The leader intron of *Arabidopsis thaliana* genes encoding cytochrome c oxidase subunit 5c promotes high-level expression by increasing transcript abundance and translation efficiency

Graciela C. Curi, Raquel L. Chan and Daniel H. Gonzalez*

Cátedra de Biología Celular y Molecular, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, CC 242 Paraje El Pozo, 3000 Santa Fe, Argentina

Received 4 March 2005; Accepted 20 June 2005

Abstract

The involvement of regions located upstream of the translation start site in the expression of two *Arabidopsis thaliana* nuclear COX5c genes encoding subunit 5c of mitochondrial cytochrome c oxidase has been analysed. It was observed that these regions, which include a leader intron, direct the tissue-specific expression of the *gus* reporter gene, mainly in root and shoot meristems, actively growing tissues and vascular strands. Expression was also observed in flowers, specifically localized in anthers, stigma, and the receptacle, and in developing seeds. GUS activity measurements in protein extracts from transformed plants indicated that expression levels are higher than those observed with the constitutive CaMV 35S promoter. Removal of the leader intron produced a significant decrease in expression to values only slightly higher than those observed with a promoterless *gus* gene. Histochemical staining of plants transformed with the intronless construct revealed expression only in pollen, suggesting that regulatory elements capable of directing pollen-specific expression are present upstream of the intron. The COX5c-2 intron also increased GUS expression levels when fused in the correct orientation with the promoter of the unrelated COX5b-1 gene. Comparison of GUS activity values with the transcript levels suggests that the intron also increases translation efficiency of the corresponding mRNA. The results obtained point to an essential role of the intron present in the 5′-non-coding region of all known COX5c genes in directing the expression of these genes in plants.

Key words: Cytochrome c oxidase, gene expression, leader intron, mitochondrion, promoter analysis.

Introduction

Cytochrome c oxidase (COX) is a multimeric complex composed of several different subunits, two or three of them encoded by the mitochondrial genome and the rest encoded in the nucleus (Capaldi, 1990; Jünsch et al., 1996). Three different nuclear-encoded subunits, COX5b, COX6a, and COX6b, have been identified in plants through sequence comparisons with yeast and animal counterparts (Kadowaki et al., 1996; Ohtsu et al., 2001; Curi et al., 2003). A fourth subunit, COX5c, is the smallest plant COX subunit and has been recognized by protein purification studies (Nakagawa et al., 1987, 1990). Recent studies using 2D gel electrophoresis combined with mass spectrometry indicated the presence of additional plant-specific subunits (Millar et al., 2004).

It is generally assumed that the expression of components of the plant mitochondrial respiratory chain must somehow be co-ordinated. It is now well established that most mitochondrial components show enhanced expression in flowers (Huang et al., 1994; Landschu¨tze et al., 1995; Felitti et al., 1997; Heiser et al., 1997; Zabaleta et al., 1998). Expression in flowers is mainly localized in anthers as indicated by *in situ* hybridization experiments (Smart et al., 1994; Ribichich et al., 2001; Elorza et al., 2004). Expression studies in *Arabidopsis thaliana* have shown similar responses for the nuclear genes encoding cytochrome c and COX subunits 5b, 6a, and 6b (Welchen et al.,
2002; Curi et al., 2003). Notably, a different behaviour has been observed for genes encoding COX subunit 5c (COX5c), at least in sunflower (Curi et al., 2002).

The first COX5c cDNA and gene were isolated from sweet potato (Nakagawa et al., 1990, 1993). However, no functional studies have been performed on the cis-acting sequences required for the expression of COX5c genes from any species. In the present study, it has been demonstrated that sequences located upstream of the transcription start site of two Arabidopsis COX5c genes direct specific expression in pollen and that an intron located within the 5′-non-coding region of all known COX5c genes is responsible for high-level expression throughout the plant.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Heynh. ecotype Columbia (Col-0) was purchased from Lelehe 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 0.5×Murashige and Skoog medium and 0.8% agar. The dishes were kept at 4 °C for 2 d and then transferred to growth chamber conditions and kept in complete darkness for 7 d.

Isolation of genomic clones

Arabidopsis EST clones encoding COX5c-1 and COX5c-2 (clones 245H10T7, accession no. N97140, and 248L23T7, accession no. AA713295) were obtained from ABRC. For the isolation of genomic clones, a mixture of these cDNAs was used to screen 1 × 105 plaques 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. Isolated phage DNA from purified clones was characterized by restriction analysis and hybridization. A 1.8 kbp EcoRI fragment from COX5c-1 and a 3.2 kbp EcoRI/NheI fragment from COX5c-2, comprising the entire transcribed region and upstream sequences, were subcloned into pBlueScript SK− digested with EcoRI or EcoRI and XbaI, respectively. Positive clones were checked by partial sequencing and named VCAT1 and VCAT2.

RNA isolation and analysis

Total RNA was isolated as described by Carpenter and Simon (1998). For northern blot analysis, specific amounts of RNA were electro-phoresed through 1.5% (w/v) agarose/6% formaldehyde gels. The integrity of the RNA and equality of RNA loading were verified by ethidium bromide staining. RNA was transferred to Hybond-N nylon membranes (Amersham Corporation) and hybridized overnight at 68 °C to a 32P-labelled cDNA probe, comprising the entire GUS coding region isolated from vector pBI101.3, in buffer containing 6× SSC, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.2% (w/v) SDS, and 10% (w/v) polyethylene glycol 8000. Filters were washed with 2× SSC plus 0.1% (w/v) SDS at 68 °C (4 times, 15 min each), 1× SSC plus 0.1% (w/v) SDS at 37 °C for 15 min, dried, and exposed to Kodak BioMax MS films. To check the amount of total RNA loaded in each lane, filters were then re-probed with a 25S rDNA from Vicia faba under similar conditions as those described above, except that hybridization was performed at 62 °C and the wash with 0.1× SSC was omitted.

Reporter gene constructs and plant transformation

A 1.3 kb BglII/SalI fragment, comprising sequences upstream of the ATG initiation codon (i.e. exon 1, the intron and part of exon 2, plus non-transcribed upstream sequences) from COX5c-1, was amplified from clone VCAT1 using oligonucleotide COX3C: 5′-GGCGAGATCTCTCCTCTTCTTTCTTCTC-3′ (BglII site underlined) and universal primer −40 and cloned in plasmid pBI101.3 digested with BamHI and SalI. A similar construct for COX5c-2 was made by amplifying a 2.2 kbp fragment from VCAT2 with primers COXC41: 5′-GGCTCTAGATCATCTCACCACCTGAC-3′ (XbaI site underlined) and −40 and cloning in pBI101.3 digested with XbaI and HindIII. Constructs containing exon 1 and upstream sequences were obtained in a similar way by amplification with either COXC3C: 5′-GGCGGATCCCAAGTGGCTTGAGG-3′ (COX3C-1) or COXC42: 5′-GGCGGGATCCAGTCGTGAGCTTGATTGTGTAGA-3′ (COX3C-2), followed by cloning in the SalI/BamHI or HindIII/BamHI sites of pBI101.3, respectively. A construct containing the entire COX5c-2 5′-non-coding region without the intron was obtained by amplification with primers COXC45: 5′-GGCTCTAGATCATCTCACCACCTGAC-3′ and −40 followed by cloning into the XbaI and HindIII sites of pBI101.3. To test the effect of the intron on an exogenous promoter, the COX5c-2 intron and transcribed 5′-non-coding sequences were amplified with primers COXC45: 5′-GGCTCTAGATCATCTCACCACCTGAC-3′ and COXC41 and cloned in both orientations into the XbaI site of a construct containing a 609 bp promoter fragment from the COX5b-1 gene fused to gus (Welchen et al., 2004). In this way, the intron was placed between the COX5b-1 promoter and the gus coding region. The different 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 COX5c-1 or -2 and the gus-specific primer 5′-TTGGGTTCTCAAGGAG-3′. Five to ten independent lines (depending on the construct) were further propagated and homozygous T3 and T4 plants were used to analyse gus expression. Plants transformed with pBI101.3 or pBI121 were obtained in a similar way and used as negative or positive controls of expression, respectively.

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, and 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.

Specific GUS activity in protein extracts was measured using the fluorogenic substrate 4-methylumbelliferone (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 Na₂CO₃ and the amount of 4-methylumbelliferone was calculated by relating relative fluorescence units with those of a standard of known
Results

COX5c related sequences in the Arabidopsis genome

A search for COX5c coding regions in the Arabidopsis genome using sunflower COX5c protein sequences (Curi et al., 2002) and the program TBLASTN revealed the existence of four genomic regions from which proteins with COX5c-related sequences could be deduced. For three of them (At2g47380, At3g62400, and At5g61310) mRNA sequences are also deposited in data banks, indicating that they are expressed. The fourth region, located in chromosome 5, encodes a protein more distantly related for which no transcripts have been detected, suggesting that it may be a pseudogene. Accordingly, the genes present in chromosomes 2, 3 and 5 have arbitrarily been named COX5c-1, COX5c-2, and COX5c-3, respectively. Upon comparing the corresponding genomic and cDNA sequences, it becomes evident that the three Arabidopsis COX5c genes contain a single intron located within the 5’-non-coding region, at variable distances with respect to the ATG start codon (Fig. 1A). An intron in the same location is also present in the other COX5c genes for which sequences are available, the single genes from rice (Oryza sativa; BAC clone accession number AB027123), sweet potato (Ipomoea batatas; Nakagawa et al., 1993), and Lotus corniculatus (BAC clone accession number AP006137).

Sequences upstream of the translation start site of COX5c-1 and COX5c-2 promote high-level expression of a reporter gene in specific cell-types

Two Arabidopsis thaliana clones containing the COX5c-1 and -2 genes and additional genomic regions were isolated by direct screening of a library using a mixture of ESTs derived from these genes. Subclones of these lambda clones were used to amplify fragments containing sequences located upstream of the translation start site which were introduced in vector pBI101.3 in front of the gus coding region (Fig. 1B). These constructs (pBI5c1 and pBI5c2 for COX5c-1 and -2, respectively) were introduced into Arabidopsis by Agrobacterium-mediated transformation. An initial screening of kanamycin-resistant lines was carried out by histochemistry to define expression characteristics common to most of them. Plants from at least five independent representative transgenic lines carrying each construct were then analysed in detail. The results were essentially the same for all plants analysed, carrying either pBI5c1 or pBI5c2.

Seedlings grown on Petri dishes on MS medium showed strong staining along roots and hypocotyls, while activity in cotyledons was detected only in plants older than 3 d post-germination (Fig. 2A, B). Activity in roots was progressively localized to the vascular cylinder and the root meristem upon growth (Fig. 2B). After 15 d, strong expression was also detected in developing secondary roots (Fig. 2C). In hypocotyls, activity was also progressively localized to vascular tissues upon development (Fig. 2D). Cotyledons displayed GUS activity in the lamina and specially in vascular tissues (Fig. 2D, E). A similar expression pattern was evident in developing leaves (Fig. 2D, E). Strong activity was also observed in the shoot apical meristem (Fig. 2F).

Adult (45-d-old) plants grown on soil displayed activity in roots, leaves, and flowers. Expression in roots was similar to that described for younger plants. In leaves, vascular tissues and, to a lesser extent, mesophyll tissues were stained (Fig. 2G). In flowers, strong expression was detected in anthers, specially in reproductive tissues and pollen grains when these were formed (Fig. 2H, I). Activity was also detected in the stigma, receptacle, and petal and sepal veins, and in siliques and developing seeds (Fig. 2I, J).

To estimate the relative expression levels produced by both constructs, fluorometric assays of GUS activity in protein extracts from transformed 20-d-old plants were performed. Plants transformed with pBI5c1 and pBI5c2 displayed activities of 35 000 pmol min⁻¹ mg⁻¹ and 66 000 pmol min⁻¹ mg⁻¹, respectively. These values are even higher than those observed with plants transformed with

---

Fig. 1. COX5c genes contain an intron within the 5’-non-coding region. (A) Scheme of the six sequenced COX5c genes available in data banks: three genes from Arabidopsis thaliana (AtCOX5c) and one each from Ipomoea batatas (IbCOX5c), Oryza sativa (OsCOX5c), and Lotus corniculatus (LcCOX5c). All genes contain a single intron (white boxes) located in the 5’-non-coding region. The OsCOX5c intron is not drawn to scale due to its length (2.4 kbp). Non-coding and coding regions of exons are shown in light and dark grey, respectively. (B) Scheme of the different constructs used to analyse COX5c regions required for expression. Different regions of the COX5c-1 and COX5c-2 genes, containing untranscribed upstream regions (black boxes), untranslated portions of exons 1 and 2 located upstream of the start codon (light grey boxes), and the leader intron (white box) were fused to the gus coding region and introduced into plants. In a similar way, COX5c-2 untranslated leader sequences were fused to the COX5b-1 promoter (striped boxes) in both orientations.
pBI121 (i.e. the gus gene under the control of the strong constitutive CaMV 35S promoter), 18 000 pmol min\(^{-1}\) mg\(^{-1}\), indicating that the sequences contained within the constructs direct high-level expression. Activity measurements using extracts from different organs indicated that highest expression was attained in leaves followed by flowers, siliques, and roots (not shown).

Removal of the leader intron originates plants with pollen-specific expression

The presence of a conserved intron in the leader region led to the investigation of its role in expression of COX5c genes. Seedlings of plants transformed with constructs in which the respective leader introns from COX5c-1 or COX5c-2 and downstream sequences were removed (5c1-I/E2 and 5c2-I/E2 in Fig. 1B) showed no GUS activity when analysed by histochemical staining (Fig. 2K). Analysis of adult plants revealed the presence of GUS activity only in pollen grains (Fig. 2M, N), while no staining was evident in leaves, siliques, or flower organs other than anthers (Fig. 2L–O). Similar results were observed for both genes under study, indicating that the leader intron is essential to direct high-level expression throughout the plant. Indeed, activity in pollen was also reduced in these plants, since longer incubation times were required to reach similar staining: 18 h with plants transformed with the intronless constructs versus 3–5 h with plants transformed with the entire fragments. The levels of GUS activity present in protein extracts from plants transformed with the intronless constructs were extremely low: 800 and 1400 pmol min\(^{-1}\) mg\(^{-1}\), respectively, for COX5c-1 and COX5c-2, that is 40–50 times lower than those observed when the intron was present.

Untranslated exon sequences influence gus expression

The effect of 5\(^\prime\) untranslated exon regions on expression was also analysed. For this purpose, plants transformed with a construct in which the COX5c-2 leader intron was removed, but the promoter and 5\(^\prime\) non-coding sequences from exons 1 and 2 were conserved (5c2-I in Fig. 1B), were used. These plants showed considerably lower GUS activity levels than plants carrying the construct with the intron (Fig. 3). They consistently showed, however, slightly higher activities than plants bearing only the promoter and exon 1 fused to gus (Fig. 3, inset). The different expression produced by the inclusion of exon 2 non-coding sequences was also observed by histochemical staining. Indeed, activity, although low, was detected in cotyledon veins and tips and also in leaf veins, trichomes, and hydathodes (Fig. 2P–R). In reproductive tissues, expression was only detected in pollen, as with the construct in which...
the intron and exon 2 were removed (Fig. 2S, T). These results, on one side, confirm the importance of the leader intron in determining high level expression and, on the other side, indicate that non-coding exon sequences, though slightly, also influence gene expression.

The COX5c-2 leader intron and adjacent regions increase expression from an unrelated promoter

The effect of the inclusion of the COX5c-2 intron between the promoter of an unrelated gene and the gus coding region was also tested. For this purpose, a region covering the entire intron and surrounding untranslated transcribed sequences from COX5c-2 was inserted between the promoter of the Arabidopsis gene COX5b-1 and the gus coding region, either in the sense or antisense orientation (5b+Is and 5b+Ias, respectively, in Fig. 1B). The portion of the COX5b-1 promoter used (−1 to −609 relative to the translation start site) directs relatively low expression localized in meristems, root, and cotyledon vascular tissues, the leaf central vein, and in anthers (Welchen et al., 2004). Inclusion of the COX5c-2 intron in the correct orientation resulted in considerably higher expression levels throughout the plant, in a pattern similar to those observed with the COX5c-1 and COX5c-2 promoter plus intron fragments (Fig. 4). As a consequence, GUS activity was extended from vascular tissues and anthers, as observed in plants bearing only the COX5b-1 promoter fragment without the intron (Fig. 4A, B, G, J, M, N), to the lamina of cotyledons and leaves (Fig. 4D, E, H, K) and to petals and siliques (Fig. 4O, P). GUS activity measurements in protein extracts from transformed plants indicated that the intron produces a 6-fold increase in expression levels from the COX5b-1 promoter (Fig. 5).
observed in plants transformed with the T-DNA region of these plants, however, were considerably lower than those from COX5c-1 levels of the intron was also observed when analyzing transcript levels. The increase in expression promoted by the presence of leader introns and adjacent regions increase translation efficiency. Six spurious ATG codons are introduced when the intron is placed in the reverse orientation, thus probably affecting the recognition of the correct start codon by the translation machinery. In addition, it is well-known that transcripts with premature termination stop codons in phase affect the stability or translation efficiency. An effect of the intron on translation could also be observed when analyzing transcript levels of plants transformed with the intron on translation could also be observed when analyzing transcript levels of plants transformed with the CaMV 35S promoter fusion. Since plants transformed with the entire COX5c fragments showed 3.5 times higher GUS activities than the latter (see above), this suggests that transcripts containing COX5c 5'-non-coding sequences are more efficiently translated, producing a 25-fold increase in the protein/transcript ratio. Although protein levels were not directly quantified, it can be assumed that enzyme activity measurements constitute an appropriate estimation, since all constructs must produce proteins with the same amino acid sequence. An effect of the intron on translation could also be observed when analyzing transcript levels of plants transformed with the COX5b-1 gene promoter with or without the COX5c-2 intron (Fig. 6, lanes 4, 6). In this case, the increase in transcript levels promoted by the presence of the intron was very low compared with the 6-fold increase in GUS activity observed in plants with the intron in the correct orientation. On the other hand, plants with the intron in the antisense orientation showed undetectable levels of gus transcripts (Fig. 6, lane 5). Although this may be due to a lower transcription efficiency, a more likely explanation is that the unspliced RNA is rapidly degraded.

**Discussion**

COX5c is a polypeptide of about 63 amino acids with sequence similarity to yeast COX VIIa and mammalian
COX VIII (Nakagawa et al., 1990). COX5c cDNAs have been isolated from sweet potato, rice, and sunflower (Nakagawa et al., 1990; Hamanaka et al., 1999; Curi et al., 2002), and ESTs from several species are available. The first COX5c gene was also isolated from sweet potato (Nakagawa et al., 1993), and related sequences can be detected in the totally or partially sequenced genomes from Arabidopsis, rice, and Lotus corniculatus. Expression studies in rice and sunflower indicated that COX5c genes are expressed at different levels throughout the plant (Hamanaka et al., 1999; Curi et al., 2002). However, no detailed analysis on the tissue specificity of expression or on the gene sequences involved in directing this expression have been performed for any plant COX5c gene.

In the present work, it has been determined that sequences from two Arabidopsis COX5c genes located upstream of the respective translation start sites, including an intron present in the 5′-non-coding region, direct similar tissue-specific expression patterns. Expression is observed throughout development, specially in vascular and meristematic tissues, and in pollen grains and siliques. The tissue-specific patterns of COX5c expression may be the consequence of responses to cell-specific factors or to the metabolic status of these tissues, which undergo constant cell proliferation. COX5b-1, a gene encoding a different COX subunit, shows a similar expression pattern, although more strictly localized to young proliferating tissues (Welchen et al., 2004).

It is noteworthy that removal of the leader intron produces a pronounced decrease in expression levels for both genes, making reporter gene activity barely detectable, except in pollen grains. Enhancement of expression by introns has been reported for several genes from monocot (Callis et al., 1987; McElroy et al., 1990; Christensen et al., 1992; Xu et al., 1994; Jeon et al., 2000; Morello et al., 2002) and dicot plants (Norris et al., 1993; Gidekel et al., 1996; Rose and Last, 1997; Plesse et al., 2001; Mun et al., 2002). Introns that influence expression are more frequently located near the translation start site within non-coding regions, as is the case for COX5c genes. The exact role of introns in promoting an increase in expression levels is not clear. Some introns seem to contain transcriptionally active regulatory elements (Gidekel et al., 1996), while others seem to act post-transcriptionally (Rose and Last, 1997), suggesting the existence of different mechanisms of action. It has recently been proposed that many introns would act by increasing the processivity of the transcription machinery (Rose, 2004). Besides the quantitative enhancement of expression, some introns direct tissue-specific patterns of expression (Bolle et al., 1996; Jeon et al., 2000). In some cases, like those of the Petunia actin-depolymerizing factor (Mun et al., 2002), the rice α-tubulin OstubA1 (Jeon et al., 2000) and the Arabidopsis polyubiquitin Ubi.U4 genes (Plesse et al., 2001), expression is specifically observed in vascular tissues and/or metabolically active dividing cells. These expression patterns are similar to those observed here for the COX5c genes, probably indicating that these introns operate by similar mechanisms or respond to similar factors. To test if the COX5c introns contain sequences that direct tissue-specific expression or transcriptional enhancers, plants carrying fusions to a minimal CaMV 35S promoter will be obtained and analysed.

Comparison of GUS activities with the respective transcript levels indicate that COX5c 5′-non-coding sequences also increase translation efficiency. This observation could be made with constructs that possess the COX5c leader introns, but not with those that only carry non-coding exon sequences, due to the low expression levels produced by the latter. It should be emphasized that the context of the start codon, which could affect translation efficiency, is the same in all constructs analysed, since they use the ATG provided by the pBI101.3 vector which is placed several nucleotides downstream of the cloning sites. The differences in translation efficiency are then produced by the intron itself or by the presence of translational enhancers in the 5′-non-coding region. So far, similarities with other known translational enhancers (Yamamoto et al., 1995; Dickey et al., 1998) within COX5c untranslated regions have not been observed. Although the involvement of introns in translation seems an unexpected effect, similar observations have been made in animal and plant systems (Le Hir et al., 2003; Rose, 2004).

In addition, removal of the intron highlights the existence of elements that direct pollen-specific expression within the non-transcribed upstream region of both COX5c genes. This is not unexpected, since transcript levels for a set of mitochondrial genes are considerably higher in flowers and, specifically, in anther tissues (Smart et al., 1994; Zabaleta et al., 1998; Ribichich et al., 2001). Mascarenhas and Hamilton (1992) reported that GUS expression in pollen may be an artefact originated in the diffusion of dye produced in other parts of the anther. This kind of artefact arises when high-level expression is present and produces uneven pollen staining (i.e. both stained and unstained pollen grains are visible even when homozygous plants are used). Since staining of all pollen grains and very low expression levels was observed, it is highly unlikely that GUS expression in pollen is an artefact in this case. In addition, GUS staining of pollen isolated from anthers before performing the histochemical assay was observed. 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 the three genes. Analysis of the promoter regions of both COX5c genes showed the presence of G+T-rich elements (TGGGCC and TGGTGG for COX5c-1 and -2), located at −208 and −210, respectively, from the putative transcription start site. The first of these elements is identical to those observed in one of the Complex I genes and in the tomato LAT52 and LAT56 genes, specifically expressed in pollen (Twell et al., 1991). Both genes also possess two close copies of site II elements (TGGGCC/T), located at −109/−90 and −84/−71, respectively, known to be present in genes preferentially expressed in cycling cells (Kosugi et al., 1995; Trémousaygue et al., 2003). The functional significance of these sequences must be assessed by mutagenesis experiments.

The relevance of the elements directing pollen-specific expression in the COX5c genes is not clear, since the presence of the intron seems to abolish this specificity. It may well be that these elements help to respond to pollen-specific factors, coupled with intron sequences that promote high-level expression. Alternatively, acquisition of the leader intron by a gene that was predominantly expressed in pollen may have occurred later in evolution. The fact that these elements remain functional in both genes suggests, however, that they play an active role in determining the expression characteristics of present-day COX5c genes.

Acknowledgements

We thank the Arabidopsis Biological Resource Center for providing the COX5c EST clones. This work was supported by grants from CONICET, ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica), Fundación Antorchas, and Universidad Nacional del Litoral. RLC and DHG are members of CONICET (Argentina).

References


A leader intron is involved in COX5c gene expression 2571