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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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 GA₃ 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.
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.) Histochmical 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.
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
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 man-
nitol, 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 respira-
tory 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 respira-
tion. 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, GA3 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 respira-
tory chain (Welchen and Gonzalez 2005, Welchen et al.
2009) and may be associated with the promotion of cell pro-
liferation 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 induc-
tion 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 induc-
tion by etiolation and, for AtCOX6b-1 and AtCOX6b-3, by
sucrose (Supplementary Fig. S2). The putative element(s)
required for the response to GA3 are located further
upstream, between –458 and –239 of the AtCOX6b-1 pro-
moter (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 ele-
ment 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. 3](https://academic.oup.com/pcp/article-abstract/50/7/1393/1889003)
AtCOX6b-2 gene in three central nucleotides that have been shown to be essential for the function of SURE2 in the pata-
tin 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 dark-
ness (Fig. 4B) or under illumination (not shown). Interest-
ingly, 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). Mutagen-
genesis of this sequence also abolished sucrose responsive-
ness 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) embed-
ded in a 19 bp repeated sequence (Fig. 4A). Plants with
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 evolution-
ary 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 effec-
tors 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 iso-
form 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** Heynh. ecotype Columbia plants were
grown on soil at 22–24°C under long-day photoperiods (16 h
illumination by a mixture of cool-white and GroLux fluo-
rescent 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 T3 and T4 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-32P]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).

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**References**


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