Isocitrate lyase of *Ashbya gossypii* – transcriptional regulation and peroxisomal localization

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Abstract The isocitrate lyase-encoding gene *AgICL1* from the filamentous hemiascomycete *Ashbya gossypii* was isolated by heterologous complementation of a *Saccharomyces cerevisiae* icl1d mutant. The open reading frame of 1680 bp encoded a protein of 560 amino acids with a calculated molecular weight of 62 584. Disruption of the *AgICL1* gene led to complete loss of AgIcl1p activity and inability to grow on oleic acid as sole carbon source. Compartmentation of AgIcl1p in peroxisomes was demonstrated both by Percoll density gradient centrifugation and by immunogold labeling of ultrathin sections using specific antibodies. This fitted with the peroxisomal targeting signal AKL predicted from the C-terminal DNA sequence. Northern blot analysis with mycelium grown on different carbon sources as well as *AgICL1* promoter replacement with the constitutive *AgTEF* promoter revealed a regulation at the transcriptional level. *AgICL1* was subject to glucose repression, derepressed by glycerol, partially induced by the C_2 compounds ethanol and acetate, and fully induced by soybean oil.

Key words: *Ashbya gossypii*; Isocitrate lyase; Immunoelectron microscopy; Peroxisome

1. Introduction

The filamentous hemiascomycete *Ashbya gossypii* (Ashby and Novell) [1–6] is a natural overproducer of vitamin B_2 (riboflavin) [7]. Plant oils which are degraded to fatty acids and glycerol by an extracellular lipase [8] are the preferred substrates for riboflavin production because of their yield-enhancing effect [9,10]. The ability of plants and microorganisms to grow on fatty acids as carbon source is based on the function of the glyoxylate cycle. Since the precursors of riboflavin, GTP and ribulose 5-phosphate, originate from carbohydrate metabolism, the glyoxylate cycle in concert with gluconeogenesis plays an essential role with respect to growth and riboflavin synthesis of the fungus. Isocitrate lyase (Iclp), the key enzyme of this metabolic pathway, catalyzes the cleavage of isocitrate to glyoxylate and succinate diverting isocitrate through a carbon-conserving pathway. A correlation of riboflavin production and AgIcl1p specific activity was recently demonstrated [11].

Based on sequence analysis of 18S rDNA, 25S rDNA and the highly variable internal transcribed spacer regions ITS1 and ITS2 *A. gossypii* was recently classified into the Saccharomycetaceae family [12]. High homology of the *THR4* and *TEF* genes isolated from *A. gossypii* [13,14] with the corresponding genes from *Saccharomyces cerevisiae* and conservation of gene order, as described for the *THR4* locus [13], emphasize a close relationship of the two fungi. In consequence, the functional complementation of a *S. cerevisiae* ICL1 defect mutant with a *A. gossypii* genomic library was a promising approach to isolate the *AgICL1* gene.

The current paper reports on the isolation and disruption of the *AgICL1* gene of *A. gossypii*. Since cytosolic localization of ScIcl1p was shown recently in *S. cerevisiae* [15,16], we focused on its compartmentation in *A. gossypii* accompanied by studies on enzyme regulation.

2. Materials and methods

2.1. Microorganisms and cultivation

2.1.1. A. gossypii. Strain ATCC 10895 was obtained from BASF (Ludwigshafen, Germany). The fungus was grown in a complex medium (1% yeast extract, 1% peptone, 0.3% glycine, 1% soybean oil) or in a medium consisting of 1% yeast extract and 200 mM potassium buffer pH 6.5 with either 1% soybean oil (YS) or 2% glucose (YG), ethanol, acetate or glycerol as the carbon source. The minimal medium consisted of 0.67% yeast nitrogen base, 0.5% ammonium sulfate with 0.1% oleic acid as carbon source. Cultivation conditions have been described previously [8]. Geneticin (G418, Boehringer Mannheim) at a concentration of 400 μg/ml was used for the selection of transformants.

2.1.2. S. cerevisiae. icl1d (MATα *leu*2-3, 112 *icl1*::*LEU2 ura*3-(fs)) [17] was grown at 30°C on rich YPD medium (1% yeast extract, 2% peptone, 2% glucose) or on minimal YNB medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate) with 2% glucose or ethanol as carbon source.

2.2. Recombinant DNA methods

The method of Dohmen et al. [18] was used for *S. cerevisiae* transformation. Reisolation of plasmids from *S. cerevisiae* was performed according to Nasmyth and Reed [19]. *Escherichia coli* transformation and plasmid amplification were done by standard protocols [20].

For *A. gossypii* transformation mycelium grown for 16 h on YG was harvested by vacuum filtration through filter paper, washed and incubated for 30 min at 30°C in 50 mM phosphate buffer pH 7.5 including 25 mM DTT. The mycelium was washed once with a buffer composed of 10 mM Tris-HCl pH 7.5, 0.27 M sucrose, 1 mM EDTA and resuspended in a minimal volume of the same buffer. Aliquots of this suspension mixed with 1–5 μg DNA were pulsed in 2 mm cuvettes at 1.5 kV/cm, 100 Ω and 25 μF using a Gene Pulser (Bio-Rad). After 6 h of non-selective growth at 30°C the YG agar plates were overlaid with 5 ml top agar containing 9 mg of geneticin. Homokaryotic transformants were obtained by selection of geneticin-resistant spores [21].

Southern blot and Northern blot analyses were done by standard techniques [20].

For genomic DNA isolation, mycelium of *A. gossypii* grown for 16 h on YG was harvested by vacuum filtration, washed with distilled

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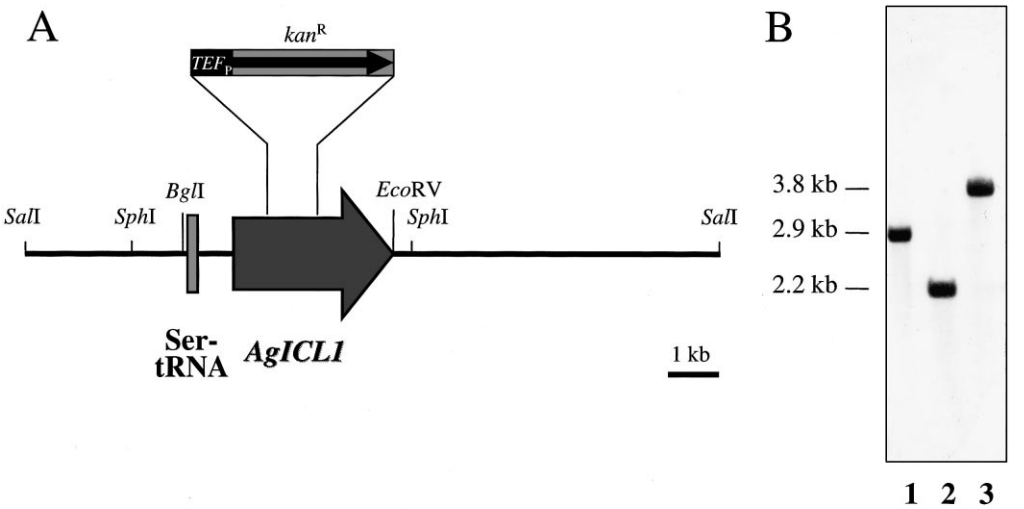

Fig. 1. Partial restriction map of the *A. gossypii ICL1* locus and strategy for gene disruption (A) and Southern blot analysis (B) (lane 1: wild type *Sph*I, 2: wild type *Eco*RV/*Bgl*I, 3: *AgΔICL1*::*kan*^{R} *Eco*RV/*Bgl*I).

water and resuspended in 0.1 mM sodium citrate pH 7.5 containing 1 M sorbitol, 60 mM EDTA, and 0.1% (v/v) β-mercaptoethanol. Protoplasts were formed after adding Lysing Enzyme (Sigma, 2 mg/ml) during an incubation for 30 min at 30°C. The addition of 1/10 vol each of 0.5 mM EDTA and 10% (v/v) SDS led to protoplast breakage followed by proteinase K treatment for 1 h at 50°C. The resulting suspension was treated with 1 vol of 5 M ammonium acetate and centrifuged, and genomic DNA was precipitated from the supernatant with ethanol. The DNA was purified by an RNase A treatment followed by phenol/chloroform (1:1 v/v) extraction and re-precipitation with ethanol, and finally resuspended in TE buffer.

To confirm *AgICL1* gene disruption, a digoxigenin-labeled DNA fragment corresponding to the entire open reading frame was used as a probe. Labeling and detection were performed with a DIG labeling and detection kit according to the user's manual (Boehringer Mannheim).

Total RNA was isolated according to a yeast protocol [22].

For Northern blot analyses the *AgICL1* gene and the *AgACT1* actin gene (EMBL accession number AJ131713) from *A. gossypii* were labeled with α[^{32}P]dCTP (Amersham) by random priming. Hybridization signals were quantified by storage phosphor screen technology with a PhosphoImager (Bio-Rad).

DNA sequence analysis was conducted on both strands using the dideoxynucleotide termination method of Sanger et al. [23]. Sequences were aligned using the BESTFIT program (EMBL, Heidelberg).

2.3. Isolation, disruption and promoter replacement of the AgICL1 gene

To clone the *AgICL1* gene, an *A. gossypii* genomic library was constructed using the *S. cerevisiae*/*E. coli* shuttle vector YEp352 [24]. Partial *Sau*3A digests (>8 kb) of genomic *A. gossypii* DNA were separated on a sucrose density gradient [20] and ligated into the *Bam*HI site of YEp352.

To construct an *AgICL1* gene disruption, an internal 500 bp fragment was replaced by a 2.1 kb *Sal*I fragment of the plasmid pAG100 carrying the geneticin resistance cassette [14].

For *AgICL1* promoter replacement the *A. gossypii TEF* promoter was amplified by PCR (P1: 5′-CGCCAGGGATCCAAGCTTGCCTCGTCCCGCCGG-3′, P2: 5′-AGCGATGGATCCAACGTGGGTACCTTCCTTCTCGAGTTTTGTTTATGTTCGGATGTG-3′) (Expand Long Template PCR System, Boehringer Mannheim) with pAG110 as template, creating *Bam*HI, *Kpn*I and *Xho*I restriction sites respectively (underlined). The amplified fragment was cloned into the *Bam*HI-digested pUC18, generating the plasmid pST10. After introducing a *Xho*I site (underlined) upstream of the start codon by PCR (template: YEpAgICL1, P3: 5′-GTGAACCTCGAGATGTCCCCTTCCGTCAGAGAC-3′; P4: 5′-TGTCGGGGTACCCGATGTTAT-3′; *Kpn*I site underlined) the *AgICL1* gene was inserted into *Xho*I/*Kpn*I cut pST10, forming the pST11 plasmid. Next, the geneticin resistance cassette, isolated from pAG231 [25] as a *Sal*I fragment, was introduced into the *Sal*I site (pST11). Another PCR-derived *Sph*I site at −166 bp upstream of the *AgICL1* gene (template YEpAgICL, P5: 5′-TTAAAAGCATGCCTTGCAGCGGCAATTGCCTCC-3′, P6: 5′-GGC-AACGCATGCGGCCCGGACCAAGTACACTTG-3′) allowed the integration of the −787 to −166 bp upstream region of *AgICL1* into the *Sph*I site, thus completing the construction (pST12). A *Kpn*I/*Nde*I fragment of pST12 was applied for transformation of *A. gossypii*. To confirm promoter replacement by PCR the primers P7 (5′-TGACGTCGCACCGGAGTCGGTGCCGTGAAT-3′) and P8 (5′-GGTTACACCCTTACCCATCGAGGCTGTCGA-3′) were used.

2.4. Cell fractionation

Cell organelles were isolated from protoplast homogenate in an isotonic, self-generating Percoll density gradient. Mycelium grown for 48 h on YS was harvested by vacuum filtration and washed with distilled water at room temperature. The hyphae were resuspended in buffer A (50 mM MES/KOH, pH 7.2, containing 1 M sorbitol, 0.5 mM EDTA) with 2 mg/ml Lysing Enzyme (Sigma) and gently shaken at 28°C until protoplast formation was complete. The following steps were carried out at 4°C. Protoplasts were recovered by a low-speed centrifugation step (5 min, 1500×*g*), washed once, resuspended in buffer A with 1 mM PMSF and homogenized using a Potter homogenizer (1000 rpm). The homogenate was centrifuged at 1500×*g* for 5 min to remove cell debris. Organelles were sedimented at 27000×*g* for 15 min and suspended in 14 ml buffer B (50 mM MES/KOH pH 7.2, 0.25 M sucrose, 33% (v/v) Percoll (Sigma), 1 mM KCl, 0.5 mM EDTA, 1 mM PMSF). The gradient was formed during an 80 min centrifugation at 30000×*g* in a Ti50 rotor (Beckman). Fractions of 1 ml were collected from the bottom of the tube and analyzed for enzyme activities.

2.5. Enzyme assays and electron microscopy

Iclp, catalase and cytochrome *c* oxidase activity were assayed as described previously [26–28]. One unit of enzyme activity was defined as the amount of enzyme required to form 1 μmol of product per min. Specific activities are given as U/mg protein. The method of Bradford

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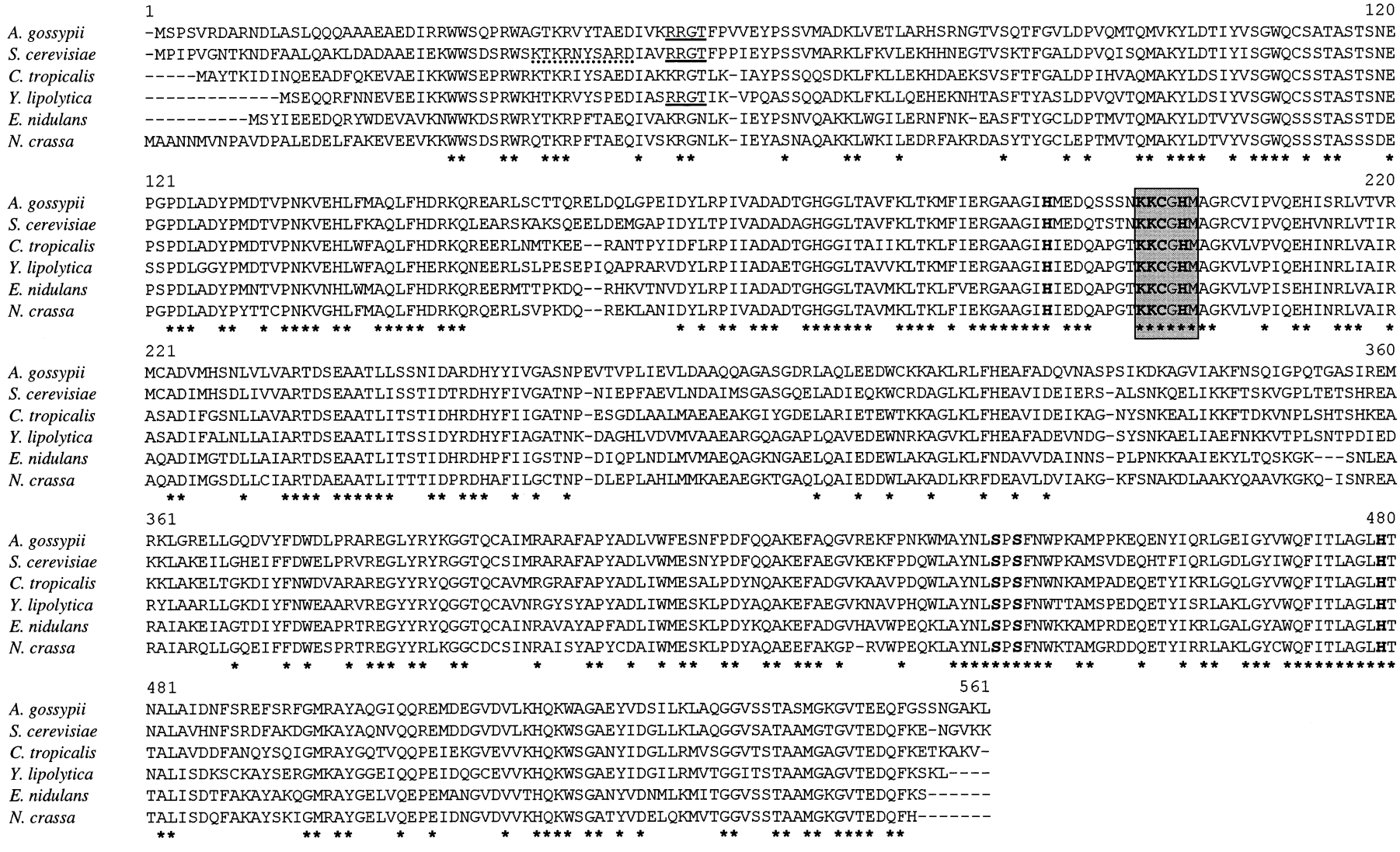
Fig. 2. Alignment of Icl amino acid sequences from various fungi. Identical amino acid residues in all sequences are indicated by asterisks. The conserved hexapeptide sequence used as an identification pattern of Iclp is boxed. Amino acids which proved to be functional residues in the Iclp of *E. coli* are in bold face. The putative cAMP-dependent protein kinase phosphorylation site is underlined by a solid line. The decapeptide signal for glucose-triggered inactivation in *S. cerevisiae* is underlined with a dotted line.


1 120
A. gossypii -MSPSVRDARNDLASLQQQAAAEAEDIRRWWSQPRWAGTKRVYTAEDIVKRRGTFPVVEYPSSVMADKLVETLARHSRNGTVSQTFGVLDPVQMTQMVKYLDTIYVSGWQCSATASTSNE
S. cerevisiae -MPIPVGNTKNDFAALQAKLDADAAEIEKWWSDSRWSKTKRNYSARDIAVRRGTFPPIEYPSSVMARKLFKVLEKHHNEGTVSKTFGALDPVQISQMAKYLDTIYISGWQCSSTASTSNE
C. tropicalis -----MAYTKIDINQEEADFQKEVAEIKKWWSEPRWRKTKRIYSAEDIAKKRGTLK-IAYPSSQQSDKLFKLLEKHDAEKSVSFTFGALDPIHVAQMAKYLDSIYVSGWQCSSTASTSNE
Y. lipolytica ----------MSEQQRFNNEVEEIKKWWSSPRWKHTKRVYSPEDIASRRGTIK-VPQASSQQADKLFKLLQEHEKNHTASFTYASLDPVQVTQMAKYLDSIYVSGWQCSSTASTSNE
E. nidulans ----------MSYIEEEDQRYWDEVAVKNWWKDSRWRYTKRPFTAEQIVAKRGNLK-IEYPSNVQAKKLWGILERNFNK-EASFTYGCLDPTMVTQMAKYLDTVYVSGWQSSSTASSTDE
N. crassa MAANNMVNPAVDPALEDELFAKEVEEVKKWWSDSRWRQTKRPFTAEQIVSKRGNLK-IEYASNAQAKKLWKILEDRFAKRDASYTYGCLEPTMVTQMAKYLDTVYVSGWQSSSTASSSDE
121 220
A. gossypii PGPDLADYPMDTVPNKVEHLFMAQLFHDRKQREARLSCTTQRELDQLGPEIDYLRPIVADADTGHGGLTAVFKLTKMFIERGAAGIHMEDQSSSNKKCGHMAGRCVIPVQEHISRLVTVR
S. cerevisiae PGPDLADYPMDTVPNKVEHLFKAQLFHDRKQLEARSKAKSQEELDEMGAPIDYLTPIVADADAGHGGLTAVFKLTKMFIERGAAGIHMEDQTSTNKKCGHMAGRCVIPVQEHVNRLVTIR
C. tropicalis PSPDLADYPMDTVPNKVEHLWFAQLFHDRKQREERLNMTKEE--RANTPYIDFLRPIIADADTGHGGITAIIKLTKLFIERGAAGIHIEDQAPGTKKCGHMAGKVLVPVQEHINRLVAIR
Y. lipolytica SSPDLGGYPMDTVPNKVEHLWFAQLFHERKQNEERLSLPESEPIQAPRARVDYLRPIIADAETGHGGLTAVVKLTKMFIERGAAGIHIEDQAPGTKKCGHMAGKVLVPIQEHINRLIAIR
E. nidulans PSPDLADYPMNTVPNKVNHLWMAQLFHDRKQREERMTTPKDQ--RHKVTNVDYLRPIIADADTGHGGLTAVMKLTKLFVERGAAGIHIEDQAPGTKKCGHMAGKVLVPISEHINRLVAIR
N. crassa PGPDLADYPYTTCPNKVGHLFMAQLFHDRKQRQERLSVPKDQ--REKLANIDYLRPIVADADTGHGGLTAVMKLTKLFIEKGAAGIHIEDQAPGTKKCGHMAGKVLVPIQEHINRLVAIR
221 360
A. gossypii MCADVMHSNLVLVARTDSEAATLLSSNIDARDHYYIVGASNPEVTVPLIEVLDAAQQAGASGDRLAQLEEDWCKKAKLRLFHEAFADQVNASPSIKDKAGVIAKFNSQIGPQTGASIREM
S. cerevisiae MCADIMHSDLIVVARTDSEAATLISSTIDTRDHYFIVGATNP-NIEPFAEVLNDAIMSGASGQELADIEQKWCRDAGLKLFHEAVIDEIERS-ALSNKQELIKKFTSKVGPLTETSHREA
C. tropicalis ASADIFGSNLLAVARTDSEAATLITSTIDHRDHYFIIGATNP-ESGDLAALMAEAEAKGIYGDELARIETEWTKKAGLKLFHEAVIDEIKAG-NYSNKEALIKKFTDKVNPLSHTSHKEA
Y. lipolytica ASADIFALNLLAIARTDSEAATLITSSIDYRDHYFIAGATNK-DAGHLVDVMVAAEARGQAGAPLQAVEDEWNRKAGVKLFHEAFADEVNDG-SYSNKAELIAEFNKKVTPLSNTPDIED
E. nidulans AQADIMGTDLLAIARTDSEAATLITSTIDHRDHPFIIGSTNP-DIQPLNDLMVMAEQAGKNGAELQAIEDEWLAKAGLKLFNDAVVDAINNS-PLPNKKAAIEKYLTQSKGK---SNLEA
N. crassa AQADIMGSDLLCIARTDAEAATLITTTIDPRDHAFILGCTNP-DLEPLAHLMMKAEAEGKTGAQLQAIEDDWLAKADLKRFDEAVLDVIAKG-KFSNAKDLAAKYQAAVKGKQ-ISNREA
361 480
A. gossypii RKLGRELLGQDVYFDWDLPRAREGLYRYKGGTQCAIMRARAFAPYADLVWFESNFPDFQQAKEFAQGVREKFPNKWMAYNLSPSFNWPKAMPPKEQENYIQRLGEIGYVWQFITLAGLHT
S. cerevisiae KKLAKEILGHEIFFDWELPRVREGLYRYRGGTQCSIMRARAFAPYADLVWMESNYPDFQQAKEFAEGVKEKFPDQWLAYNLSPSFNWPKAMSVDEQHTFIQRLGDLGYIWQFITLAGLHT
C. tropicalis KKLAKELTGKDIYFNWDVARAREGYYRYQGGTQCAVMRGRAFAPYADLIWMESALPDYNQAKEFADGVKAAVPDQWLAYNLSPSFNWNKAMPADEQETYIKRLGQLGYVWQFITLAGLHT
Y. lipolytica RYLAARLLGKDIYFNWEAARVREGYYRYQGGTQCAVNRGYSYAPYADLIWMESKLPDYAQAKEFAEGVKNAVPHQWLAYNLSPSFNWTTAMSPEDQETYISRLAKLGYVWQFITLAGLHT
E. nidulans RAIAKEIAGTDIYFDWEAPRTREGYYRYQGGTQCAINRAVAYAPFADLIWMESKLPDYKQAKEFADGVHAVWPEQKLAYNLSPSFNWKKAMPRDEQETYIKRLGALGYAWQFITLAGLHT
N. crassa RAIARQLLGQEIFFDWESPRTREGYYRLKGGCDCSINRAISYAPYCDAIWMESKLPDYAQAEEFAKGP-RVWPEQKLAYNLSPSFNWKTAMGRDDQETYIRRLAKLGYCWQFITLAGLHT
481 561
A. gossypii NALAIDNFSREFSRFGMRAYAQGIQQREMDEGVDVLKHQKWAGAEYVDSILKLAQGGVSSTASMGKGVTEEQFGSSNGAKL
S. cerevisiae NALAVHNFSRDFAKDGMKAYAQNVQQREMDDGVDVLKHQKWSGAEYIDGLLKLAQGGVSATAAMGTGVTEDQFKE-NGVKK
C. tropicalis TALAVDDFANQYSQIGMRAYGQTVQQPEIEKGVEVVKHQKWSGANYIDGLLRMVSGGVTSTAAMGAGVTEDQFKETKAKV-
Y. lipolytica NALISDKSCKAYSERGMKAYGGEIQQPEIDQGCEVVKHQKWSGAEYIDGILRMVTGGITSTAAMGAGVTEDQFKSKL----
E. nidulans TALISDTFAKAYAKQGMRAYGELVQEPEMANGVDVVTHQKWSGANYVDNMLKMITGGVSSTAAMGKGVTEDQFKS------
N. crassa TALISDQFAKAYSKIGMRAYGELVQEPEIDNGVDVVKHQKWSGATYVDELQKMVTGGVSSTAAMGKGVTEDQFH-------

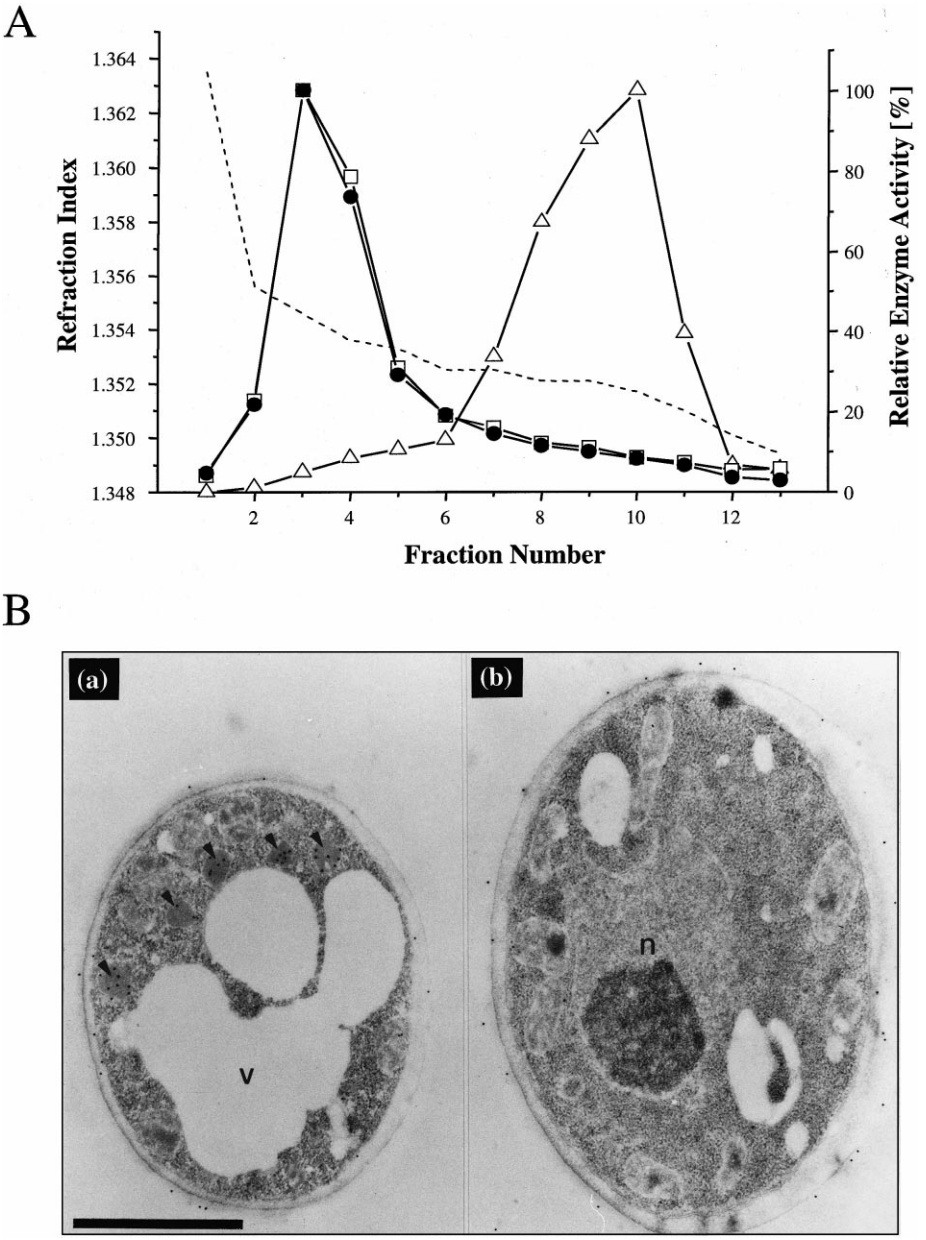


Fig. 3. Distribution of AgIcl1p (●), catalase (□) and cytochrome *c* oxidase (△) activity in fractions of a Percoll density gradient (A) and immunogold labeling of AgIcl1p (B) in soybean oil- (a) and glucose- (b) grown *A. gossypii* mycelium (n = nucleus, v = vacuole; bar represents 1 μm).

[29] with bovine serum albumin as the standard was used to quantitate protein concentrations.

For electron microscopy mycelium grown on YS and YG was fixed with 2% formaldehyde, 0.05% glutaraldehyde (60 min, 4°C), embedded in agarose, dehydrated with ethanol at progressively lowered temperatures and finally embedded in Lowicryl HM20 [30]. Labeling with anti-AgIcl1p antibodies [11] and protein A-13 nm gold as well as final heavy metal staining was performed according to Steverding et al. [31].

3. Results

3.1. Isolation and identification of AgICL1

To isolate the *AgICL1* gene from *A. gossypii*, heterologous complementation of *S. cerevisiae* icl1d, which is unable to grow on ethanol as carbon source, was performed using a genomic *A. gossypii* DNA library. Transformants, obtained by selection for uracil prototrophy, were replica-plated on YNB with ethanol as carbon source to screen for a functional AgIcl1p. Two positive isolates were obtained in a screen of approximately 2000 uracil prototrophic yeast colonies. However, in only one case was the reisolation of the corresponding plasmid (YEpAgICL1) successful. Restriction mapping, retransformation and AgIcl1p specific activity in retransformants (0.3 U/mg protein) conducted with this plasmid indicated that the *AgICL1* gene is located on a 2.2 kb *Bgl*I/*Eco*RV fragment (Fig. 1A). The authenticity of the *Sph*I fragment was confirmed by DNA sequencing (EMBL database, accession number AJ010727). The *AgICL1* consisted of 1680 bp, corresponding to 560 amino acid residues. The deduced polypeptide had a predicted molecular weight of 62 584, which is

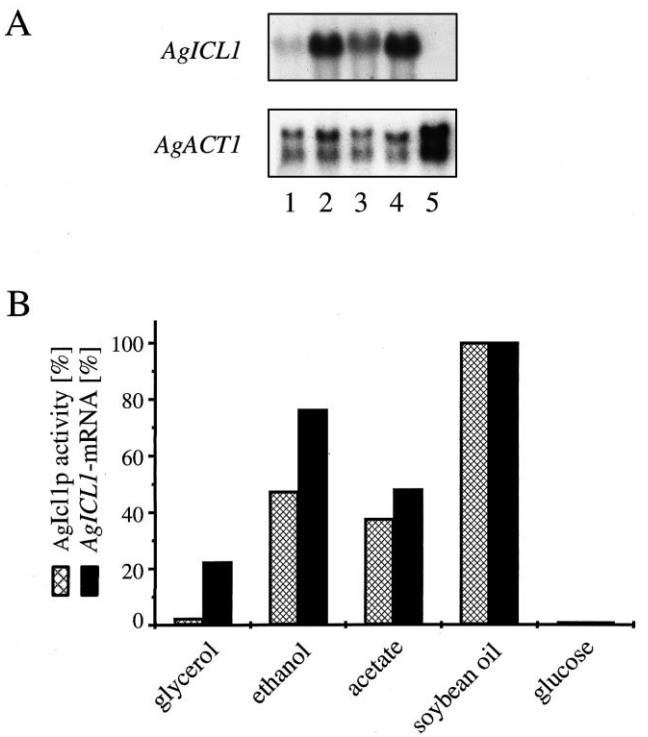


Fig. 4. Northern blot analysis of *AgICL1* from mycelium grown on different carbon sources. Blots were probed with the *AgICL1* and the *AgACT1* gene as a constitutive mRNA control (A) (lane 1: glycerol, 2: ethanol, 3: acetate, 4: soybean oil, 5: glucose). *AgICL1* signals, in relation to the *AgACT1* signals, and enzyme activities were compared with soybean oil data set at 100% (B).

similar to the estimated value for the purified protein [26]. The predicted amino acid sequence was compared to Iclps of *S. cerevisiae*, *Candida tropicalis*, *Yarrowia lipolytica*, *Emericella nidulans* (anamorph: *Aspergillus nidulans*) and *Neurospora crassa* (Fig. 2) revealing 70.0%, 61.1%, 59.0%, 57.5% and 54.6% identity, respectively. The functional residues K193, K194, C195 and H197 of the *E. coli* enzyme [32], which are comprised by the hexapeptide KKCGHM conserved in all known Icl peptides, and H184, S319, S321 and H356 [32–34] were found within the *A. gossypii* protein. The carboxy-terminal amino acids alanine-lysine-leucine, representing a putative peroxisomal targeting signal (PTS1 [35]), suggest a peroxisomal localization of Icl1p in *A. gossypii*. A putative TATA box was located within the 5′ non-coding region at position −83 bp to −90 bp. The upstream sequence from −397 bp to −478 bp showed complete identity to the Ser-tRNA (UGA) gene from *S. cerevisiae*. Interestingly, the *ICL1* and Ser-tRNA are also proximal genes in *S. cerevisiae*.

To disrupt the *AgICL1* gene a 500 bp genomic *AgICL1* fragment was replaced with the 2.1 kb geneticin resistance cassette (Fig. 1A). The substitution was verified by Southern blot analysis using digoxigenin-labeled *AgICL1* gene as a probe (Fig. 1B). In the case of *AgΔICL1*::*kan*^{R} the wild-type signal shifted by the expected 1.6 kb, additionally showing that *AgICL1* is a single copy gene. Disruption led to the inability to grow on minimal medium with oleic acid as sole carbon source. No AgIcl1p specific activity (<0.005 U/mg protein) was detectable in the knockout mutant on complex medium, whereas in the wild type, transformed with pAG100 to exclude an influence of the geneticin resistance, 0.24 U/mg protein was measured under the same conditions.

3.2. AgICL1 subcellular localization

To study the subcellular localization of Icl1p in *A. gossypii*, an organelle preparation isolated from wild-type mycelium grown on soybean oil was subjected to Percoll density gradient centrifugation (Fig. 3A). AgIcl1p activity co-segregated with the peroxisomal marker enzyme catalase, showing a distinct separation from mitochondria, identified by the marker enzyme cytochrome *c* oxidase. To exclude a potential cytosolic localization of AgIcl1p immunogold labeling of ultrathin sections of *A. gossypii* mycelium with anti-AgIcl1p antibodies was performed (Fig. 3B). Whereas no labeling was found in glucose-grown cells (Fig. 3Bb), gold particles labeled organelles of higher electron density in soybean oil-grown cells (Fig. 3Ba). These results indicate a peroxisomal Iclp in *A. gossypii* and, furthermore, suggest a glucose repression of *AgICL1* which is in accordance with the enzyme data reported for other sources.

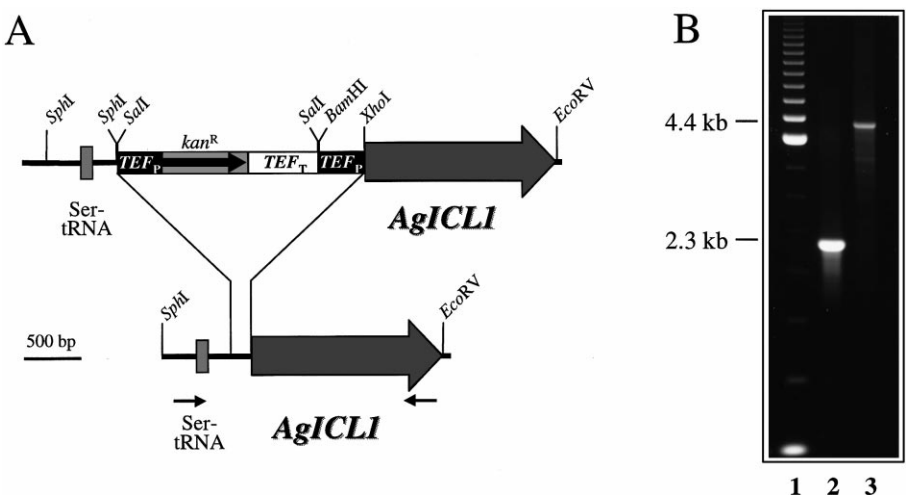


Fig. 5. Replacement of *AgICL1* promoter region. Scheme for genomic integration of a construct carrying the *A. gossypii TEF* promoter and the geneticin resistance cassette (*kan*^{R}) by direct flanking genomic DNA (A). Arrows indicate the primers for PCR amplification of the *AgICL1* locus (B) to confirm the replacement (lane 1: 500 bp ladder, 2: wild type, 3: *AgTEF$_p$ICL*).

3.3. Regulation of AgICL1

In order to analyze *AgICL1* regulation specific mRNA levels were examined by Northern blot analysis after growth on glycerol, ethanol, acetate, soybean oil or glucose and compared to the enzyme level (Fig. 4). Enzyme specific activity as well as the *AgICL1* mRNA amount were highest during cultivation on soybean oil. Neither *AgICL1* transcript ($<1\%$) nor AgIcl1p specific activity ($<1\%$) was detectable during growth on glucose. The carbon sources glycerol, ethanol or acetate revealed intermediate levels of mRNA (22%, 76%, 48%, respectively), correlating with the enzyme specific activities (2%, 47%, 38%, respectively).

To confirm that this correlation was due to a repression or induction of transcription rate the *AgICL1* upstream region from -1 to -166 bp was replaced by the constitutive *TEF* promoter from *A. gossypii* (Fig. 5A). The replacement was verified by PCR (Fig. 5B). As expected the amplified fragment shifted by 2.1 kb. Cultivation of this mutant on YG or YS revealed no significant difference in AgIcl1p specific activity (0.25 ± 0.02 U/mg protein or 0.22 ± 0.03 U/mg protein, respectively), indicating that glucose repression of the *AgICL1* gene was attributed to a promoter-dependent regulation of transcription.

4. Discussion

This paper reports on the molecular identification and regulation of the *AgICL1* gene which encodes a peroxisomal Iclp. The peroxisomal localization of Icl1p in *A. gossypii*, suggested by the carboxy-terminal tripeptide A-K-L, was confirmed both by density gradient centrifugation and immunoelectron microscopy. Interestingly, although high sequence identity and syntenic gene order concerning Ser-tRNA, Iclp compartmentation differs in *A. gossypii* and *S. cerevisiae*. Cytosolic localization of the *S. cerevisiae* Icl1p has been confirmed by immunocytochemical studies [15] as well as by plasma membrane permeabilization of protoplasts and green fluorescent protein fusions [16]. Peroxisomal Iclps are described for *C. tropicalis* [36], *Y. lipolytica* [37] and *A. nidulans* [38] and a glyoxisomal Iclp for *N. crassa* [39]. However, the enzymes of the last two fungi mentioned lack a PTS1 resembling the carboxy-terminal amino acids S(A,C)-K(H,R)-L [35]. Whether the tripeptide of AgIcl1p is both necessary and sufficient for protein translocation into peroxisomes remains to be elucidated.

In accordance with Iclps of various species, Schmidt et al. [11] observed for the *A. gossypii* Icl1p a repressing effect of glucose by means of activity measurements and Western blotting. In agreement with these data investigation of *AgICL1* mRNA amounts revealed a regulation of *AgICL1* expression in response to different carbon sources on the transcriptional level. Levels of mRNA can be affected both by the rate of transcription of the corresponding gene and by the stability of the mRNA. The promoter replacement study indicated a regulation of the *AgICL1* gene due to different transcription rates. Within the upstream region of the *ScICL1* gene of *S. cerevisiae* several transcription-regulating elements are described. In addition to the transcription-activating carbon source response element (CSRE, consensus sequence CCRTYSRNCCG) [40], an upstream repressing sequence element (URS, 5′-AGTCCGGACTAGCATCCCAG-3′) [41] was identified. However, none of these sequences was found in the *AgICL1* upstream region. Further binding sites of the transcription activator Pip2p or so-called oleate response elements (ORE) [42] were identified in several promoters of genes encoding peroxisomal enzymes in *S. cerevisiae*. A putative ORE is present at position -615 to -637 upstream of the *AgICL1* start codon, its functionality remains to be elucidated. Taking peroxisomal localization and induced formation of *A. gossypii* Icl1p by peroxisome proliferating carbon sources such as ethanol, acetate and fatty acids into consideration, an involvement of the transcription factor Pip2p in regulation seems possible. Besides glucose repression, a second mechanism of catabolite control of Iclps of *S. cerevisiae* [43] and *Phycomyces blakesleeanus* [44] is described, namely glucose-induced inactivation. In *S. cerevisiae* this control mechanism consists of a short-term inactivation mediated by a c-AMP-dependent protein kinase which phosphorylates ScIcl1p at amino acid position 53 (RRGT) [45], and a long-term inactivation leading to proteolytic degradation of ScIcl1p which is triggered by phosphorylation of other unidentified residues [45] and/or the internal decapeptide KTKRNYSARD [46]. Because no significant loss of the AgIcl1p specific activity was observed within the first hour after setting a glucose pulse to a soybean oil-grown culture an inactivation by phosphorylation was ruled out for the enzyme of *A. gossypii* [11], resembling the situation in *A. nidulans* [47–49]. In contrast to these results the phosphorylation target of the *S. cerevisiae* enzyme (RRGT) was also found in the predicted amino acid sequence of *AgICL1*. Since only six amino acid residues of the decapeptide, which functions as both a necessary and sufficient signal for proteolytic ScIcl1p degradation in *S. cerevisiae*, are conserved within the sequence of *A. gossypii* it has to be investigated whether this sequence triggers degradation in *A. gossypii* too. As no differences in AgIcl1p specific activities on glucose in comparison to soybean oil were observed under the control of the *TEF* promoter, the enzyme of *A. gossypii* is probably not regulated at the activity level.

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