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The γ-actin encoding gene from the β-carotene producer *Blakeslea trispora*

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Abstract

We determined the nucleotide sequence of a 4599-bp DNA genomic fragment including the γ-actin encoding gene from *Blakeslea trispora*, showing an open reading frame of 1561 bp interrupted by four introns with fungal consensus splice-site junctions. The untranslated regions of the *actA* gene contain a consensus TATA box, a CCAAT motif, a large pyrimidine stretch, and the polyadenylation sequence AATAAA. The predicted protein (375 amino acids) revealed high identity to γ-actins from fungi (>90%), and gene phylogenies support the grouping of *B. trispora* actin close to those from the majority of the filamentous fungi. *actA* transcript (1.4 kb) level in β-carotene producing conditions was faintly higher than *carRA* (1.9 kb) and slightly lower than *carB* (1.8 kb) β-carotene biosynthetic genes. The use of the *actA* promoter (P*actA*) for heterologous gene expression was ascertained by the transformation of gene fusions with the bleomycin resistance gene (*ble*^{R}) from *Streptoalloteichus hindustanus* and the geneticin resistance marker (*aphI*) from Tn903, into *Escherichia coli* and *Acremonium chrysogenum*.

Keywords: Actin; Intron; β-Carotene; Lycopene; *Blakeslea trispora*

1. Introduction

Actin is one of the most abundant proteins found in eukaryotic cells, characterized as α, β, or γ isotypes, and being involved in crucial cellular processes as motility, regulation of cell growth and differentiation, endocytosis, and exocytosis and structural stability [1]. γ-Actin is present in most cell types as a component of the cytoskeleton and mediator of internal cell motility [2]. During septation of *Aspergillus nidulans*, actin first appears at the septum site as a circumferential ring and, later, it broadens and invaginates forming an hourglass-shaped structure coincident with septal wall synthesis [3]. Almost no increase in actin levels is observed after 20-fold augmentation in the number of hyphal tips in *Neurospora crassa*, suggesting that the amount of actin monomers within the hyphae is enough to support this augment [4].

All reported actins include from 374 to 379 amino acid residues and can be used in eukaryotic phylogenetic studies [5,6] because they are conserved [7]. Higher eukaryotes have multiple actin genes, whereas only

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γ-actin has been described in fungi [8]. Promoters from actin-encoding genes have been used to direct gene expression in, among others, the mucoralean fungus *Absidia glauca* [9], the cellulolytic filamentous fungus *Trichoderma reesei* [10], the pathogenic fungus *Cryptococcus neoformans* [11], *Saccharomyces cerevisiae* [12], the methylotrophic yeast *Hansenula polymorpha* [13], Baker's yeast strains [14], a wine yeast strain [15], and the pathogenic yeast *Candida albicans* [16].

Blakeslea trispora is the microorganism industrially used for β-carotene and lycopene production by fermentation in stirred submerged cultures [17]. However, genetic characterization of this fungus is very scarce, existing only recent descriptions on β-carotene biosynthetic genes *carB* and *carRA* [18], *pyrG* gene [19], and genes for rRNA [20]. Although efforts have been made to develop a transformation method (protoplasts, electroporation) of *B. trispora*, it has not been yet achieved, thus delaying the use of molecular biology techniques to improve β-carotene production. Since expression of fungal genes is regulated by promoter regions and intron/exon splicing signals [21,22] and a limited number of *B. trispora* promoters is available, we searched for new promoters among the genes involved in primary metabolism in order to use them for gene expression. This work reports the structure and expression of the gene from *B. trispora* encoding γ-actin (*actA*), and the use of its promoter (P*actA*) for heterologous gene expression.

2. Materials and methods

2.1. Microorganisms, phages, and plasmids

The β-carotene producing strain *B. trispora* F-816 (+) [17] was used as source of DNA. *Escherichia coli* SURE (Stratagene) and *E. coli* LE392 [23] were the hosts for λGEM12 (Promega) phage derivatives. Phage λGEM12 was used to construct a genomic library of *B. trispora* F-816 (+). *E. coli* DH5α [23] was the recipient for high-frequency plasmid transformation. pBluescript I KS (+) and pBC KS (+) phagemids (Stratagene) were used for subcloning and sequencing. General procedures for plasmid DNA purification, cloning, and transformation of *E. coli* were those of Sambrook et al. [23].

2.2. Construction and screening of a B. trispora genomic library

B. trispora F-816 (+) was grown [17] and total DNA was purified [24] according to described methods. Fragments of genomic DNA (17–22 kb) were selected from *Sau*3AI partially digested DNA, ligated to λGEM12, and packaged with "Gigapack II Gold" Kit (Stratagene) to yield approximately 5×10^4 pfu. For screening, the library was amplified in *E. coli* LE392, plated to obtain around 4×10^4 pfu, and hybridized according to standard methods [23] using an 888-bp *Nco*I–*Cla*I fragment from the γ-actin encoding gene of *A. nidulans* [25] as a probe. Recombinant phages were amplified in liquid medium to purify their DNA [23].

2.3. Nucleic acid hybridization and sequencing

Southern hybridizations were made with ^{32}P-dCTP labeled probes according standard procedures [23]. A pilot-scale fed-batch fermentation was carried out by mating the two sexual forms (+ and −) of *B. trispora* to maximize β-carotene production [26]. Mycelium after 72, 96, and 120 h was frozen in liquid nitrogen and total RNA recovered [27]. RNA was resolved by agarose-formaldehyde gel electrophoresis and blotted onto Hybond-N$^+$ membranes (Amersham Biosciences) according to standard procedures [23]. In order to use equimolar concentration of probes, two hybrid probes were constructed in pBluescript I KS (+) by subcloning the following DNA fragments: (i) a 372-bp *Bgl*II–*Xmn*I fragment from the *actA* gene of *B. trispora* and a 466-bp *Eco*RV–*Bgl*II fragment from the *carRA* gene [18] originating the plasmid pALBT123 (*carRA–actA*) and (ii) same fragment from *actA* and a 415-bp *Eco*RV–*Nde*I fragment from the *carB* gene [18] generating pALBT128 (*carB–actA*). Each plasmid was linearized (pALBT123 with *Eco*RV and pALBT128 with *Bgl*II) and used as template for the synthesis of an antisense DIG-labeled RNA probe with the "DIG RNA Labeling Kit" (Roche Molecular Biochemicals). The filters were hybridized at 68 °C overnight with "DIG-Easy Hyb Buffer" (Roche Molecular Biochemicals) and subsequently washed for 15 min at room temperature in 2× SSC, 0.1% SDS, followed by two washes of 15 min at 68 °C in 0.1× SSC, 0.1% SDS. Immunological detection with CDP-Star chemiluminescent substrate (Roche Molecular Biochemicals) was carried out following manufacturer advices. Intensity of hybridization bands was estimated with Gene Tools Analysis Software (Syngene). Clones to be sequenced were constructed with the "Erase a base" system (Promega) and sequenced with an ABI PRISM 377 DNA sequencer (Applied Biosystems). DNA sequences were analyzed with the Dnastar and Winstar packages (DNASTAR Inc.), and compared to known sequences by using the BLASTP and BLASTX programs [28]. The codon-preference algorithm (Geneplot, Winstar) was used to detect the presence of ORFs. Multiple protein alignments were made with the Clustal V algorithm and pairwise alignments with the Lipman–Pearson method (MegAlign, Winstar). The nucleotide sequence corresponding to the gene encoding γ-actin has been deposited in the GenBank with the accession No. AY694422.

2.4. ble^R and aphI gene expression using PactA

Plasmid pALfleo9 was constructed to express the bacterial phleomycin resistance gene (*ble*^{R}) from *Streptoalloteichus hindustanus* under the control of P*actA*. P*actA* was amplified by PCR with primers #88 (5′ GGATGGTGAATTCACATGTATAAAAGCTTTACTGCC 3′; *Eco*RI site underlined) and #89 (5′ GTCCTCCATGGTGAGTGATATTATATATTAAAG 3′; *Nco*I site underlined). Optimized amplification reactions (100 μl) contained about 20 ng of pALBT5 as template, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 20 pmol of each primer, 1.25 U of Taq DNA polymerase, and dNTPs 200 mM each. A Perkin–Elmer DNA Thermal Cycler 480 was used for amplification with a program of 25 cycles [94 °C, 60 s (2 min for the first cycle); 50 °C, 60 s; 72 °C, 90 s (extending 3 s per cycle)]. After sequencing to verify the absence of punctual mutations, P*actA* was subcloned as an 835-bp *Nco*I–*Eco*RI fragment into pALfleo5 [29] to give pALfleo9 (P*actA*–*ble*^{R}–T*trpC*). DNA sequence showed the correct in-frame fusion P*actA*–*ble*^{R}. The transcriptional terminator signal (T*trpC*) is from the *trpC* gene of *A. nidulans*. pALfleo9 confers phleomycin–bleomycin resistance in fungi and ampicillin resistance in *E. coli*. Plasmid pALgen1 expresses the geneticin–kanamycin resistance gene (*aphI*) from Tn903 under the control of P*actA*. It was constructed by subcloning an 1865-bp *Nco*I–*Sac*I fragment from pALG418 (including *aphI* and T*trpC*) [30] under P*actA*. pALgen1 contains the cassette P*actA*–*aphI*–T*trp*C for geneticin (G418) resistance in fungi, and kanamycin and ampicillin resistance in *E. coli*. Protoplasts transformation of *Acremonium chrysogenum* was as previously described [31]. *A. chrysogenum* transformants were selected in tryptic soy agar (Difco) with sucrose (10.3%) supplemented with 3 μg ml^{-1} phleomycin (Cayla) or 7 μg ml^{-1} G418 (Geneticin, Life Technologies) after incubation at 28 °C for 10 days.

3. Results and discussion

3.1. Cloning and characterization of the γ-actin encoding gene from B. trispora

To identify the *actA* gene, a genomic library of *B. trispora* was screened with a probe from the γ-actin encoding gene of *A. nidulans* [25]. Ten positive clones were identified and characterized by restriction mapping and Southern analysis. A common 4.6-kb *Pvu*II fragment detected in most of the phages was subcloned in the *Eco*RV site of pBC KS (+) to yield pALBT5 and pALBT6. A DNA region of 4599 bp including the *actA* gene was sequenced by both strands and analyzed with the codon-preference algorithm (Geneplot), showing the presence of five exons (at positions 1929–1949, 2078–2143, 2290–2963, 3057–3192, and 3262–3494) and four intervening sequences (Fig. 1). The presence

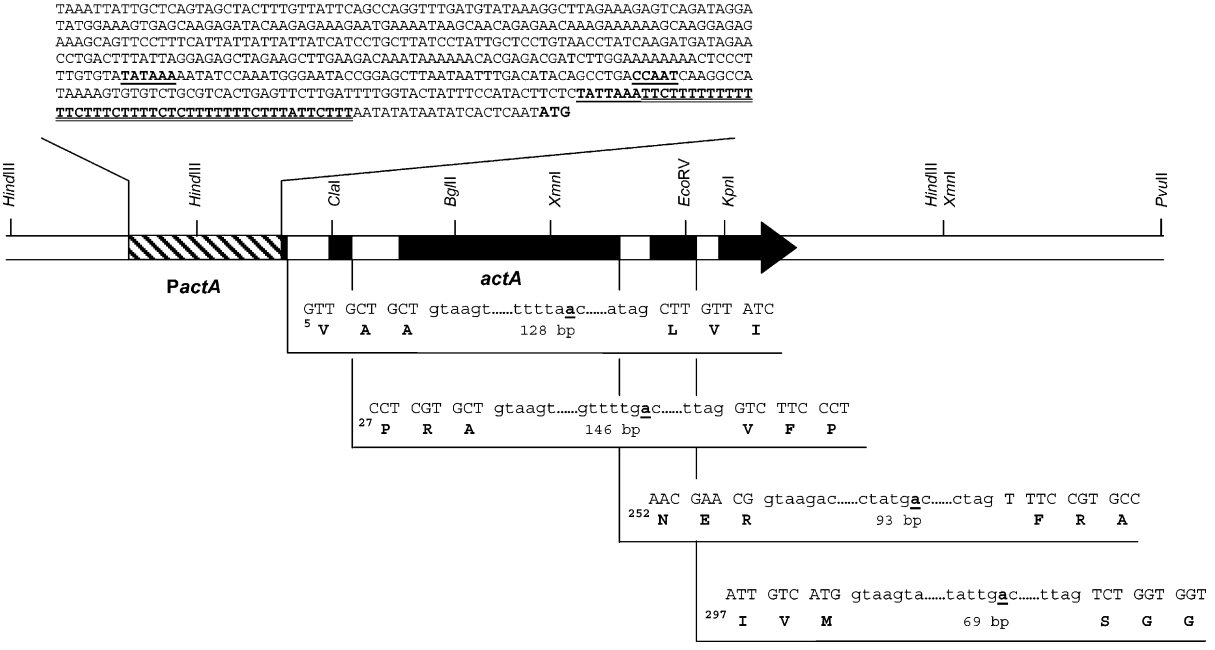

Fig. 1. Restriction map of the genomic DNA region of *B. trispora* including the *actA* gene. The *actA* gene is represented by an arrow, P*actA* is dashed, and white boxes inside the arrow represent four introns. Potential regulatory sequences identified in the 5′ non-coding region such as pyrimidine rich stretch (bold and double underlined), TATA box, TATA-like box, and CCAAT box (bold and underlined) are shown. Location and partial sequence of exons (upper case) and introns (low case) in the deduced protein are indicated. Fungal consensus intron processing sites for intron/exon boundaries at 5′ (g/GTAHGTY) and 3′ (MYAG/g) and splice signal for lariat formation (WRCTRAC) are conserved. H is A or C or T, D is G or A or T, Y is C or T, W is A or T, R is A or G, M is C or A, and K is G or T, according to the IUB codes. The conserved adenosine at the lariat branch site is bold and underlined.

of introns is a common phenomenon in most of actin encoding fungal genes [8,25,32–34]. The exons code for 7, 22, 225, 45, and 76 amino acids, respectively, and the introns are 128-, 146-, 93-, and 69-bp long. Fungal consensus sequences described for intron/exon boundaries and splice signal for lariat formation [21] were detected (Fig. 1). The consensus of the lariat sequences maintains an adenosine (bold and underlined in Fig. 1) at the branch site, which has been shown to be crucial for the chemical steps of splicing [32]. The consensus lariat sequence for the *actA* gene of *B. trispora* (YWWTRAC) is similar to that found in higher plants (CTRAY). Intron comparisons were done with γ-actin encoding genes from the taxonomically related fungi (Zygomycota, Zygomycetes, Mucorales) *A. glauca* (AJ287135), *Mucor circinelloides* (AJ287173), and *Rhizomucor pusillus* (AJ287192). Unfortunately only partial gene sequence (including the region corresponding to third and fourth introns of *B. trispora*) from these species is available. Whereas the location of both introns in *M. circinelloides* and *R. pusillus* matches that of *B. trispora*, only the position of the fourth intron is analogous in *A. glauca*. Interestingly, the location of the first three introns of *B. trispora* (Fungi, Zygomycota, Zygomycetes, Mucorales) does not match the described for the γ-actin encoding genes from *A. nidulans* (Fungi, Ascomycota, Pezizomycotina, Eurotiomycetes), *Penicillium chrysogenum* (Fungi, Ascomycota, Pezizomycotina, Eurotiomycetes), and *A. chrysogenum* (Fungi, Ascomycota, Pezizomycotina, Sordariomycetes), showing a clear divergence between Zygomycota and Ascomycota; only the position of the fourth intron of *B. trispora* coincides exactly with the fifth intron of these fungi. The existence of introns is also usual in the scarce number of *B. trispora* genes described: *carB* (AY176662 and AY176663), *carRA* (AY176662, and AY176663), *pyrG* (AY262090), and *gpd* (AJ278318).

3.2. Analysis of the 5′ and 3′ flanking sequences of the actA gene

Potential regulatory elements were found in the promoter region of the *actA* gene. A large (44 bp) pyrimidine-rich region was detected between positions 1865 and 1908 (Fig. 1), suggesting a possible transcription start site. Usually, the region immediately upstream of the transcription start point is pyrimidine rich in the coding strand, particularly in highly expressed genes [22]. A consensus TATA box (TATAAA) and TATA-like box (TATTAAA) (positions 1734 and 1858, respectively) were located upstream (189 and 64 bp, respectively) from the translation initiation site ATG (position 1929) (Fig. 1). The majority of promoters in higher eukaryotes contain this box 25–30 bp upstream from the transcription initiation site [22]. Furthermore, a CCAAT box was found at position 1789. This box is present in the promoter region of about the 30% of known eukaryotic genes located 50–200 bp upstream of the transcription start site [22]. The consensus sequence AATAAA, potentially involved in the polyadenylation of the 3′ terminus, was found at position 4242 and the polyadenylation-like sequence (ATATAAA) was at 3630, within an AT rich region.

3.3. Analysis of the deduced amino acid sequence encoded by the actA gene

The 375 amino acid deduced protein has a predicted molecular mass of M_r 41.76 kDa and an isoelectric point of 5.39. Comparison to SwissProt & PIR databases gave high identity scores with fungal γ-actins, especially with those from *Absidia blakeesleana* (97%), *Schizosaccharomyces pombe* (92%), *A. chrysogenum* (92%), *N. crassa* (91%), *P. chrysogenum* (91%), and *A. nidulans* (91%). Most residues were absolutely conserved in all these sequences and minimal differences were found.

Actins are highly conserved proteins present in all eukaryotic cells that can be used in phylogenetic studies. A phylogenetic tree was constructed with actin sequences from fungal representatives of the different taxonomical classes and some examples of higher eukaryotes (Fig. 2). Actin phylogenies perfectly agree with taxonomical classifications, discriminating among Fungi, Metazoa, and Viridiplantae. Based on the analyzed sequences, Fungi are divided in three main lineages: Zygomycota (*B. trispora* and *A. glauca*), Ascomycota (including filamentous fungi as *P. chrysogenum* and budding yeasts as *S. cerevisiae*), and Basidiomycota (*Coprinus cinereus*). Correspondingly, Metazoa are classified in Arthropoda (*Drosophila melanogaster*), Nematoda (*Onchocerca volvulus*), and Chordata (including Amphibia as *Xenopus laevis* and Mammalia as *Homo sapiens*).

Three signature patterns have been developed for actin identification in the Prosite database: (1) ^{54}[FY]-[LIV]-G-[DE]-E-A-Q-X-[RKQ]-[RKQ]-G^{64}, (2) ^{106}[LM]-[LIVM]-T-E-[GAPQ]-X-[LIVMFYWHQ]-N-[PSTAQ]-X-X-N-[KR]^{118}, and (3) ^{357}W-[IV]-[STA]-[RK]-X-[DE]-Y-[DNE]-[DE]^{365}. The first two are specific to actins, whereas the last matches both to actins and actin-like proteins. The three signatures have been located in the actin of *B. trispora*: ^{53}YVGDEAQSKRG^{63}, ^{104}LLTEA- PLNPKSNR^{116}, and ^{356}WISKQEYDE^{364}. Likewise, it matches the N-terminal sequence identifying the γ-actin isotype ^{1}MEEEXXXXXI^{10} described for the γ-actin of *A. nidulans* [25], but showing D at the third position.

3.4. actA, carB, and carRA gene expression

A comparison of the *actA* gene expression and the β-carotene biosynthetic genes *carB* and *carRA* was made in order to investigate the expression level of a constitutive

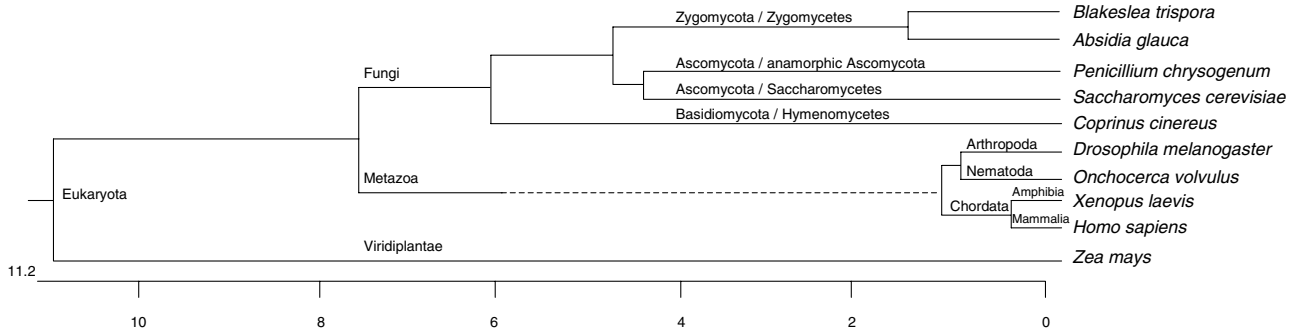

Fig. 2. Phylogenetic tree of actins. The protein sequence of *B. trispora* (Zygomycota) was compared with sequences from the fungi *A. glauca* (Zygomycota), *P. chrysogenum* (Ascomycota), *S. cerevisiae* (Ascomycota), and *C. cinereus* (Basidiomycota), the Metazoa *D. melanogaster* (Arthropoda), *O. volvulus* (Nematoda), *X. laevis* (Nematoda), and *H. sapiens* (Chordata), and the Viridiplantae *Z. mays* (Streptophyta). The alignment was performed with the program Megalign by using the Clustal method with the PAM250 residue weight table. Multiple alignment parameters were: gap penalty 10 and gap length penalty 10. Pairwise alignment parameters were: ktuple 1, gap penalty 3, window 5, and diagonals 5. The length of each pair of branches represents the distance between sequence pairs. The units at the bottom of the tree indicate the number of substitution events.

house-keeping gene (*actA*) in contrast to regulated genes involved in secondary metabolism (*carB* and *carRA*) [18]. Their transcription level was analyzed by Northern hybridization, using total RNA samples obtained from pilot-scale feed-batch fermentations (after 72, 96, and 120 h) to produce β-carotene [26]. The size of each transcript detected corresponded to the expected according to sequence data: 1.8 kb for *carB*, 1.9 kb for *carRA*, and 1.4 kb for *actA* (Fig. 3). While maximum *carB* and *carRA* transcription levels occur after 72–96 h in old solid-medium cultures of *M. circinelloides* and *Phycomyces blakesleeanus* [35–37], steady-state levels of *carB* and *carRA* transcripts were present in *B. trispora* after 120 h in a fermenter. At least two explanations exist for these differences: (i) *B. trispora* cultures were mated and cultured in a fermenter, while single strains of *M. circinelloides* and

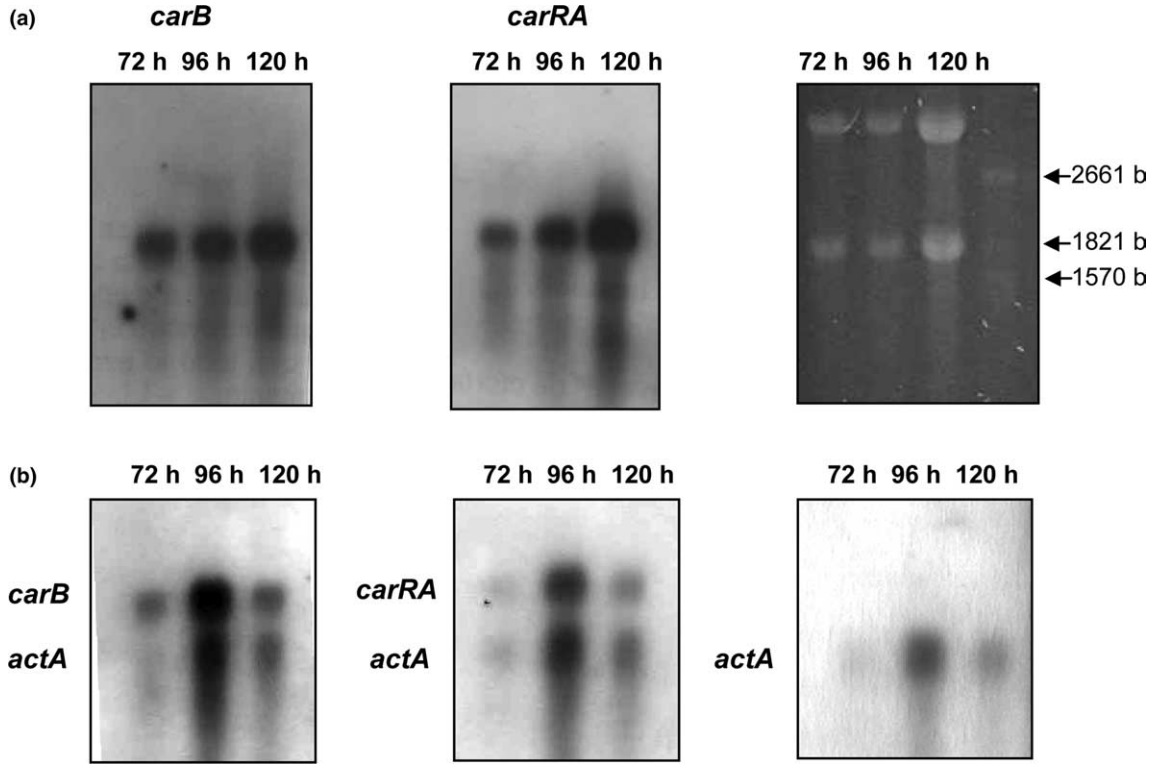

Fig. 3. Transcription of *actA*, *carB*, and *carRA* genes of *B. trispora*. Northern analyses were done with total RNA (around 5 μg per well) purified after 72, 96, and 120 h from pilot-scale fermentors in β-carotene production conditions. (a) Estimation of transcript size for *carB* (1.8 kb) and *carRA* (1.9 kb) genes, and stained gel showing rRNAs and M_w markers. (b) Hybridization with *carB–actA* or *carRA–actA* hybrid probes to guarantee equimolar concentration of each biosynthetic gene with reference to the *actA* (1.4 kb) gene.

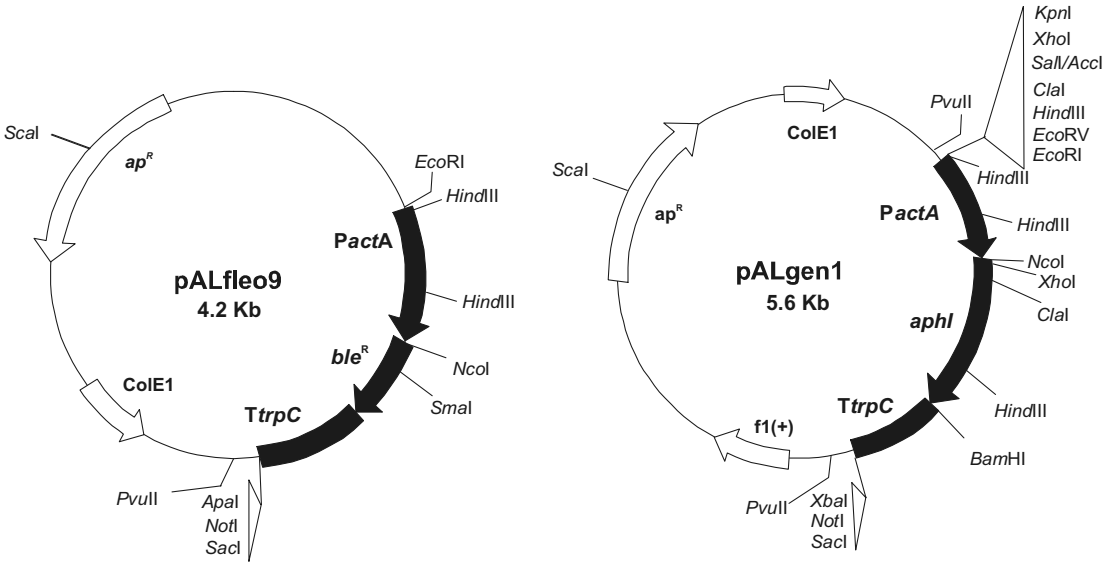

Fig. 4. Restriction map of the plasmids pALfleo9 and pALgen1 used, respectively, for *ble*^{R} and *aphI* gene expression under P*actA* control.

P. blakesleeanus were grown on solid-medium and (ii) the nutrient feeding protocol in the fermenter extends fungal vegetative growth and β-carotene production. An estimation of *carB* and *carRA* transcription levels with reference to *actA* was done (Fig. 3(b)) with the hybrid probes *carB–actA* or *carRA–actA* (see Section 2.3) to guarantee equimolar concentration of the β-carotene biosynthetic genes and *actA*. Transcription of *actA* is assumed to be maintained throughout the growth phase in the fermentor. Changes in band intensity were due to loading variability (e.g., 96 h). The estimated relative intensities with reference to *actA* were 0.7–0.8 for *carRA* and 1.1–1.4 for *carB*, showing a fairly higher transcript level for *carB*.

3.5. Gene expression under the control of PactA

There is no transformation system for *B. trispora*, so P*actA* functionality was tested in *E. coli* and *A. chrysogenum* (a fungi routinely transformed in our laboratory) using the phleomycin and geneticin–kanamycin reporter systems encoded respectively by the *ble*^{R} determinant from *S. hindustanus* and the *aphI* gene from Tn903. Plasmids pALfleo9 and pALgen1 (Fig. 4) contain the cassette P*actA–ble*^{R}–T*trpC* and P*actA–aphI*–T*trpC*, respectively, and both include the ampicillin resistance gene for selection in *E. coli*. Most of *E. coli* DH5α transformants containing pALgen1, selected by ampicillin resistance, were able to grow on plates containing 50 μg ml^{-1} kanamycin, showing that *aphI* gene is correctly expressed under the control of P*actA*. As expected, transformation efficiency (transformants per μg of pALgen1) in *E. coli* DH5α was above two orders of magnitude lower when transformants selection was done directly by kanamycin resistance instead of ampicillin resistance. We were unable to select any stable geneticin resistant transformant of *A. chrysogenum* using pALgen1. This could be due to inefficient function of the protein encoded by *aphI*, as recently described for *A. chrysogenum* transformation using P*gpd–aphI*–T*trpC* [30]. On the contrary, pALfleo9 was able to confer phleomycin resistance (3 μg ml^{-1}) to *A. chrysogenum*. Although phleomycin resistance levels are dependent on the strain tested and the number of functional copies of the P*actA–ble*^{R}–T*trpC* cassette integrated in their chromosomes, higher resistance levels were obtained in *A. chrysogenum* by expressing *ble*^{R} under the control of P*pcbC* (5–10 μg ml^{-1}) or P*gdhA* (10–20 μg ml^{-1}), both from *P. chrysogenum* [29]. These results show that P*actA* is able to direct heterologous *ble*^{R} and *aphI* gene expression in *E. coli* and *A. chrysogenum*. *ble*^{R} and *aphI* expression cassettes for transformation of lower and higher eukaryotes have been previously described [30,38,39].

As a conclusion, here is described a new gene from *B. trispora*, a microorganism with a scarce genetic knowledge but with a great importance from a commercial point of view because it is industrially used for the biotechnological production of β-carotene and lycopene. Although at present neither gene dosage nor gene sequence in *B. trispora* can yet be altered by transformation, the *actA* promoter region described here can be used as a tool to guide gene expression when a transformation protocol is available.

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