The Journal of Microbiology (2010) Vol. 48, No. 2, pp. 188-198
DOI 10.1007/s12275-010-0096-8

Differential Expression of *citA* Gene Encoding the Mitochondrial Citrate Synthase of *Aspergillus nidulans* in Response to Developmental Status and Carbon Sources

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(Received March 12, 2010 / Accepted April 19, 2010)

As an extension of our previous studies on the mitochondrial citrate synthase of *Aspergillus nidulans* and cloning of its coding gene (*citA*), we analyzed differential expression of *citA* in response to the progress of development and change of carbon source. The cDNA consisted of 1,700 nucleotides and was predicted to encode a 474-amino acid protein. By comparing the cDNA sequence with the corresponding genomic sequence, we confirmed that *citA* gene contains 7 introns and that its transcription starts at position -26 (26-nucleotide upstream from the initiation codon). Four putative CreA binding motifs and three putative stress-response elements (STREs) were found within the 1.45-kb *citA* promoter region. The mode of *citA* expression was examined by both Northern blot and confocal microscopy using green fluorescent protein (sGFP) as a vital reporter. During vegetative growth and asexual development, the expression of *citA* was ubiqiutous throughout the whole fungal body including mycelia and conidiophores. During sexual development, the expression of *citA* was quite strong in cleistothecial shells, but significantly weak in the content of cleistothecia including ascospores. Acetate showed a strong inductive effect on *citA* expression, which is subjected to carbon catabolite repression (CCR) caused by glucose. The recombinant fusion protein $CitA_{40}$::sGFP (sGFP containing the 40-amino acid N-terminal segment of CitA) was localized into mitochondria, which supports that a mitochondrial targeting signal is included within the 40-amino acid N-terminal segment of CitA.

Keywords: *A. nidulans*, citrate synthase, *citA*, differential expression, carbon catabolite repression, mitochondrial targeting

The Krebs (TCA) cycle plays an essential role not only in utilization of non-fermentable carbon source by oxidative generation of reducing equivalents that derive mitochondrial aerobic respiration to yield ATP but also in biosynthetic metabolism by providing the carbon skeletons used in various biosynthesis. Citrate synthase (EC 4.1.3.7) catalyzes the first step of the TCA cycle, the condensation reaction between acetyl-CoA and oxaloacetate to form citrate, and thus functions as a rate-limiting enzyme of the cycle (Suissa *et al.*, 1984). In mammalian cells, this reaction seems to be exclusively located in mitochondria. In yeast and plants cells, a second enzyme localized in peroxisomes is involved in the glyoxylate cycle, an anaplerotic pathway of the TCA cycle (Velot *et al.*, 1999).

A few citrate synthase genes have been isolated from fungi and other higher eukaryotic organisms, such as *Saccharomyces cerevisiae* (Suissa *et al.*, 1984; Kim *et al.*, 1986; Rosenkrantz *et al.*, 1986), *Neurospora crassa* (Ferea *et al.*, 1994), *Aspergillus nidulans* (Park *et al.*, 1997), and human (Goldenthal *et al.*, 1998). In yeast *S. cerevisiae*, two mitochondrial (Cit1p and Cit3p) and one peroxisomal (Cit2p) isoforms of citrate synthase are encoded by three distinct nuclear genes. Cit1p is the major mitochondrial enzyme that functions as the rate-limiting enzyme of the TCA cycle (Suissa *et al.*, 1984), and Cit2p is the peroxisomal form that is involved in biosynthesis of glutamate and/or the glyoxylate cycle (Kim *et al.*, 1986; Rosenkrantz *et al.*, 1986; Lewin *et al.*, 1990). Cit3p is the minor mitochondrial form and is important for growth on glycerol in the presence of *CIT1* deletion although poorly expressed under normal conditions (Jia *et al.*, 1997). The expression of yeast *CIT1* is subject to glucose repression and is further repressed by glucose plus glutamate, which reflects the catabolic and biosynthetic roles of citrate synthase (Kim *et al.*, 1986). The expression of *CIT1* requires Hap2,3,4,5p transcription complex coded by *HAP* genes under normal conditions (Rosenkrantz *et al.*, 1994). However, when the cells' respiratory function is reduced or eliminated, the expression of *CIT1*, together with the other three TCA cycle genes, *ACO1*, *IDH1*, and *IDH2*, switches from *HAP* control to control by the three genes, *RTG1*, *RTG2*, and *RTG3* (Liu and Butow, 1999). The expression of *CIT2* is sensitive to the functional state of mitochondria, and is also subject to *RTG*-dependent control as *CIT1* (Chelstowska and Butow, 1995; Jia *et al.*, 1997).

While there has been considerable study on the regulation of the citrate synthase genes in *S. cerevisiae*, less is known about the regulation of the corresponding genes of other eukaryotic organisms including the filamentous fungus *A. nidulans*. We have reported the purification and characterization of the citrate synthase from *A. nidulans* (Maeng *et al.*, 1993), and also have analyzed the nucleotide sequence of the citrate synthase gene, named *citA*, cloned from the chromosome-

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specific genomic library of this fungus (Park *et al.*, 1997). It has also been reported that deletion of *citA* results in poor growth on glucose but not on derepressing carbon sources, and that methyl citrate synthase (McsA) can substitute for the loss of CitA activity (Murray and Hynes, 2010). In the present study, we isolated the cDNA of *citA* and analyzed its nucleotide sequence to confirm the primary structure of the citrate synthase. We also examined the mode of *citA* expression under different nutritional conditions and at different developmental stages by using sGFP (Fernandez-Abalos *et al.*, 1998) as a vital reporter as well as by real-time RT-PCR. We further found that a mitochondrial targeting signal (MTS) is included within the 40-amino acid N-terminal segment of CitA by analyzing the subcellular localization of the fusion protein $CitA_{40}$::sGFP.

Materials and Methods

Strains, media, cultivation, and transformation

A. nidulans FGSC A26 [*biA1*] (Fungal Genetics Stock Center, Kansas City, Kansas, USA) and *A. nidulans creA*^{d}30/argB [*biA1*, *creA*^{d}30] (Arst *et al.*, 1990) were used for RNA preparation. *A. nidulans* Wx24 [*npgA1 biA1; sB3; chaA1 trpC801*] (Han *et al.*, 2001) was used as a recipient strain for transformation. The *A. nidulans* strains were maintained on *Aspergillus* complete medium (CM) (Kim *et al.*, 2001) or its derivatives: the transformants from Wx24 on CM, and Wx24 on CMW (CM+4 mM tryptophan) medium.

For transformation of *A. nidulans* Wx24, 1.0×10^8 conidia were inoculated in 100 ml of CMW broth and grown at 37°C for 16 to 18 h with agitation. The mycelia were then harvested by filtration through a Miracloth filter (Calbiochem, USA), and washed with distilled water and osmotic buffer (0.6 M KCl, 10 mM NaCl, pH 7.5). Transformation mediated by polyethylene glycol (PEG) was performed by the method of (Yelton *et al.*, 1984), and the protoplasts of transformants were regenerated on 1% glucose minimal medium (MM) (Kim *et al.*, 2001) supplemented with 0.1 μM biotin, 4 mM methionine, and 0.6 M KCl (MBMK). When necessary, the transformants were grown on minimal medium supplemented with 0.1 μM biotin and 4 mM methionine (MBM), and the parental strain, Wx24, on MBM supplemented with 4 mM tryptophan (MBMW).

To prepare primary-cultured vegetative mycelia, spores of *A. nidulans* strains were inoculated in a complete broth medium, and grown for approximately 15 h at 37°C. Asexual differentiation was induced by spreading the primary-cultured mycelia onto appropriate agar plates and incubating at 37°C. To induce sexual differentiation, the primary-cultured mycelia were transferred onto agar plates, incubated for another 24 h while the plates were sealed closely, and incubated thereafter under unsealed conditions.

For media replacement experiments, strains were pregrown in a supplemented liquid MM with 0.1% fructose and 5 mM urea as a sole carbon and nitrogen sources, respectively, at 37°C for 15 h. Mycelia were then harvested by filtration, washed with sterile ice-cold water, and resuspended in equal volume of supplemented liquid MM containing 5 mM urea as a sole nitrogen source and one of the following sole carbon sources or their combinations: 1% glucose (MMG), 50 mM acetate (MMA), 50 mM acetate+1% glucose (MMAG). After appropriate time intervals, mycelia were harvested by filtration and use for extraction of total RNAs or proteins.

Plasmids were amplified in *Escherichia coli* DH5α [*supE44 ΔlacU169* (*Φ80lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*], and *E. coli* cells were grown in Luria-Bertani (LB) medium. *E. coli* transformation was done by the standard method (Sambrook and Russell, 2001).

Cloning of *citA* cDNA

To isolate the cDNA of *citA* encoding the citrate synthase, 24-h developmental cDNA library from *A. nidulans* constructed in λ ZAP vector was purchased from FGSC. Recombinant phages were transfected into *E. coli* LE392. Plaques of 1.0×10^5 were transferred onto nylon membranes (Hybond-N$^+$; Amersham, UK) and screened by using the Gene Image CDP-Star kit (Amersham) with a 0.58-kb *citA* probe which was amplified from pJNC14 using a pair of primers, PC4s (5′-GGTCTCTTCTGGTTGCTTCTGACC-3′) and PC5a (5′-AATCCATGGAGAGGACCGGC-3′). Single positive plaques were confirmed by repeating plaque hybridization three times under the same conditions as above.

For phagemid excision, *E. coli* XL1-Blue MRF' was infected with 1:100 mixture of the positive phage clone (1×10^3 pfu/ml) and the EXASSIST helper phage (1×10^3 pfu/ml). Plasmid DNA prepared from the colonies grown on LB agar plates containing both tetracyclin and ampicillin were amplified in *E. coli* DH5α.

Construction of vectors

To construct *citA-p::sgfp* hybrid gene, a DNA fragment containing the presumptive full-length *citA* promoter region, *citA-p* (from nucleotide -1 to -1,450 in which the number indicates the distance from the translation start site), was first amplified from pJNC14 (Park *et al.*, 1997) using a pair of primers, PC1s (5′-GGAATTCCTTGAGAGCCA GC-3′) and PC2a (5′-CGGGATCCTCTGATCAAACAAC-3′). The resulting PCR fragment was cloned into the *Eco*RV site of pT7Blue(R) (Novagen, USA) to yield pT7/citA-p. The 1.45-kb *Eco*RI-*Bam*HI fragment containing the presumptive promoter region was excised from pT7/citA-p and cloned into *Eco*RI-*Bam*HI-digested pTsgfp vector (Fig. 2A; Lee *et al.*, 2004) upstream of *sgfp* in the correct orientation to yield pTcitA-p::sgfp.

Chimeric gene *citA40::sgfp* was constructed as follows. First, an *sgfp* variant with a deletion of the initiation codon (*sgfpΔ1*) was amplified from pRS31 using a pair of primers PG3s (5′-CGCAGATCTGTGAG CAAGGGCGAG-3′) and PG2a (5′-CGCGGATCCTTACTTGTACA GCTCGTCC-3′). The resulting PCR fragment was cloned into the *Eco*RV site of pT7Blue(R) in the opposite direction against the *lacZ* to yield pT7/sgfpΔ1. In parallel, the 1.58-kb *citA40* fragment containing the full-length presumptive *citA* promoter region and the coding sequence for the N-terminal 40 amino acids of CitA protein was amplified from pJNC14 using a pair of primers, PC1s and PC3a (5′-CGGGATCCAAGACGAACCTTGGTCTTGCC-3′). The PCR product was cloned into pT7Blue(R) to yield pT7/citA40 from which the *Eco*RI-*Bam*HI *citA40* fragment was excised and ligated into *Eco*RI-*Bgl*II-digested pT7/sgfpΔ1 upstream of the *sgfpΔ1* to yield pT7/citA40::sgfp. To construct pTcitA40::sgfp vector the 2.38-kb *Eco*RI-*Bam*HI *citA40::sgfp* fragment excised from pT7/citA40::sgfp was inserted into *Eco*RI-*Bam*HI-digested pTT which had been constructed by insertion of the 0.53-kb PCR-amplified *Hin*dIII (blunted)-*Bam*HI *trpC-t* fragment into the *Xba*III (blunted)-*Bam*HI-digested pSH96.

Nucleic acid hybridizations and DNA sequencing

Chromosomal DNAs from *A. nidulans* strains were isolated by using miniprep procedures (Yelton *et al.*, 1984). Southern blot analysis was performed by the standard method (Sambrook and Russell, 2001) using ECL Labeling and Detection kit (Amersham). Total RNAs were prepared from the liquid nitrogen-frozen and ground mycelia of *A. nidulans* at the vegetative growth, asexual development, or sexual

development stages by modified guanidine thiocyanate/CsCl density gradient ultracentrifugation (Sambrook and Russell, 2001). Northern hybridization was performed according to standard procedures (Sambrook and Russell, 2001), in which the DNA probes were labeled with ^{32}P using a Random Primed DNA Labeling kit (Boehringer Mannheim, Germany). The nucleotide sequences of the cloned DNAs were determined by using an automatic DNA sequencer ABI 377 (Perkin-Elmer).

Confocal microscopy

Confocal microscopic observation of *A. nidulans* cells was performed with a Leica TCS SP2 Confocal system (Leica, Switzerland). To observe sGFP fluorescence, fungal cells were excited with the wavelength of 488 nm, and the fluorescent emission with the wavelength of 500-520 nm was detected. All sGFP images were generated under standardized conditions in which the main parameters are adjusted as follows: PMT gain, 690-710; PMT offset, -10; Pinhole, 150; Number of section, 8; Number of scan for each section, 8. For visualization of mitochondria, cells were stained with 100 nM MitoTracker Red CMXRos (Molecular Probes, USA) for 30 min at 37°C and fixed with 3% formaldehyde for 30 min. The cells were excited with 568 nm, and the red fluorescence of 580-600 nm was detected.

Quantitative real-time RT-PCR

Real-time RT-PCR for measuring relative expression levels of *citA* was performed with the primers, PC6s (5′-TTATGGTGGTGCCCGT GGCGTGA-3′) and PC7a (5′-ATGAACTTGGGGAGGTCGGAGCG-3′), and relative levels of gene expression were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For normalization of gene expression data, *A. nidulans tubC* gene was used as an internal standard (Gelmini *et al.*, 2001).

Results

Sequence analysis of cDNA and promoter of *citA*

By screening of the cDNA library from *A. nidulans* with the 0.40-kb *Nco*I *citA* fragment, we isolated a λZAP phage clone containing the 1.70-kb *citA* cDNA. Then a plasmid containing the *citA* cDNA, pBS/*citA*, was isolated from the phage DNA by phagemid excision, and the entire *citA* cDNA was sequenced (GenBank accession no. AF468824).

The cDNA sequence of *citA* was found to contain an open reading frame (ORF) encoding a protein with 474 amino acids (Fig. 1). The molecular mass of CitA protein was deduced to be 52.2 kDa, which is quite similar to those of corresponding proteins from *S. cerevisiae* (53.5 kDa) (Rosenkrantz *et al.*,

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-26                                                          attcaaaaaagttgttttgatcagat
  1  ATGGCTTCTACACTCAGACTTAGTACCTCTGCTCTCCGCTCCAGCACTCTTGCTGGCAAGCCCGTTGTTCAG
  1  M  A  S  T  L  R  L  S  T  S  A  L  R  S  S  T  L  A  G  K  P  V  V  Q
 73  TCTGTTGCCTTCAATGGCCTGCGCTGCTACTCTACCGGCAAGACCAAGTCCCTTAAAGAGACCTTCGCCGAC
 25  S  V  A  F  N  G  L  R  C  Y  S  T  G  K  T  K  S  L  K  E  T  F  A  D
145  AAGCTTCCCGGCGAGCTCGAGAAGGTCAAGAAGCTCCGAAAGGAGCATGGCAACAAGGTCATTGGCGAGCTC
 49  K  L  P  G  E  L  E  K  V  K  K  L  R  K  E  H  G  N  K  V  I  G  E  L
217  ACCCTTGACCAGGCTTATGGTGGTGCCCGTGGCGTGAAGTGCCTCGTGTGGGAGGGTTCCGTCCTTGATTCC
 73  T  L  D  Q  A  Y  G  G  A  R  G  V  K  C  L  V  W  E  G  S  V  L  D  S
289  GAGGAGGGTATCCGTTTCCGTGGTCTCACGATTCCCGAGTGCCAGAAGCTTCTTCCCAAGGCCCCCGGTGGT
 97  E  E  G  I  R  F  R  G  L  T  I  P  E  C  Q  K  L  L  P  K  A  P  G  G
361  GAGGAGCCTCTCCCTGAAGGTCTCTTCTGGTTGCTTCTGACCGGCGAGGTTCCCTCTGAGCAGCAGGTTCGT
121  E  E  P  L  P  E  G  L  F  W  L  L  L  T  G  E  V  P  S  E  Q  Q  V  R
433  GACCTGTCTGCTGAGTGGGCCGCCCGCTCCGACCTCCCCAAGTTCATTGAGGAGCTTATCGACCGCGTGCCC
145  D  L  S  A  E  W  A  A  R  S  D  L  P  K  F  I  E  E  L  I  D  R  V  P
505  AGCACTCTCCACCCCATGGCTCAGTTCTCTCTCGCTGTCACTGCTCTTGAGCACGAGTCTGCTTTTGCAAAG
169  S  T  L  H  P  M  A  Q  F  S  L  A  V  T  A  L  E  H  E  S  A  F  A  K
577  GCTACGGCCAAGGGTATCAACAAGAAGGAGTACTGGCACTACACCTTCGAGGACTCTATGGACCTTATTGCC
193  A  T  A  K  G  I  N  K  K  E  Y  W  H  Y  T  F  E  D  S  M  D  L  I  A
649  AAGCTGCCCACCATCGCCGCCAAGATCTACCGCAATGTTTTCAAGGACGGCAAGGTTGCTCCTATCCAGAAG
217  K  L  P  T  I  A  A  K  I  Y  R  N  V  F  K  D  G  K  V  A  P  I  Q  K
721  GACAAGGACTACTCCTACAACTTGGCCAACCAGCTCGGCTTTGCCGACAACAAGGACTTCGTTGAGCTGATG
241  D  K  D  Y  S  Y  N  L  A  N  Q  L  G  F  A  D  N  K  D  F  V  E  L  M
793  CGCCTGTACCTCACCATCCACTCCGATCACGAGGGTGGAAATGTTAGCGCTCACACTACCCACCTTGTTGGT
265  R  L  Y  L  T  I  H  S  D  H  E  G  G  N  V  S  A  H  T  T  H  L  V  G
865  AGTGCTCTGAGCTCCCCCATGCTTTCCCTGGCGGCTGGTCTCAACGGTCTGGCCGGTCCTCTCCATGGATTG
289  S  A  L  S  S  P  M  L  S  L  A  A  G  L  N  G  L  A  G  P  L  H  G  L
937  GCCAACCAGGAAGTCCTTAACTGGCTCACCGAAATGAAGAAGGTCGTCGGCAACGACCTCAGCGACCAGTCC
313  A  N  Q  E  V  L  N  W  L  T  E  M  K  K  V  V  G  N  D  L  S  D  Q  S
1009 ATCAAGGACTATCTCTGGTCTACCCTCAACGCTGGTCGCGTTGTTCCTGGATACGGTCACGCCGTTCTTCGC
337  I  K  D  Y  L  W  S  T  L  N  A  G  R  V  V  P  G  Y  G  H  A  V  L  R
1081 AAGACCGACCCTCGCTACACGTCCCAGCGCGAGTTTGCTCTCCGCAAGTTGCCTGATGACCCCATGTTCAAG
361  K  T  D  P  R  Y  T  S  Q  R  E  F  A  L  R  K  L  P  D  D  P  M  F  K
1153 TTGGTCAGCCAGGTTTACAAGATCGCTCCTGGCGTCCTGACCGAGCACGGCAAGACCAAGAACCCTTACCCC
385  L  V  S  Q  V  Y  K  I  A  P  G  V  L  T  E  H  G  K  T  K  N  P  Y  P
1225 AATGTTGATGCTCACTCTGGTGTCCTCCTGCAGTACTACGGCCTCACTGAGGCTAACTACTACACCGTCTTG
409  N  V  D  A  H  S  G  V  L  L  Q  Y  Y  G  L  T  E  A  N  Y  Y  T  V  L
1297 TTCGGTGTCTCGCGTGCTCTTGGTGTCCTTCCTCAGCTCATCATTGACCGTGCTTTTGGTGCTCCTATCGAG
433  F  G  V  S  R  A  L  G  V  L  P  Q  L  I  I  D  R  A  F  G  A  P  I  E
1369 CGTCCCAAGAGTTTCAGCACTGAGGCTTACGCCAAGCTTGTTGGCGCTAAGCTATAAatttcttgaacgact
457  R  P  K  S  F  S  T  E  A  Y  A  K  L  V  G  A  K  L  *
1441 gaggataatgggggtattatatcttttatgaataacaaaacagccatttttgtatttatctaacgtcttct
1513 ttggatgaggacgtaccaagtgttgggtttagttcgattaatgtatgataggacttgttcgagcaggccttg
1585 taacaacacttcgttttcatgtcagtcattcttttaaggttagatccaatgctccctgtctggcctttgcaa
1657 gcccaaggacaaaaaaaa
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Fig. 1. Nucleotide sequence of the *citA* cDNA and the deduced amino acid sequence of CitA protein. A putative cleavage and polyadenylation motifs for the 3′ end of the primary transcripts are boxed. A putative R-3 type motif (R-X-Y↓S/A) for proteolytic cleavage of the MTS (mitochondrial targeting signal) is boldfaced. This sequence data is available from GenBank under the accession number AF468824.

1986) and *N. crassa* (52.0 kDa) (Ferea *et al.*, 1994). The amino acid sequence deduced from the cDNA showed identity of 92% to *A. niger* citrate synthase (GenBank accession no. D63376), 81% to *N. crassa* enzyme (M84187) (Ferea *et al.*, 1994), 71% to *S. cerevisiae* mitochondrial enzyme (Cit1p, X00782) (Suissa *et al.*, 1984), and 66% to *S. cerevisiae* peroxisomal enzyme (Cit2p, Z11113) (Rosenkrantz *et al.*, 1986).

By comparing the sequence of *citA* cDNA with its genomic DNA sequence (Park *et al.*, 1997), we confirmed that the *citA* gene has seven introns (69, 65, 64, 51, 51, 74, and 53 bp, respectively) and that five of them are clustered in the first quarter of the coding sequence. We also found that the cDNA sequence starts at 26-bp upstream of the initiation codon, which suggests that the transcription start point (tsp) of *citA* is located at this site.

The nucleotide sequence of the 1.45-kb DNA fragment that was expected to carry the whole promoter region of *citA* was analyzed (data not shown; GenBank accession no. U89675). A long CT box (pyrimidine-rich segments), which has been shown to be important for determining the position of transcription initiation, was found in the range of -105 to -43 [positions of the 5′ end of sequence elements are given in bp from the A(+1) of the translation initiation codon]. Four putative binding motifs for CreA repressor (consensus 5′SYGGRG3′) (Kulmburg *et al.*, 1993; Cubero and Scazzocchio, 1994) that are responsible for carbon catabolite repression (CCR) (Dowzer and Kelly, 1989) were found at positions -1449, -1424, -540, and -203. Three copies of a stress responsive element, STRE (5′CCCCT3′ or 5′AGGGG3′), which is responsive to a variety of stress signals were found at positions -912, -1115, -1202.

Construction of *A. nidulans* transformants

To analyze the expression of *citA-p::sgfp* reporter gene and the intracellular localization of $CitA_{40}$::sGFP fusion protein carrying the N-terminal 40-amino acid segment of CitA, each of the two plasmids, pTcitA-p::sgfp and pTcitA40::sgfp was individually introduced into *A. nidulans* Wx24, and the transformants which grew in the absence of tryptophan were selected. Because the wild type trpC hybrid can be formed only by homologous recombination of trpC*Δ176* (5′ 1.76-kb segment of *trpC*) contained in the transforming vectors with chromosomal *trpC801* allele, all the transformants were expected to harbor at least one copy of the vectors at *trpC* locus. To confirm correct single-copy integration of the vectors at *trpC* locus, EcoRI-digested chromosomal DNAs of the transformants were analyzed by Southern blotting in comparison with those of pTsgfp transformant (Lee *et al.*, 2004) and Wx24 (Fig. 2B). The DNA from *A. nidulans* Wx24 showed a single 2.40-kb fragment hybridized with the probe, a 0.40-kb *Sal*I-digested *trpC* fragment (Fig. 2B). On the other hand, the transformants exhibited differently sized DNA fragments hybridized with the trpC probe: specifically, pTsgfp transformant had 3.20- and 4.50-kb fragments; pTcitA-p::sgfp transformant 4.50- and 4.65-kb fragments; and pTcitA40::sgfp transformant 4.50- and 4.78-kb fragments. All of the positive fragments from the transformants showed similar intensity. These results support a correct single-copy integration of

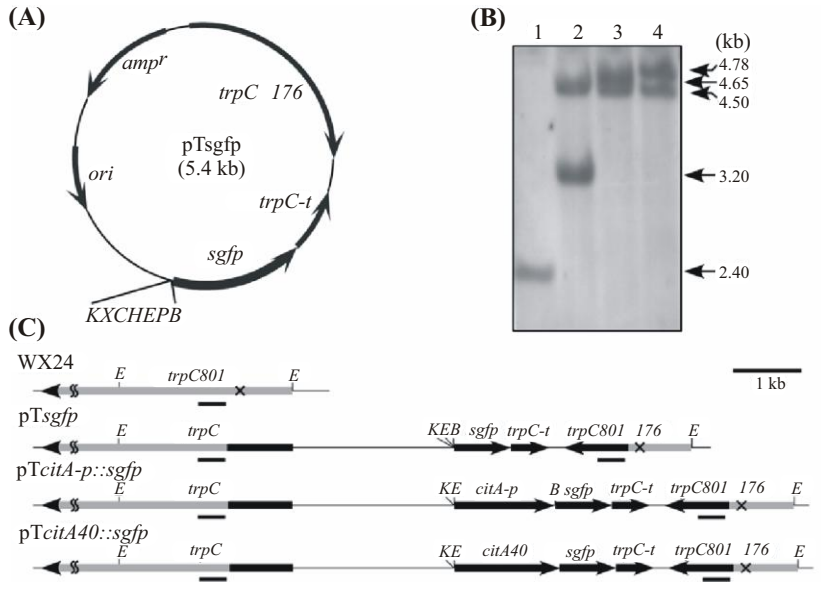

Fig. 2. Integration of the targeting vectors, pTsgfp, pTcitA-p::sgfp, and pTcitA40::sgfp, into the *trpC* locus. (A) Structure of the promoter analysis vector, pTsgfp. The 5′ 1.76-kp segment of *trpC* is designated as *trpCΔ176*, the 0.53-kb *trpC* terminator as *trpC-t*, and the cDNA encoding sGFP as *sgfp*. (B) For Southern blot analysis, the genomic DNAs from the host strain and the transformants were digested with *Eco*RI (lanes 1-5), and hybridized with the probe, a 0.40-kb *Sal*I-digested *trpC* fragment. Lanes: 1, Wx24; 2, pTsgfp transformant; 3, pTcitA-p::sgfp transformant; 4, pTcitA40::sgfp transformant. (C) Restriction maps of the *trpC* loci of the host strain (Wx24) and the transformants were predicted on the basis of the results of Southern blot analysis. Dark arrows or bars represent the essential components from the recombinant vectors, and the gray ones in the chromosomes of the host strain. The region homologous to the probe, 0.40-kb *Sal*I-digested *trpC* fragment, are shown as bars. Abbreviations for restriction enzymes: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I.

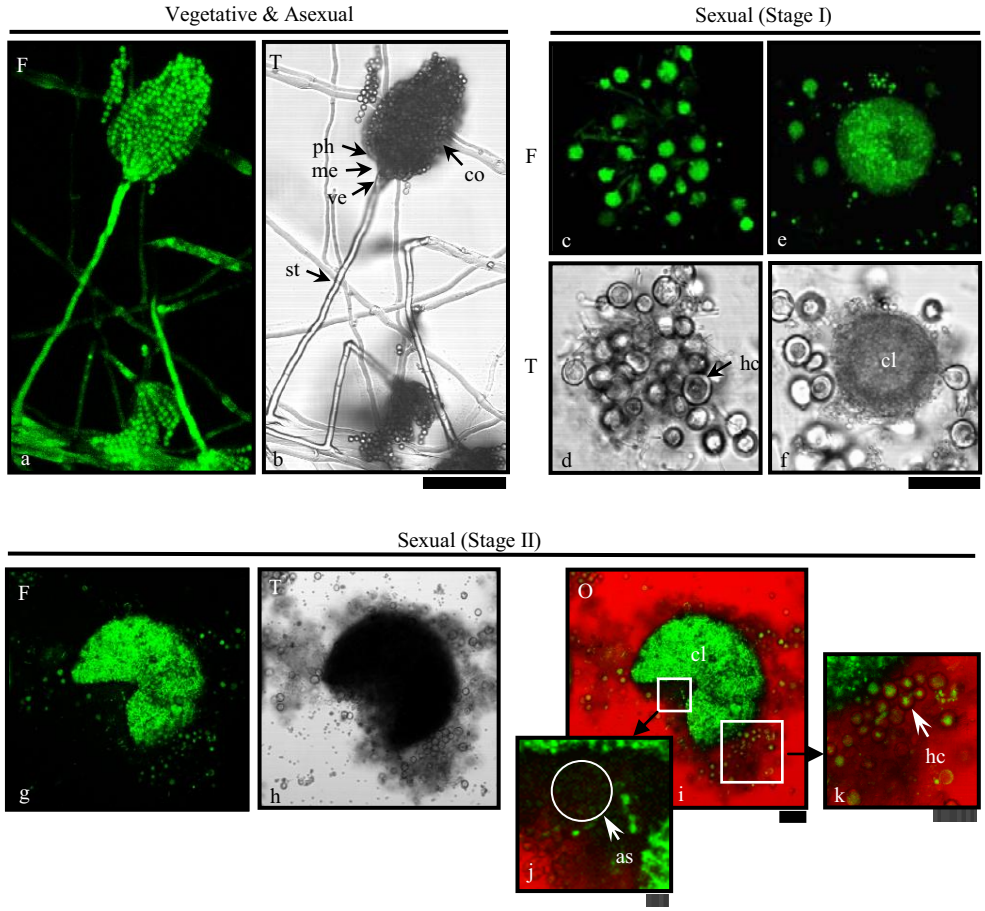

Fig. 3. Analysis of *citA* expression during different developmental stages by using sGFP as a vital reporter. To support vegetative growth and asexual development, pTcitA-p::sgfp transformant was slide-cultured for about 2 days on 1% glucose MBM agar plates (a and b). For induction of sexual differentiation, the mycelia of pTcitA-p::sgfp transformant grown in CM broth were transferred onto 1% glucose MBM plates, incubated for another 24 h while the plates were sealed closely, and incubated thereafter under unsealed conditions (c-r). The expression of sGFP was monitored by confocal microscopy. Stages of sexual differentiation: Stage I, 0-2 days after induction (c-f); Stage II, 3-5 days after induction (g-l); Stage III, 6-8 days after induction (m-r). Asexual or sexual structures: st, stalk; ve, vesicle; me, metula; ph, phialide; co, conidium; cl, cleistothecium; hc, Hülle cell; as, ascospore. Images: F, sGFP images generated by excitation with 488 nm and detection of 500-520 nm emission; T, transmission images; O, overlapping of sGFP and transmission images. Scale bars: black or white bars, 50 μm; striped bars, 10 μm.

pTcitA-p::sgfp or pTcitA40::sgfp at the *trpC* locus in each transformant.

Differential expression of *citA* during different developmental stages

To analyze the differential expression of *citA* during vegetative growth, asexual development, and sexual development, *A. nidulans* Wx24 and the transformants were slide-cultured on minimal media (MBMW for Wx24 and MBM for the transformants), and the sGFP fluorescence in the cells of the *A. nidulans* strains were observed by confocal microscopy. The host strain Wx24 and pTsgfp transformant did not show any visible level of sGFP fluorescence at all in any part of the fungal bodies (data not shown). On the contrary, pTcitA-p::sgfp transformant showed a considerable level of sGFP fluorescence throughout the whole fungal body including substrate mycelia, conidiophores, and conidia when slide-cultures on 1% glucose MBM plates. The intensity of sGFP fluorescence observed at different stages of hyphal growth and asexual development seemed to remain relatively constant (Figs. 3a and b). sGFP fluorescence was also examined during sexual development, which was divided into three arbitrary stages: Stage I when Hülle cells and young cleistothecia were formed (0-2 days after induction), Stage II when cleistothecia enlarged and ascospore formation occurred (3-5 days after induction), and Stage III when mature asci were liberated from the spontaneously rupturing cleistothecia (6-8 days after induction) (Lee *et al.*, 2004). At the early moment of Stage I, the transformant exhibited considerable level of sGFP fluorescence in Hülle cells, but relatively weak fluorescence in substrate mycelia (Figs. 3c and d). As development proceeded, quite strong sGFP fluorescence was also observed in young cleistothecia at the later moments of Stage I (Figs. 3e and f). The level of sGFP in cleistothecia, more exactly cleistothecial shells, was increased throughout stage II (Figs. 3g-i), reached its maximum at the beginning of Stage III, and declined

during the latest period of sexual development (data not shown). Remarkably, significantly weak sGFP fluorescence was visible in the cleistothecial contents, i.e., asci and ascospores, which were spilled out from the sexual structures, at any stages of sexual development (Figs. 3j and k).

Northern blot analysis of the mRNAs from the cells of different developmental phases was performed using the 0.58-kb intron-free *citA* fragment as a probe (Fig. 4). The size of *citA* transcript was estimated approximately 1.7 kb, which is in good agreement with the size of *citA* cDNA sequenced in this study (Fig. 1). During vegetative growth, the level of *citA* transcript increased gradually to reach to its maximum after 12-15 h of submerged culture, and slowly decreased thereafter. However, the level of *citA* transcript fluctuated within relatively a narrow range, suggesting that *citA* functions as one of the members of house-keeping genes. During asexual differentiation, the amount of *citA* transcript was retained at quite a high level until conidiation occurred, i.e., about 12 h after induction, and declined thereafter down to half the level of the maximum after about 22 h. During sexual differentiation, the amount of *citA* transcript slightly increased in accordance with the progress in morphogenesis of sexual structures and reached to the highest level at the latest moment. However, the level of *citA* transcript was considerably lower than those observed during vegetative growth or asexual differentiation.

The results of confocal microscopy and Northern blot analysis as a whole suggest that citA is expressed ubiquitously throughout the fungal body at considerably high level and is relatively independent of the change in developmental status of fungal cells during vegetative growth and asexual differentiation. In addition, the results clearly support that the expression of *citA* is retained throughout sexual differentiation. Interestingly, it turns out that the expression of *citA* is spatially differential in sexual structures: considerably high in the shells of cleistothecia, but significantly low in their contents bearing ascospores.

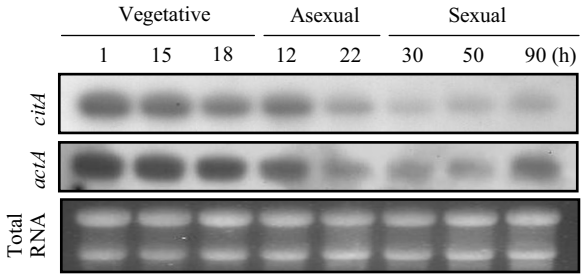

Fig. 4. Northern blot analysis of *citA* expression during different developmental stages. Total RNAs were prepared from the mycelia of *A. nidulans* FGSC A26 at the three different developmental status, i.e., vegetative growth, asexual differentiation, and early stage of sexual differentiation (Stage I). For preparation of vegetative mycelia, conidia of the strain were inoculated in CM broth and grown for approximately 18 h at 37℃. Induction of asexual differentiation was performed by spreading the vegetative mycelia onto agar plates and incubation at 37℃. For induction of sexual differentiation, the vegetative mycelia were transferred onto agar plates, incubated for another 24 h while the plates were sealed closely, and incubated thereafter under unsealed conditions. Each lane was loaded with 20 μg of total RNA based on reading of absorbance at 260 nm (A_{260}). The RNAs were hybridized with the PCR-amplified 0.58-kb *citA* probe labeled with ^{32}P. The blot of RNAs was rehybridized with a restriction fragment containing the whole ORF of the *A. nidulans actG* gene as an internal control. However, the expression of *actG*, a member of house-keeping genes, was not constant but fluctuated during sexual development as reported previously (Jeong *et al.*, 2001).

Regulation of *citA* expression by carbon sources

To analyze the effect of nutritional conditions on the expression of *citA*, pTcitA-p::sgfp transformant was slide-cultured on minimal media containing either glucose or sodium acetate, and the sGFP fluorescence in the cells of the *A. nidulans* strains were monitored by confocal microscopy.

In the cells of pTcitA-p::sgfp transformant grown on 1% glucose, a moderate level of sGFP was seen throughout the whole fungal bodies including vegetative mycelia, conidiophores, and conidia (Figs. 5a-d). When the concentration of glucose was increased to 3%, lower level of sGFP fluorescent was observed than on 1% glucose in vegetative mycelia. On the other hand, the intensity of sGFP in conidiophores remained almost constant despite of the increase of glucose concentration. This result suggests that the expression of *citA* gene is repressed to some extent by increased concentrations of glucose in substrate mycelia, but is affected little in the asexually differentiated structures including stalks, vesicles, metulae, phialides, and conidia. When grown on MBM containing 1% or 3% sodium acetate as a sole non-sugar carbon source instead of 1% glucose, the transformant showed significantly higher level of sGFP fluorescence throughout the fungal bodies than on 1% glucose (Figs. 5i-p). This result suggests that acetate can enhance the expression of *citA*.

Together with the confocal microscopic observation, media replacement experiments followed by by real-time RT-PCR were performed to analyze the effect of carbon sources on the expression of *citA* (Fig. 6). The mycelia of FGSC A26 grown in neutral liquid media containing 0.1% fructose as a sole carbon source (MMF) for 15 h were exposed to different types of sole carbon sources contained in liquid MM, and the change of *citA* expression was monitored by real-time RT-PCR. When exposed to glucose (MMG), the mycelia showed only a limited extent of increase in *citA* expression. On the other hand, the level of *citA* transcript increased 15-fold during the first 2 h of exposure to 50 mM of acetate. This result suggests that the expression of *citA* gene is strongly induced by acetate. To examine the effect of glucose on the inducible expression of *citA* by acetate, the level of *citA* expression in MMAG containing both acetate (50 mM) and glucose (1%) were also determined. After 2-h exposure to MMAG, the amount of *citA* transcript was estimated to be only about 10% of that obtained in the absence of additional glucose. This result suggests that the induction of *citA* expression by acetate is significantly repressed by glucose.

To determine whether CreA, a DNA-binding transcriptional repressor mediating CCR in *A. nidulans* (Ruijter and Visser, 1997), is responsible for the glucose-repression of *citA* induction by acetate, we also analyzed the expression of *citA* in the mycelia of a CreA-loss of function mutant strain, *creA^{d}30* (Fig. 6). The mycelia of *creA^{d}30* strain showed similar

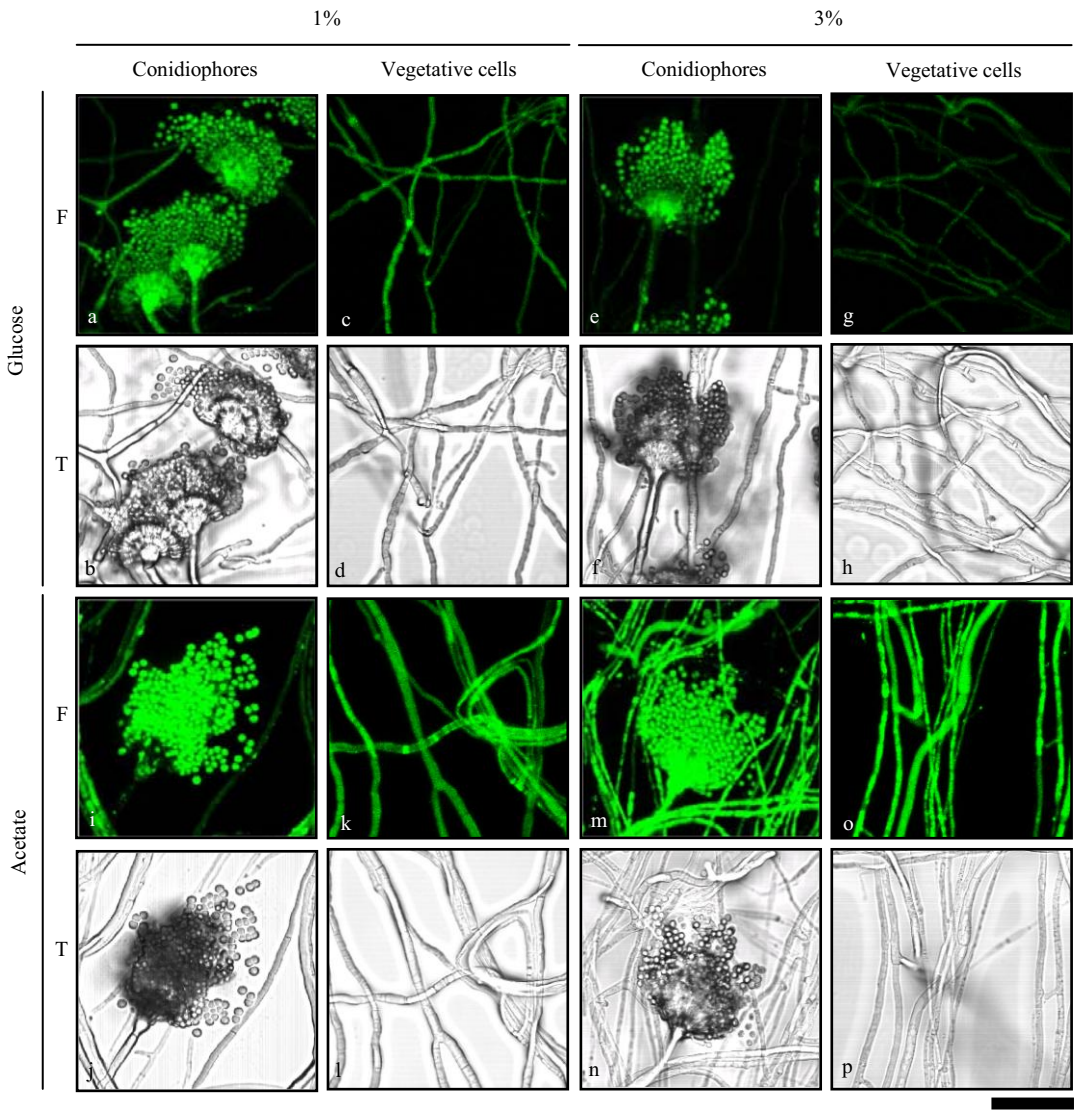

Fig. 5. Analysis of the effect of carbon sources on *citA* expression by using sGFP as a vital reporter. pTcitA-p::sgfp transformant was slide-cultured for about 3 days on MBM agar plates containing 1 or 3% glucose (a-h) or sodium acetate (i-p), and the expression of sGFP was monitered by confocal microscopy. Images: F, sGFP images generated by excitation with 488 nm and detection of 500-520 nm emission; T, transmission images. Scale bar=50 μm.

mode of *citA* expression as the wild-type strain when shifted to MMG and MMA for 2 h. On the contrary, when the mycelia of *creA*^{d}*30* strain were incubated for 2 h in MMAG, the level of *citA* transcript were about 35-40% of that obtained MMA. Thus the *creA*^{d}*30* strain showed approx. 4-fold higher level of *citA* expression under glucose-repressed conditions than the wild-type strain did. These results indicate that the loss of glucose-repression of *citA* is caused by *creA*^{d}*30* mutation, and accordingly that expression of *citA* gene is, at least partially, subject to regulation by CreA-dependent CCR.

Function of the N-terminal segment of CitA protein as a mitochondrial targeting signal

To analyze the function of the N-terminal region of CitA protein as a signal for intracellular organelle targeting of the protein, we constructed a hybrid gene encoding $CitA_{40}$::sGFP fusion protein containing the 40-amino acid N-terminal segment of CitA fused to sGFP. Then the intracellular localization of $CitA_{40}$::sGFP protein produced in the mycelia of pTcitA40::sgfp transformant grown on 1% acetate MBM was analyzed in comparison with that of sGFP formed in pTcitA-p::sgfp transformant. As expected, the green fluorescence of sGFP was evenly distributed not only in the vegetative mycelia of pTcitA-p::sgfp transformant (Figs. 7a and b) but also in their conidia (Figs. 7c and d). On the contrary, $CitA_{40}$::sGFP fusion protein was localized into certain intracellular organelles in both vegetative mycelia (Figs. 7e and f) and conidia (Figs. 7f and h). The intracellular compartments of vegetative cells in which $CitA_{40}$::sGFP fusion protein was localized were stained with a mitochondria-specific fluorescent dye MitoTracker Red CMXRos (Figs. 7i-l). Unfortunately, we could not get confocal microscopic images

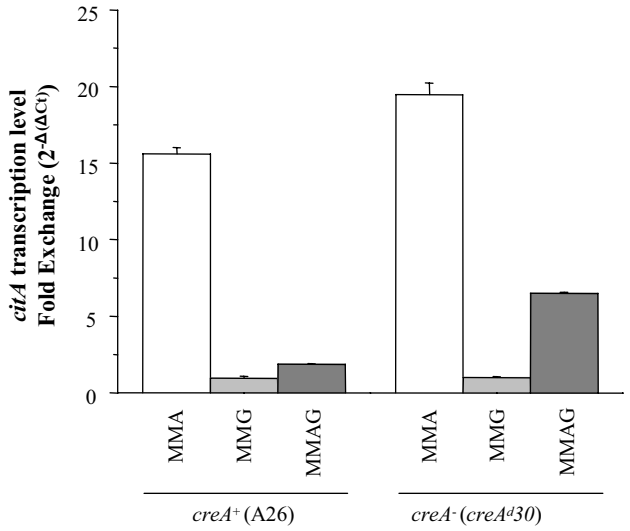

Fig. 6. Real-time RT-PCR analysis of the effect of glucose on the inducible expression of *citA* by acetate. Media replacement experiments and quantitative real-time RT-PCR were performed as described in the 'Materials and Methods' section. For preparation of total RNAs, mycelia of the wild-type (A26) and *creA^{d}30* strains pregrown in 0.1% fructose supplemented liquid was resuspended in liquid MMA, MMG, and MMAG media. After 2 h of incubation, mycelia were harvested by filtration and use for extraction of total RNAs.

of conidia stained with MitoTracker Red, which might be due to the conidial mitochondria being inactive. These results as a whole indicate that $CitA_{40}$::sGFP is effectively targeted into mitochondria in both vegetative cells and conidia, and thus that the 40-amino acid N-terminal segment of CitA precursor protein includes a mitochondrial targeting signal (MTS).

Discussion

In this paper we describe (i) cloning of the cDNA and promoter region of *A. nidulans citA* gene, (ii) differential expression of *citA* according to the change of developmental status, (iii) regulation of *citA* expression by carbon sources, such as glucose and acetate, via CCR, and (iv) visible evidence for presence of an MTS within the 40-amino acid N-terminal segment of CitA precursor protein.

By comparison of the cDNA sequence of *citA* obtained in the present study with the corresponding genomic sequence reported previously (Park *et al.*, 1997), it was confirmed that *citA* contains as many as seven small introns and that five of them are clustered in the first 25% of the coding sequence for CitA. On the other hand, the genomic sequence of *cit-1* encoding the mitochondrial citrate synthase of another filamentous fungus *N. crassa* is interrupted by just four introns (Ferea *et al.*, 1994). Only two introns are present in the closely related gene of *A. nidulans*, *mcsA*, encoding the mitochondrial methylcitrate synthase which is active towards both propionyl-CoA and acetyl-CoA (Brock *et al.*, 2000). In *Aspergilli* only a few genes, such as those encoding pyruvate kinase (de Graaff *et al.*, 1992) and α-amylases (Korman *et al.*, 1990), have been reported to include seven or more introns. It has been suggested that the genes for highly expressed and/or housekeeping enzymes usually have four or more introns that are mainly clustered in the beginning of the protein coding region while those expressed at low levels or tightly regulated often contain only one or two introns (Ferea *et al.*, 1994). The abundance and clustering of the introns in the *citA* genomic sequence is thus in good accordance with this suggestion in that the citrate synthase coded by *citA* functions as an essential rate-limiting enzyme of the TCA cycle, the central biosynthetic and catabolic metabolism, and thus is expressed at high level (Suissa *et al.*, 1984). However, there are some controversial examples, such as the gene encoding human mitochondrial citrate synthase, which contains no introns (Goldenthal *et al.*, 1998).

In yeast *S. cerevisiae*, most of the proteins required for mitochondrial function including the enzymes involved in the TCA cycle and respiratory chain are encoded by nuclear genes, and expression of many of these genes is repressed by glucose (Ronne, 1995). For example, expression of *CIT1* is repressed by glucose, and more severely by glucose plus glutamate. Unlike *CIT1*, expression of *CIT2* gene is reduced by glucose plus glutamate, but not by glucose alone (Kim *et al.*, 1986). In *A. nidulans*, three groups of genes are subject to the repression by glucose or sucrose: the genes encoding enzymes committed to catabolism of less preferred carbon sources, such as ethanol (*alcA* and *alcR*) (Kulmburg *et al.*, 1993) and proline (*prnBD*) (Cubero and Scazzocchio, 1994), those encoding gluconeogenetic and glyoxylate cycle enzymes, such as isocitrate lyase (*acuD*) (Bowyer *et al.*, 1994) and malate synthase (*acuE*) (Sandeman and Hynes, 1989), and those encoding the enzymes related to secondary metabolite, such as penicillin (*ipnA*) (Espeso and Penalva, 1992). However, unlike *S. cerevisiae*, carbon repression of the genes encoding the proteins involved in the TCA cycle and respiratory chain would not be expected in *Aspergilli* because these filamentous fungi are obligate aerobes (Ruijter and Visser, 1997). For example, expression of the *A. nidulans cycA* gene encoding cytochrome *c* is not affected by glucose repression at all, but is induced in the presence of oxygen and under heat-shock conditions (Raitt *et al.*, 1994). Transcription of *A. nidulans idpA* which encodes NADP-dependent isocitrate dehydrogenase catalyzing the third step of the TCA cycle yields two different transcripts started from two distinct transcription start point, of which the shorter one is inducible by acetate and by fatty acids while the longer one is present in higher amounts during growth in glucose containing media (Szewczyk *et al.*, 2001). In the present study, we found that the expression of *citA* yields transcript of approximately 1.7 kb, which is in good agreement with the size of *citA* cDNA, and that no variation in the size of the *citA* transcript and accordingly no shift of the transcription starting point is caused by the change of carbon source and growth conditions (data not shown). The expression of *citA* was strongly induced by acetate, however, no matter how strong the induction, it was significantly repressed in the presence of glucose. It is noteworthy that the expression of *citA* encoding one of the key enzymes of the TCA cycle is subject to both induction by acetate and CCR by glucose, and that the latter dominates over the former.

CreA is almost the only regulatory protein which has been demonstrated to mediate the carbon repression of the genes

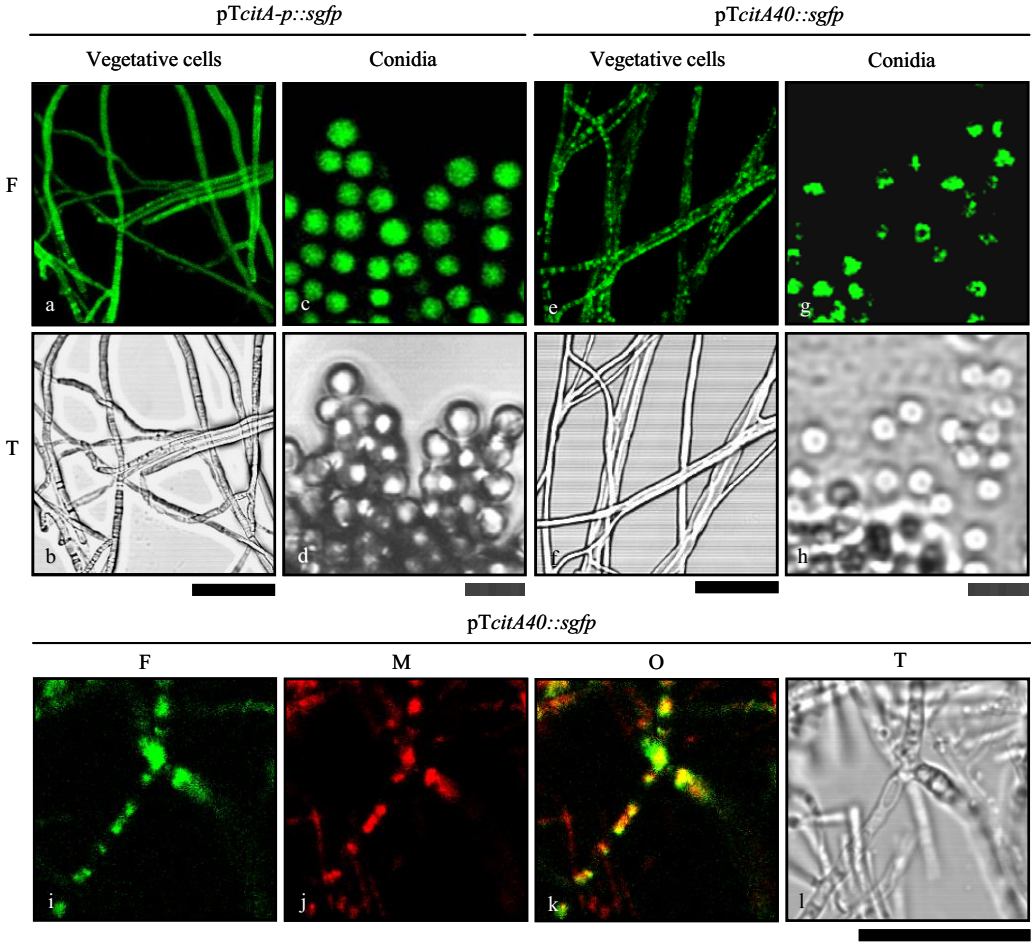

Fig. 7. Analysis of subcellular distribution of $CitA_{40}$::sGFP fusion protein by confocal microscopy. To support vegetative growth followed by asexual development, pTcitA-p::sgfp and pTcitA40::sgfp transformants were slide-cultured for about 3 days on 1% acetate MBM agar plates. To visualize mitochondria with fluorescent label, slide-cultured cells of pTcitA40::sgfp transformant were stained with MitoTracker Red CMXRos (i-l). Intracellular distributions of sGFP and $CitA_{40}$::sGFP fusion proteins was monitored by confocal microscopy. Images: F, sGFP images generated by excitation with 488 nm and detection of 500-520 nm emission; M, MitoTracker Red CMXRos images generated by excitation with 568 nm and detection of 580-600 nm emission; O, overlapping images of sGFP and MitoTracker Red CMXRos; T, transmission images. Scale bars: black bars=50 μm; striped bars=10 μm.

of *Aspergillus*, such as *alcA* and *alcR* (Kulmburg *et al.*, 1993), *prnBD* (Cubero and Scazzocchio, 1994), *acuD* (Bowyer *et al.*, 1994), and *acuE* (Sandeman and Hynes, 1989) of *A. nidulans*, *A. niger tpsA* (Wolschek and Kubicek, 1997), and *A. oryzae Taa-G2* gene (Kato *et al.*, 1996). Little is known about the CreA-independent mechanisms as in the case of *ipnA* encoding isopenicillin N synthetase (Espeso *et al.*, 1993). In the present study, we found four putative *cis*-acting binding motifs for CreA repressor in the presumptive promoter region of *citA*. Thus it is suggested that the glucose repression of *citA* induction by acetate in mycelia is mediated by CreA protein. However, a series of mutational analysis of the *citA* promoter is required to identify the genuine CreA-responsive element(s).

The life cycle of *A. nidulans* can be characterized by three developmental events, vegetative hyphal growth, asexual development, and sexual development. In the process of asexual development, conidiophores containing several types of differentiated cells, such as foot cell, aerial hyphae, vesicle, metulae, and philalides, are formed from the vegetative mycelium that acquires developmental competence, and then conidia are produced by repeated interstitial budding of phialides (Timberlake, 1990). In this study, we showed that the *citA* gene is expressed at relatively high levels if not under the conditions of carbon repression and thus plays an important role throughout the period of vegetative growth and asexual development in *A. nidulans*. During sexual development, cleistothecial primordia and thick-walled globose cells (Hülle cells) are formed from the vegetative hyphae, the intertwined network of specialized dikaryotic hyphae develops within the cleistothecial shell, and then nuclear fusion, meiosis, and ascosporogenesis occurs within the dikaryons (Champe *et al.*, 1994). The present results suggest that *citA* is specifically and strongly expressed in the mycelial network of cleistothecial shells, but is weakly expressed in the cells that are directly involved in the process of ascospore formation. However, it has been recently reported that deletion of *citA* does not

affect sexual development, i.e., cleistothecial wall and pigment formation, but causes a complete loss of ascospores in fruiting bodies (Murray and Hynes, 2010). It seems that despite of the low-level expression in ascospores, *citA* is essential for the process generation of ascospores.

It is noticeable that the expression of *citA* is regulated by the developmental status of the fungal thallus of *A. nidulans.* Considering that the mitochondrial citrate synthase CitA catalyzes the first step of the TCA cycle that plays an essential role both in generating reducing equivalents used for respiratory ATP synthesis and in supplying the carbon skeletons for biosynthesis, the present result implies that some difference in the central metabolism between the two developmental processes may exist. Although *A. nidulans* is known as an obligate aerobic organism, the process of respiratory ATP synthesis which requires the TCA cycle might occur weakly in the contents of cleistothecia at any stage of sexual development. This implication is compatible with the structural characteristics of cleistothecia that are formed as large global structures into which an adequate amount of oxygen for aerobic respiration can hardly penetrate. It is thus suggested that some compensatory metabolism for ATP generation, such as fermentation that does not require either catabolic operation of the TCA cycle or exogenous supply of a terminal electron acceptor such as oxygen, might be required for ascosporogenesis. Thus it is of interest to find biochemical and molecular biological evidence for the presence of some kinds of fermentation processes inside of cleistothecia.

Although the N-terminal MTS of the precursor proteins destined to mitochondrial targeting do not share any distinct consensus sequences, they do share at least some common features. First, they are usually rich in basic, hydrophobic, and hydroxylated residues, but lack acidic amino acids. Secondly, they contain one of the several types of conserved motif for proteolytic cleavage of the MTS during mitochondrial targeting, i.e., R-none (X↓X-S), R-2 (R-X↓X-S), R-3 (R-X-Y↓S/A), and R-10 (R-X<↓>F/I/L-S-X-X-X-X-X-X↓X), among which R-3 motif shows the highest confidence, 75% (Gavel and von Heijne, 1990). Our results described above support that the 40-amino acid N-terminal segment of CitA precursor protein includes a mitochondrial targeting signal (MTS). In agreement with this result, there exists an R(32)-C-Y-S(35) sequence which belongs to R-3 type motif in the predicted amino acid sequences of CitA precursor protein (Fig. 1). Similarly, *S. cerevisiae* Cit1 protein of which the 37 N-terminal amino acids are cleaved in the process of mitochondrial targeting (Lee *et al.*, 1994) contains an R(35)-H-Y-S(38) sequence. Accordingly, the predicted value of molecular mass of mature CitA protein (48.6 kDa) formed by removal of the 34 N-terminal amino acids is similar to that of the purified citrate synthase from this fungus that has been estimated to be approx. 48.2 kDa by SDS-PAGE (Maeng *et al.*, 1993).

Acknowledgements

This study was financially supported by research fund of Chungnam National University in 2008. We would like to thank Dr. Dong Min Han of Wonkwang University and Dr. Arst of University of Cambridge for providing the *A. nidulans* strains and the plasmids used in this study.

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