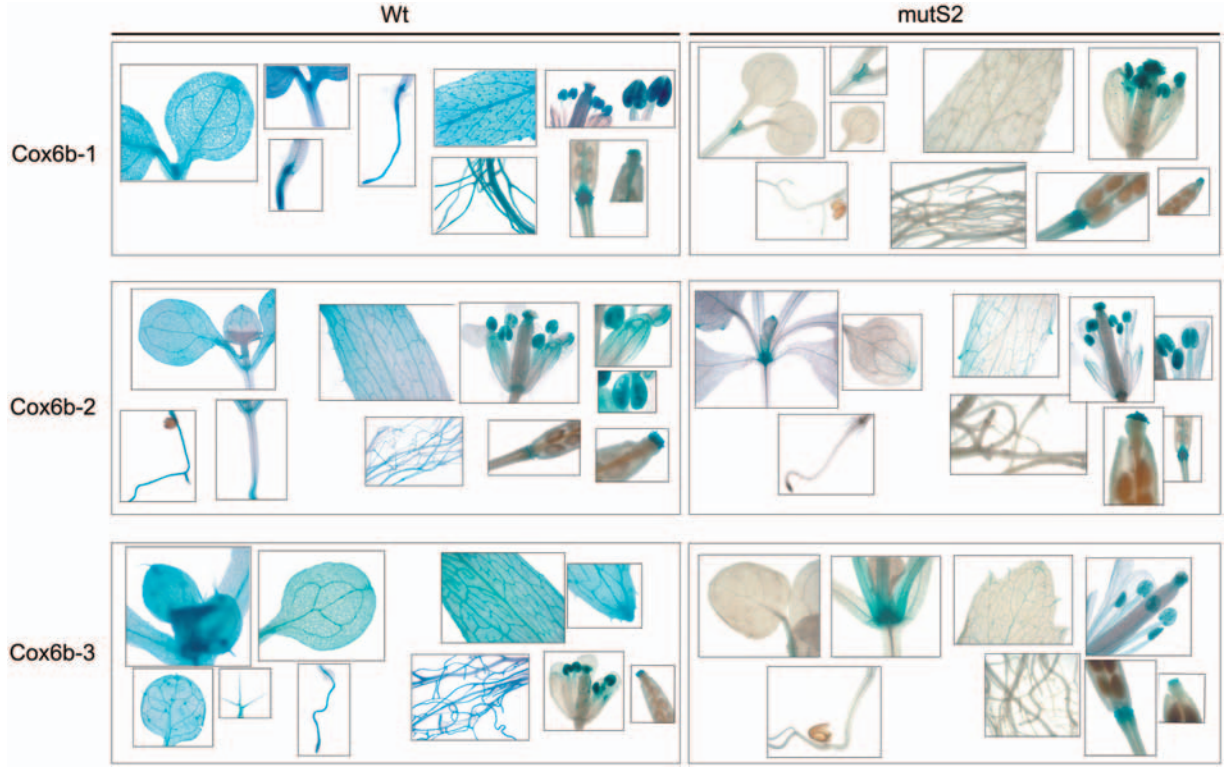

Fig. 1 Histochemical localization of GUS activity in Arabidopsis plants transformed with *AtCOX6b* promoter fragments fused to *gus* (Wt; left panels) or the same fragments with mutagenized site II elements (mutS2; right panels). The images are representative from 10 lines analyzed for each promoter construct. Incubation time in the staining solution was 8 h.

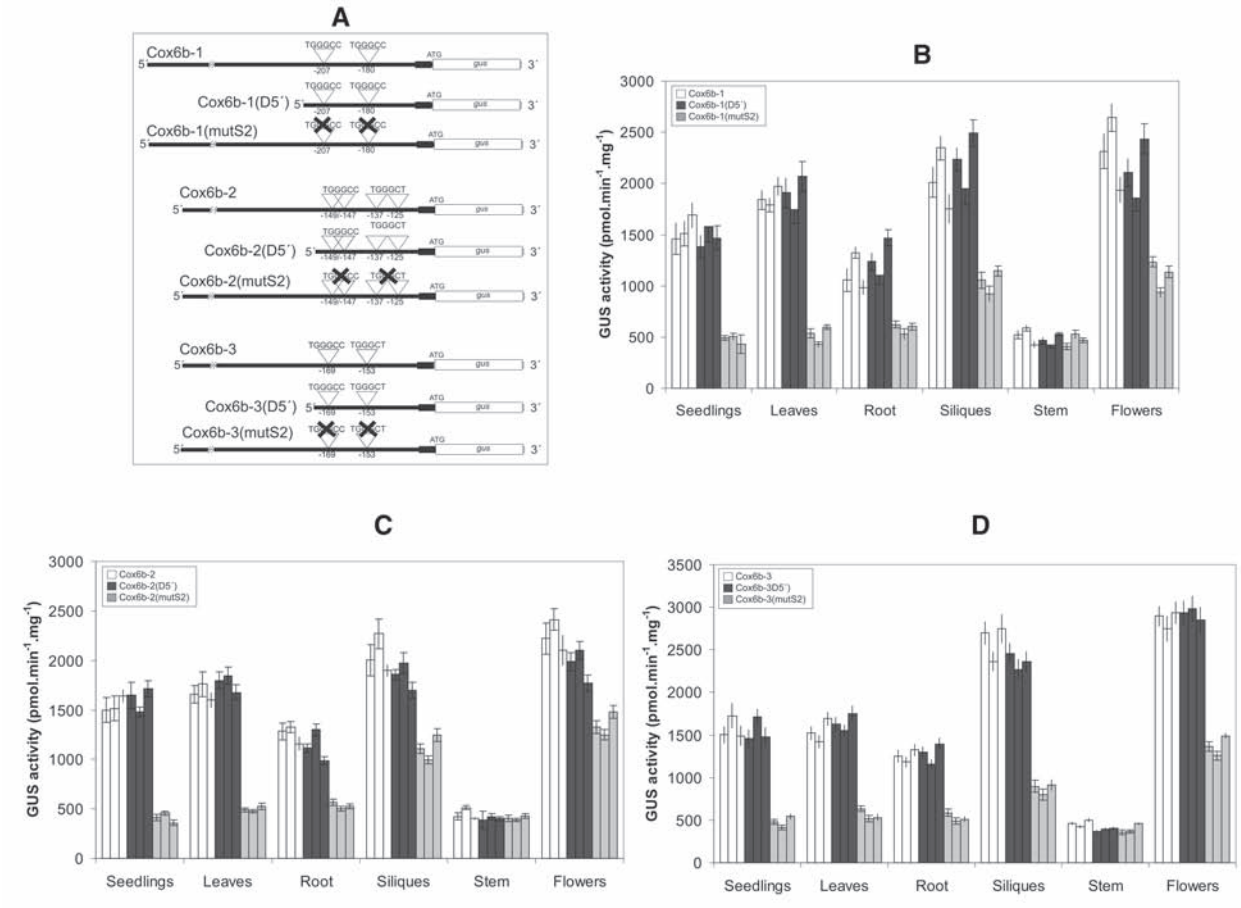

Fig. 2 Analysis of GUS activity driven by deleted and mutagenized forms of *AtCOX6b* promoters. (A) Schematic representation of different constructs used to transform Arabidopsis plants. Triangles represent site II elements and numbers indicate their position respective to the translation start site. Crosses indicate mutagenized site II elements. (B–D) GUS expression levels measured in total protein extracts from 15-day-old seedlings and organs from mature plants transformed with the different constructs. The results of three independent lines are shown for each construct. The bars represent the mean (±SD) of three replicate GUS activity measurements for each line.

and the receptacle (**Fig. 1**, right panels). The results imply that site II elements are the main determinants of the expression levels of the three *AtCOX6b* genes. The common presence of these elements may explain the conservation in expression patterns. It is noteworthy that both rice *COX6b* genes also contain several site II elements in their proximal promoter regions.

The existence of nuclear proteins able to recognize the site II elements present in the *AtCOX6b-1* promoter was analyzed in electrophoretic mobility shift assays (EMSAs). **Fig. 3A** (lanes 2 and 3) shows that a specific shifted band was observed upon addition of nuclear extract to the mix containing the labeled *AtCOX6b-1* promoter fragment, while no shift was observed when a fragment with mutagenized site II elements was used (**Fig. 3A**, lanes 6–8), suggesting that the integrity of these elements is required for specific binding of proteins to this portion of the *AtCOX6b-1* promoter. In this sense, a promoter fragment with mutagenized site II elements was considerably less effective in competing binding than the non-mutagenized fragment (**Fig. 3A**, lanes 4, 9 and 10). Nuclear proteins were also able to recognize promoter fragments from the *AtCOX6b-2* and *AtCOX6b-3* genes, and this binding was abolished when site II elements were mutated (**Fig. 3B**).

The possibility that the same nuclear proteins interact with the three *AtCOX6b* promoters was analyzed by crossed competition between the different fragments. As shown in **Fig. 3C**, unlabeled *AtCOX6b-2* and *AtCOX6b-3* promoter fragments were able to compete for binding to a labeled *AtCOX6b-1* fragment. This indicates that common nuclear proteins interact with the three *AtCOX6b* promoters, further supporting the idea that expression of these genes operates through similar mechanisms.

We also investigated the effect of incubation of plants under several conditions on promoter-dependent expression. Analysis of GUS activity in extracts from whole 15-day-old

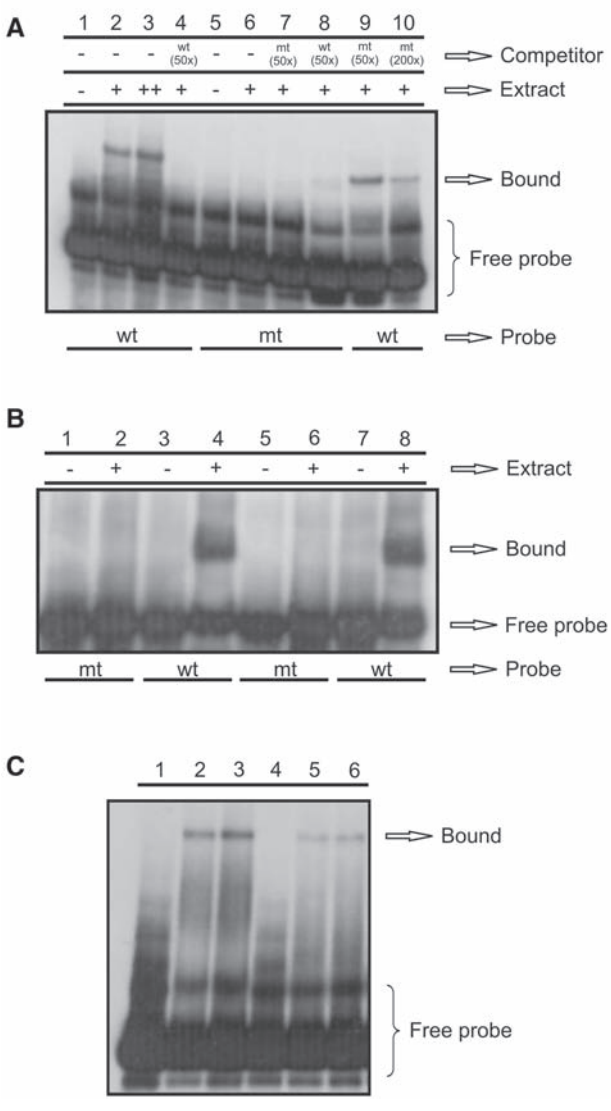

Fig. 3 Common nuclear proteins bind to the *AtCOX6b* promoter fragments containing the site II elements. (A) Nuclear extracts from cauliflower inflorescences (+, 2 μg; ++, 4 μg) were analyzed by an EMSA for the presence of proteins that bind to labeled DNA spanning nucleotides −238 to −101 from the *AtCOX6b-1* promoter. Binding was analyzed using either a labeled wild-type fragment (wt) or a similar fragment with mutagenized site II elements (mt), in the absence or presence of an excess of unlabeled wild-type or mutagenized fragment as competitor. (B) Binding of nuclear proteins to wild-type (wt) or mutagenized (mt) *AtCOX6b-2* and *AtCOX6b-3* promoter fragments spanning nucleotides −1 to −196 or −2 to −199, respectively. (C) Binding of nuclear proteins (lane 2, 2 μg; lanes 3–6, 4 μg) to the *AtCOX6b-1* promoter fragment containing site II elements was competed with a 100-fold molar excess of the same unlabeled fragment (lane 4) or with the respective fragments from the *AtCOX6b-2* or *AtCOX6b-3* promoters (lanes 5 and 6, respectively).

plants subjected to different treatments indicated that the three promoters are more active in etiolated plants than in plants grown under illumination (**Supplementary Fig. S1**). A further increase in activity was observed upon incubation in the presence of sucrose, but not in the presence of mannitol, suggesting that the effect of sucrose cannot be ascribed to an increase in osmotic potential. In addition to *AtCOX6b* genes, sucrose also induces the expression of several respiratory chain component genes (Welchen et al. 2002, Curi et al. 2003, Giegé et al. 2005). This would be part of a response to the accumulation of carbohydrates that would produce an increase in the amount of components involved in respiration. It is noteworthy that *AtCOX6b* promoters are induced by etiolation, a condition under which carbohydrate levels decrease. The effects of sucrose and light on expression may then occur through independent mechanisms. Accordingly, induction by sucrose was also observed in plants grown under illumination (not shown).

Of the other compounds tested, GA_3 induced expression from the *AtCOX6b-1* promoter, and the cytokinin 6-benzylaminopurine (BAP) was effective with *AtCOX6b-3* (**Supplementary Fig. S1**). Induction by cytokinins has been reported for other components of the mitochondrial respiratory chain (Welchen and Gonzalez 2005, Welchen et al. 2009) and may be associated with the promotion of cell proliferation by this hormone. Apart from induction with the two hormones, each specific for a given gene, the effects of sucrose and etiolation also support the idea of common regulatory mechanisms operating for the expression of the three *AtCOX6b* genes.

The approximate location of elements involved in induction by the different factors was analyzed by producing a series of nested deletions from the upstream portion of the promoters. The short promoter forms containing sequences located downstream of site II elements only retained induction by etiolation and, for *AtCOX6b-1* and *AtCOX6b-3*, by sucrose (**Supplementary Fig. S2**). The putative element(s) required for the response to GA_3 are located further upstream, between −458 and −239 of the *AtCOX6b-1* promoter (**Supplementary Fig. S3**). A similar analysis indicated that the element(s) required for induction of *AtCOX6b-3* by BAP are located between −867 and −500. Induction by all the factors analyzed was lost upon mutation of site II elements (**Supplementary Fig. S2**).

A search for elements involved in the response to sucrose of other genes in the downstream portions of the *AtCOX6b-1* and *AtCOX6b-3* promoters indicated the presence of an element with the sequence AATACTAAT, located between the two site II elements of the *AtCOX6b-3* gene. This sequence has been described as the sucrose-responsive element 2 (SURE2), which is present in the potato patatin promoter and is similar to sequences conferring sucrose inducibility in other genes (Grierson et al. 1994). We then mutagenized the

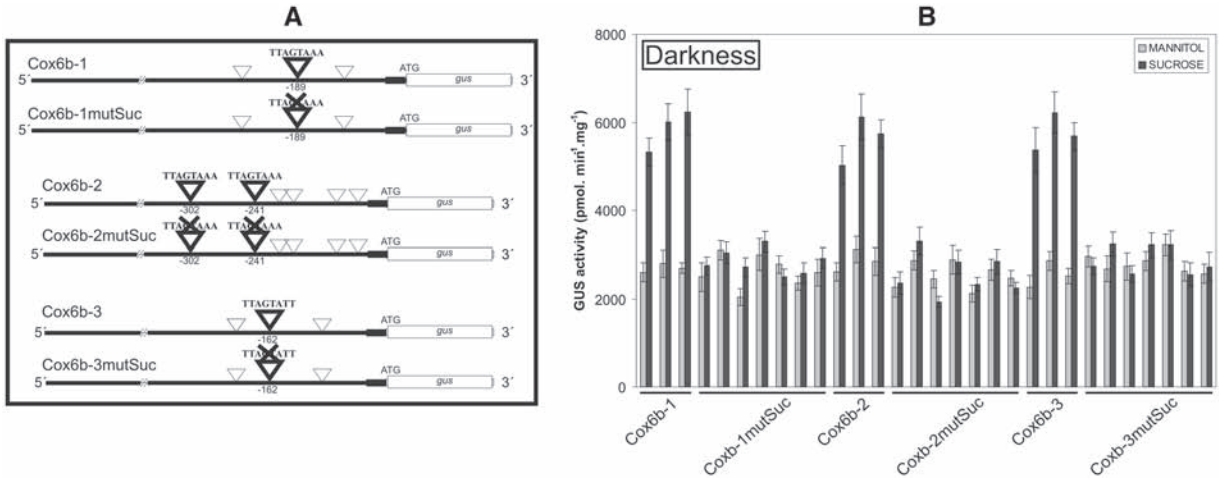

Fig. 4 Elements with the sequence TACTAA participate in the response of the three *AtCOX6b* genes to sucrose. (A) Schematic representation of promoter constructs used to evaluate the role of elements containing the SURE2 core sequence TACTAA (large triangles) in the response to sucrose. Crosses indicate constructs with mutagenized SURE2 elements. Numbers indicate the location of elements respective to the translation start site. Small triangles indicate site II elements. (B) Plants transformed with the different constructs were grown in darkness during 15 d in the presence of either 3% mannitol or 3% sucrose before GUS activity was measured in whole plant protein extracts. The results of several independent lines are shown for each construct. The bars represent the mean (±SD) of three replicate GUS activity measurements for each line and treatment.

AtCOX6b-2 gene in three central nucleotides that have been shown to be essential for the function of SURE2 in the patatin promoter (**Fig. 4A**). Plants transformed with the *AtCOX6b-3* promoter with a mutagenized SURE2 element fused to *gus* revealed no induction by sucrose either in darkness (**Fig. 4B**) or under illumination (not shown). Interestingly, the sequence TACTAA, corresponding to the six central nucleotides of SURE2, is also present between the site II elements of the *AtCOX6b-1* promoter (**Fig. 4A**). Mutagenesis of this sequence also abolished sucrose responsiveness in the *AtCOX6b-1* promoter (**Fig. 4B**). We then searched for similar sequences in the fragment of the *AtCOX6b-2* promoter located upstream of −193, and found the sequence AATACTAA repeated twice (around −241 and −302) embedded in a 19 bp repeated sequence (**Fig. 4A**). Plants with *gus* fusions to the *AtCOX6b-2* promoter mutagenized in both sequences did not show induction by sucrose (**Fig. 4B**). Our results indicate that related elements present in the three Arabidopsis *COX6b* promoters are involved in the response of these genes to sucrose.

The fact that the three *AtCOX6b* genes have acquired sucrose responsiveness through the incorporation of similar elements may indicate that these elements were already present in the ancestral form of the gene. On the other hand, the location of SURE2 elements in the promoters of the closely related *AtCOX6b-2* and *AtCOX6b-3* genes is rather different, contradicting the notion of a common evolutionary origin. Accordingly, the most plausible explanation is that SURE2 elements were independently acquired by the three *AtCOX6b* genes. Alternatively, the incorporation of a short repeated sequence that contains SURE2 elements in the upstream portion of the *AtCOX6b-2* gene may have allowed the loss of the original element present between the site II elements as in the other genes. The availability of the promoter sequences of *COX6b* genes from other species will be helpful to elucidate this point.

Since site II elements are required for responses to effectors in *AtCOX6b* genes, we favor the idea that these elements, and the proteins that interact with them, act as transducers of the signals that originate from elements specifically involved in induction by different effectors, probably through protein–protein interactions. In this sense, site II elements were shown to be involved in the magnitude of the response to effectors in the Arabidopsis *Cytc-2* gene, encoding an isoform of cytochrome *c* (Welchen et al. 2009). It is noteworthy that in two of the *AtCOX6b* genes the SURE2 element is located between the site II elements, further reinforcing the idea of a functional interaction.

Materials and Methods

Arabidopsis thaliana Heyhn. ecotype Columbia plants were grown on soil 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 μE m^{-2} s^{-1}. Plants used for the different treatments were grown in Petri dishes containing Murashige and Skoog medium, 0.8% agar and different additions, as indicated.

Fragments spanning 1,262, 1,155 or 1,162 nucleotides upstream of the initiation codon of *AtCOX6b-1*, *AtCOX6b-2* or *AtCOX6b-3*, respectively, were obtained by PCR amplification of Arabidopsis genomic DNA using the primers listed in **Supplementary Table S1** and cloned in vector pBI101.3. Deletions of upstream portions of the promoters were constructed in a similar way. Complementary primers were used for the introduction of specific mutations in putative regulatory elements using overlap extension mutagenesis by PCR (Silver et al. 1995). All constructs were checked by DNA sequencing.

Constructs were introduced into *Agrobacterium tumefaciens* strain LB4404, and transgenic Arabidopsis plants were obtained by the floral dip procedure (Clough and Bent 1998). The presence of introduced DNA in transformed plants was analyzed by PCR using 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 representative expression patterns (those common to a majority of transformants) were further reproduced, and homozygous T_3 and T_4 plants were used for detailed analysis of GUS expression. GUS activity of plants was analyzed by histochemical and fluorometric methods essentially as described (Welchen and Gonzalez 2005).

For EMSAs, aliquots of nuclear extracts were incubated with DNA (10,000 c.p.m.) obtained by amplification of the corresponding fragments with specific primers, followed by restriction enzyme cleavage and filling-in the 3′ ends with [α-^{32}P]dATP. Binding reactions were performed and analyzed as described in Welchen and Gonzalez (2005). Nuclear extracts were prepared from cauliflower buds as described by Maliga et al. (1995).

Funding

The Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina); Agencia Nacional de Promoción Científica y Tecnológica; Universidad Nacional del Litoral. E.F.M. is a fellow of CONICET; D.H.G. is a member of the same Institution.

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(Received May 20, 2009; Accepted May 31, 2009)