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ORIGINAL PAPER

Identification of elevated transcripts in a *Trichoderma reesei* strain expressing a chimeric transcription activator using suppression subtractive hybridization

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Abstract To know how the two transcription repressors Cre1 and ACEI affect their downstream genes, a new strategy was employed in which a plasmid encoding a chimeric transcription activator containing the DNA binding domains from Cre1 and ACEI and the effector domain from ACEII was constructed and transformed into *Trichoderma reesei*. Nineteen elevated transcripts were identified in the transformant against its parent strain using suppression subtractive hybridization. All of them had the consensus binding motif for Cre1 (5′-SYGGRG-3′) and ACEI (5′-AGGCA-3′), among which seven had the most representative binding motif (5′-AGGCAAA-3′) for ACEI in their 1,000-bp promoters. Cre1 could bind to the promoters of all the genes while ACEI could bind to the promoters of six out of the seven in vitro. The results provide a primary overview of a set of genes which may be associated to Cre1 or ACEI. It is the first step towards an understanding of the regulatory roles of the two repressors in cellular pathways, which would be helpful in strain improvement in *T. reesei*.

Keywords *Trichoderma reesei* · Chimeric transcription activator · Suppression subtractive hybridization · Cre1 · ACEI · ACEII

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Introduction

Trichoderma reesei is well-known for its excellent cellulase producing ability. Cre1 and ACEI are the two transcription factors that repress expression of cellulase genes (Strauss et al. 1995; Ilmen et al. 1996; Saloheimo et al. 2000; Aro et al. 2003). Disruption of *cre1* or *ace1* can improve cellulase production in *T. reesei*. However, the perturbation of the function of the repressors affects *T. reesei* growth characteristics (Ilmen et al. 1996; Saloheimo et al. 2000). This implies that both Cre1 and ACEI should affect other cellular pathways besides cellulases expression.

The search for the downstream genes of Cre1/CreA (the Cre1 equivalent in other filamentous fungi) in a genomic scale has been carried out using a *creA* deletion mutant and high density micro-array in *Aspergillus nidulans* (Mogensen et al. 2006). In *T. reesei*, little is known about how the two repressors Cre1 and ACEI affect their downstream genes. To know the functions of Cre1/ACEI in cellular pathways, like the work in *A. nidulans*, one routine method is to knock-out the corresponding gene in the genome and to determine which gene's expression is affected.

We here report an alternative new strategy to avoid the knock-out process and allow identification of the genes possibly downstream of the two repressors. A plasmid encoding a recombinant chimeric transcription activator was constructed and transformed into *T. reesei*. This chimeric protein retained the DNA binding capability and specificity of the repressors Cre1 and ACEI, while the effector domain was replaced by the one of the transcription activator ACEII. The genes that should be repressed by Cre1/ACEI, instead, would be activated by the ACEII effector domain in the chimeric protein

(Blancafort et al. 2004). The elevated transcripts were identified using suppression subtractive hybridization (SSH). The functions of the corresponding genes were diversified. As the consensus binding sequences for Cre1 (5′-SYGGRG-3′) or ACEI (5′-AGGCA-3′) have been determined, it is possible to make a preliminary in vitro exploration whether the promoters of the identified genes could be bound by either of the two transcription factors. The binding abilities of Cre1 and ACEI to their promoters were measured by electrophoresis mobility shift assay (EMSA).

Materials and methods

Microbial strains and plasmids

Escherichia coli DH5α was used as the host strain for recombinant plasmids construction. *Trichoderma reesei* strain Rut C-30 (ATCC 56765) was used as the host for pChi encoding the chimeric transcriptional activator. The gene *cre1* in Rut C-30 is truncated and loses its DNA binding ability; but *ace1* remains intact (Ilmen et al. 1996). For pChi construction, a DNA fragment encompassing the 1,360-bp promoter of the *cre1* gene and the sequence encoding Cre1 zinc fingers (amino acids 1–139) was amplified from the genomic DNA of *T. reesei* strain QM 9414 (ATCC 26921) and ligated into the *Xba*I/*Bam*HI sites of pBluescript SK-. The gene encoding ACEII effector domain (amino acids 59–341) was inserted at the *Bam*HI/*Eco*RI sites (Aro et al. 2001). A GSGGSGTS peptide was designed to link the two domains (Akopian et al. 2003). An additional *Hin*dIII site was designed immediately before the *Eco*RI site to permit the insertion of *ace1*. The gene encoding the zinc fingers (amino acids 383–529) of ACEI was then inserted into *Hin*dIII/*Eco*RI sites. The same linker peptide was designed between ACEII/ACEI. A bipartite nuclear targeting signal was located in ACEI zinc fingers (amino acid residues 387–403). A 1,496-bp *cre1* terminator was inserted at the *Eco*RI/*Sal*I sites (Fig. 1).

For prokaryotic expression of the chimeric transcriptional factor, the gene encoding Cre1-ACEII-ACEI (CAA) was amplified from pChi and ligated into pET-32a(+) between *Bam*HI and *Eco*RI sites to get pET32CAA. The DNAs for Cre1 DNA binding domain (Cre1DB) and ACEI DNA binding domain (ACEIDB) were PCR amplified from the genomic DNA of QM 9414 and ligated into pET-32a(+) between *Bam*HI and *Hin*dIII sites to get pET32Cre1 and pET32ACE1 for expression of Cre1DB and ACEIDB. The resulting plasmids were transformed into *E. coli* strain BL21(DE3). The primers used for plasmids construction were given in the Table 1.

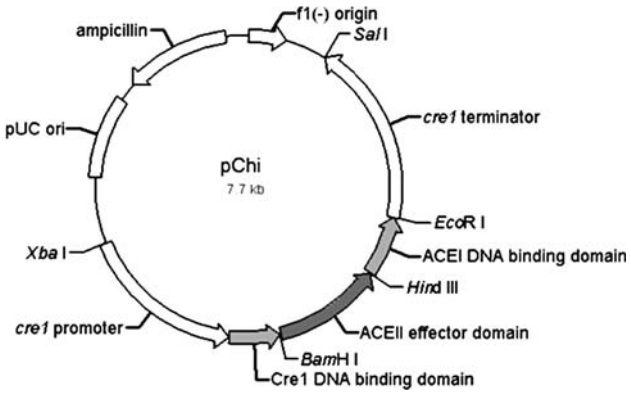

Fig. 1 Schematic drawing of pChi encoding the chimeric transcription activator CAA used in RUT C-30 transformation. The light gray arrows indicate genes encoding Cre1 and ACEI DNA binding domains, respectively; and the dark gray arrow indicates the gene encoding the ACEII effector domain

Media and culture conditions

For parallel shake flask fermentation and SSH experiments, RUT C-30 and the CAA transformants were first cultured in minimal medium (MM)-2% glycerol; and then equal amounts of mycelia were transferred to 100-ml MM-2% Avicel cellulose (Fluka) (Penttila et al. 1987). Mycelia were collected at 96 h after cellulose induction for RNA extraction for quantitative RT-PCR and at 24 h and 48 h for RNA extraction for SSH.

Prokaryotic expression of CAA, Cre1DB and ACEIDB proteins

The BL21(DE3) strain harboring pET32CAA, pET32Cre1, or pET32ACE1 plasmids was grown at 37°C to an OD_{600} of 0.6. Then IPTG was added to a final concentration of 1 mM. The induction was continued at 18°C for 12 h. The recombinant proteins were purified with a 1 ml HiTrap Chelating HP column (Amersham). Then the buffer was changed to 10 mM PBS (pH 7.4) with a 5 ml HiTrap Desalting column (Amersham).

Electrophoresis mobility shift assay

For CAA: in a 20 μl reaction mixture, CAA was mixed with 1 ng of the ^{32}P-labeled 150-bp *cbh1* promoter fragment (−853 to −704) in the buffer (10 mM HEPES-KOH, 1 mM $MgCl_2$, 20 μM $ZnSO_4$, 1 mM DTT, 10% glycerol, 50 μg/ml BSA) and incubated for 30 min at RT. This fragment contained only one binding site specific for Cre1 (5′-GTGGGG-3′, −724 to −719) and one for ACEI (5′-TGCCT-3′, −803 to −799; complement, 5′-AGGCA-3′, −799 to −803) (Saloheimo et al. 2000; Takashima et al. 1996). The gel was run at 150 V in 0.1×TBE buffer at RT.

Table 1 Primers used in this study

Gene	Primer sequence
For pChi construction	
cre1 promoter plus zinc fingers	Forward:5′-GCGGCTCTAGACTCGGTGAGAAGCGTGGAGG-3′
	Reverse:5′-ATTAGGATCCGCCATCGACGTGCATGGGGTG-3′
ace2	Forward:5′-CGTGGATCCGGCGGTAGCGGCACCAGCCATAGT CACAGTCACAA-3′
	Reverse:5′-CACGAATTCTTACTAAAGCTTCAGCAGTCTGGCACT GAC-3′
ace1	Forward: 5′-TATTAAGCTTGGCAGCGGTGGCTCCGGCACCAGCCGGTCC ATGGCCCGCCGG-3′
	Reverse: 5′-GAGGGAATTCCTAGCTGTAGCTGGGCGTGGAGG-3′
cre1 terminator	Forward:5′-GAGAATTCCTAGAATGTCCGGTACTCATGG-3′
	Reverse: 5′-GCGTCGACCACTAAAGCATCTAAAGTAGG-3′
For prokaryotic expression	
cre1-ace2-ace1	Forward: 5′-GATCGGATCCATGCAACGAGCACAGTCTGCC-3′
	Reverse: 5′-GAGGGAATTCCTAGCTGTAGCTGGGCGTGGAGG-3′
cre1	Forward: 5′-ATAAGGATCCCCTCGTCCCTACAAGTG-3′
	Reverse: 5′-TAGCAAGCTTAGCTGTGTATCCTCGAGTG-3′
ace1	Forward: 5′-ATAAGGATCCATGCGGTCCATGGCCCGCCG-3′
	Reverse: 5′-GTGGAAGCTTAGTAGCTGGGCGTGGAGG-3′
For qRT-PCR	
act	Forward:5′- TGAGAGCGGTGGTATCCACG -3′
	Reverse: 5′- GGTACCACCAGACATGACAATGTTG -3′
cbh1	Forward:5′- GCTGCCGGTGCGGCTTGAAC -3′
	Reverse: 5′- CTGGCCATTGATGAACTTCAGATCGC -3′
cbh2	Forward:5′- CCTGGTTATTGAGCCTGACTCTCTT -3′
	Reverse: 5′- ATCGCAACATTTGGAAGGTTCAG -3′
egl1	Forward:5′- GACTACACGGAGGAGCTCGAC -3′
	Reverse: 5′- TTGCGAGTAGTAGTCGTTGCTATAC -3′
xyn1	Forward:5′- CACCAAGAACAAGGTCATCAACTTC -3′
	Reverse: 5′- GCCAAAGTTCTCGACGATGTAGTAC -3′

After electrophoresis the gel was exposed to a Kodak X-ray film overnight at −70°C. For competition assays, the sequences of the competitor DNA for Cre1 and ACEI were 5′- ATTTGCCAACGGCTTGTGGGGTTGCAGAAGCA ACGGCA -3′ (−739 to −702) and 5′-AAGTACATAA GTTAATGCCTAAAGAAGTCATATACCAGC-3′ (−817 to −779), respectively.

For Cre1DB and ACEIDB: the DNA fragments corresponding to each promoter were PCR amplified or synthesized (Table 2). The labeling of these fragments with digoxigenin and the gel shift assays with Cre1DB or ACEIDB were carried out with a DIG gel shift kit (Roche).

Transformation of *T. reesei*

Transformation of RUT C-30 was carried out using 5 μg linearized pChi and $pRLM_{EX}30$ bearing the *hph* (encoding a bacterial hygromycin B phosphotransferase) gene in one experiment (Mach et al. 1994). Hygromycin resistant recombinants were selected and purified by stripping on hygromycin plates.

Quantitative RT-PCR

Total RNA was isolated using Trizol reagent (invitrogen). cDNA was synthesized using a Superscript III first-strand synthesis system (Invitrogen). All PCRs were performed in an ABI Prism 7000 real-time detection system (ABI) using a SYBR Green I RealMasterMix reagent (Tiangen). Analysis of the transcription levels was using *actin* as a reference. Primers used in qRT-PCR were given in the Table 1. The following PCR program was used: 2 min at 50°C, initial denaturation at 95°C 10 min, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C.

Biomass and enzyme activity assays

For fungus grown in cellulose medium, the biomass was determined by measuring the NaOH-extracted mycelial

Table 2 Characteristics of cDNA clones identified by SSH and the promoter fragments used in EMSA

Clone ID	cDNA length (bp)	Protein ID in the *T. reesei* genome (trire1)	(Predicted) protein function	Promoter fragment (bp) used in EMSA for Cre1/ACEI	Number of binding motifs in probes for Cre1/ACEI in EMSA
caa-1	784	32778	Unknown	−197 to −65[a]/NA	3/NA
caa-2	849	45056	Polyubiquitin	−305 to −280[b]/NA	1/NA
caa-3	325	42996	High affinity sugar transporter	−520 to −175[a]/NA	3/NA
caa-4	209	45186	Hex1	−561 to −447[a]/NA	3/NA
caa-5	690	46103	Unknown	−420 to −382[b]/NA	3/NA
caa-6	532	37375	Non-classical protein transporter	−489 to −227[a]/NA	2/NA
caa-7	261	32276	HFBII	−586 to −323[a]/NA	2/NA
caa-8	299	22728	Ubiquinol cytochrome C reductase subunit	−296 to −162[a]/−297 to −274[b]	2/1
caa-9	155	29985	Small HSP	−616 to −585[b]/NA	2/NA
caa-10	183	45923	P-type cation transporter ATPase	−487 to −152[a]/NA	4/NA
caa-11	154	39404	Ribosome biogenesis	−209 to −171[b]/NA	2/NA
caa-12	64	44282	Sucrose cleavage protein	−484 to −317[a]/−485 to −463[b]	2/1
caa-13	157	38088	Unknown	−577 to −179[a]/−346 to −317[b]	4/1
caa-14	69	44830	ATP citrate lyase subunit	−646 to −404[a]/NA	3/NA
caa-15	357	30027	Unknown	−437 to −403[b]/NA	1/NA
caa-16	797	32668	Glycyl-tRNA synthetase	−899 to −861[b]/−667 to −705[b]	1/1
caa-17	275	16354	Thioredoxin	−815 to −624[a]/−772 to −734[b]	2/1
caa-18	597	42049	Unknown	−953 to −915[b]/−843 to −810[b]	1/1
caa-19	635	42641	Unknown	−215 to −182[b]/−190 to −161[b]	1/1

NA, Not applicable

a–b: The DNA fragments were PCR amplified ([a]) or synthesized ([b])

protein as described by Jayaraman et al. (1966). And the protein concentrations were assayed using Lowery method (Lowry et al. 1951). The assays for filter paper activity, endo-β-1,4-glucanase (CMCase) and xylanase activities were measured using Whatman No.1 filter paper, sodium carboxymethyl cellulose (Hercules) and xylan (Sigma) as the substrates according to the IUPAC standard methods (Ghose 1987). To determine CBHI activity, 4-methylumbelliferyl-β-D-cellobioside (MUC, Sigma) was used as the substrate. The assay method was as described by Bailey et al. (Bailey and Tahtiharju 2003). One unit of CBHI activity was defined as the amount of enzyme which hydrolyzed 1 nmol of MUC per second.

Suppression subtractive hybridization and sequence analysis

Equal amounts of RNAs from RUT C-30 or 4C4 (the representative mutant expressing the chimeric transcription activator Cre1-ACEII-ACEI) at 24 and 48 h were combined, respectively. The synthesis of double-stranded cDNAs and subsequent SSH were carried out as described by Diatchenko et al. (1996). The cDNA from RUT C-30 and that from 4C4 were the 'driver' and 'tester' cDNA, respectively. The product of the second-round PCR was ligated into pGEM-T vector (Promega) and transformed into DH5α. The clones with DNA inserts in the SSH-library were selected randomly and cultured over night. PCR was carried out using the primers PN1/PN2 to amplify the inserts (Diatchenko et al. 1996). The PCR products were then applied to Hybond N nylon membranes. Reverse Northern blot was carried out to discover the up-regulated genes in the 4C4 transformant using digoxigenin-labeled RUT C-30 and 4C4 cDNAs as probes as described by Schmoll et al. (2004). The corresponding cDNA clones were sequenced by AUGCT Company (Beijing). The nucleotide sequences were blasted against the *T. reesei* genome (http://genome.jgi-psf.org/treesei) and the non-redundant protein sequence database in GenBank. The putative binding sites of Cre1 (5′-SYGGRG-3′) and ACEI (5′-AGGCA-3′, and the more representative 5′-AGGCAAA-3′) within the 1,000-bp promoters of these genes were screened using an online RSA-oligo-analysis tool (http://rsat.ulb.ac.be/rsat/oligo-analysis_form.cgi).

Results and discussion

Binding of CAA to the *cbh1* promoter fragment in vitro

CAA could bind to the *cbh1* promoter fragment in the EMSA assay (Fig. 2a). This binding was only partially inhibited by adding excess amounts of competitor DNA for either Cre1 or ACEI. When both competitors were added to the reaction mixture, the binding was fully inhibited, indicating that both of the two DNA binding domains in CAA were functional (Fig. 2b). It seemed that the binding of Cre1 zinc fingers was competed to a less extent than that of ACEI zinc fingers. When cold ACEI was used for competition, a low molecular weigh retardation complex was formed. We have observed that, with both Cre1 and ACEI zinc fingers, CAA was much more effective in binding the *cbh1* promoter fragment than the chimeric transcription factors Cre1-ACEII or ACEII-ACEI bearing only Cre1 or ACEI zinc fingers, and Cre1-ACEII was more effective in binding this promoter fragment than ACEII-ACEI (our unpublished data), suggesting that the zinc fingers at N- or C-terminal of CAA would stabilize each other in binding. Thus, the different extent of competition might be explained as follows: in the form of CAA, the binding of Cre1 zinc fingers to the probe might be more difficult to be competed than that of ACEI zinc fingers. When the competitor for ACEI was used, most of the ACEI zinc fingers were blocked (Fig. 2b, lane 4). This would drastically decrease the stability of the binding complex formed by CAA and the probe, thus forming the free probe and low weight molecular retardation complex with reduced stability. The residual ACEI zinc fingers which were not blocked would still co-operate with Cre1 to bind to the probe to form a stable complex appearing as the high molecular weight complex. This should be the same for Cre1 competition. However, given the low ratio of signal intensity of the low molecular retardation complex against the free probe, the low molecular weight complex might be too weak to be detected in Cre1 competition (Fig 2b, lane 3).

The transcription of cellulase and xylanase genes was enhanced in CAA transformant

From the hygromycin-resistant transformants, three strains, denoted as strain 4C1, 4C4 and 4C7, were found to have the integration of pChi into the genome by PCR. The expression of CAA was verified by RT-PCR (data not shown). A preliminary shake flask fermentation experiments showed that the general cellulase activities in the culture fluid for all the three strains (4C1: 3.3 ± 0.3 IU; 4C4: 4.1 ± 0.2 IU; 4C7, 3.3 ± 0.2 IU) at 72 h after

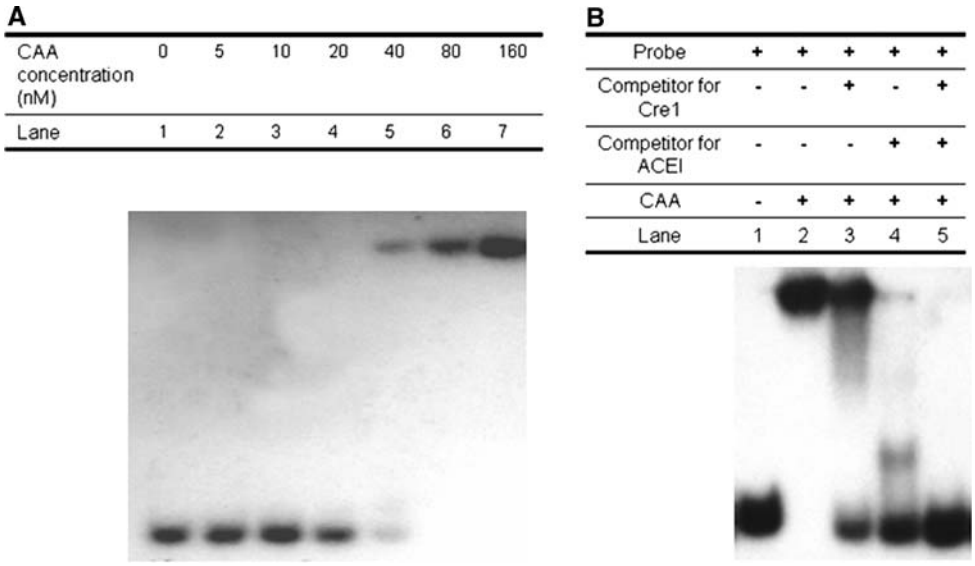

Fig. 2 CAA could specifically bind to a 150-bp *cbh1* promoter corresponding to the *cbh1* promoter sequence −853 to −704. This fragment contained only one binding site specific for Cre1 (5′-GTGGGG-3′, −724 to −719) and one for ACEI (5′-TGCCT-3′, −803 to −799, complement, 5′-AGGCA-3′,−799 to −803). **a** Gel shift assay of CAA to the ^{32}P-labeled *cbh1* promoter. The amount of probe for each lane was 1 ng. CAA was two-fold serially diluted. **b** Gel shift assay with the ^{32}P-labeled 150-bp *cbh1* promoter as a probe and the oligonucleotides corresponding to Cre1 and ACEI binding sites as competitors. The sequences of the competitor DNA for Cre1 and ACEI were 5′- ATTTGCCAACGGCTTGTGGGGTTGCAGAAGCA ACGGCA -3′ (−739 to −702) and 5′-AAGTACATAAGTTAA TGCCTAAAGAAGTCATATACCAGC-3′ (−817 to −779), respectively. The amounts of probes were 1 ng. And the protein concentrations were 80 nM. The competitors were added in 400-fold excess for both Cre1 and ACEI

induction were higher than that of RUT C-30 (2.8 ± 0.1 IU). The copy number and the position of the plasmid integration into the genome might account for the differential general cellulase activities. 4C4 showed the highest cellulase activity among the three. It was selected as the representative strain for detailed analysis of the cellulase activities in shake flask fermentation experiment. Meanwhile, the transcript levels of the cellulase and xylanase genes at 96 h after cellulose induction were analyzed. The amounts of CBHI, CBHII, EGI and Xylanase 1 mRNAs from 4C4 were much higher than those from the parent strain, indicating that CAA was functional

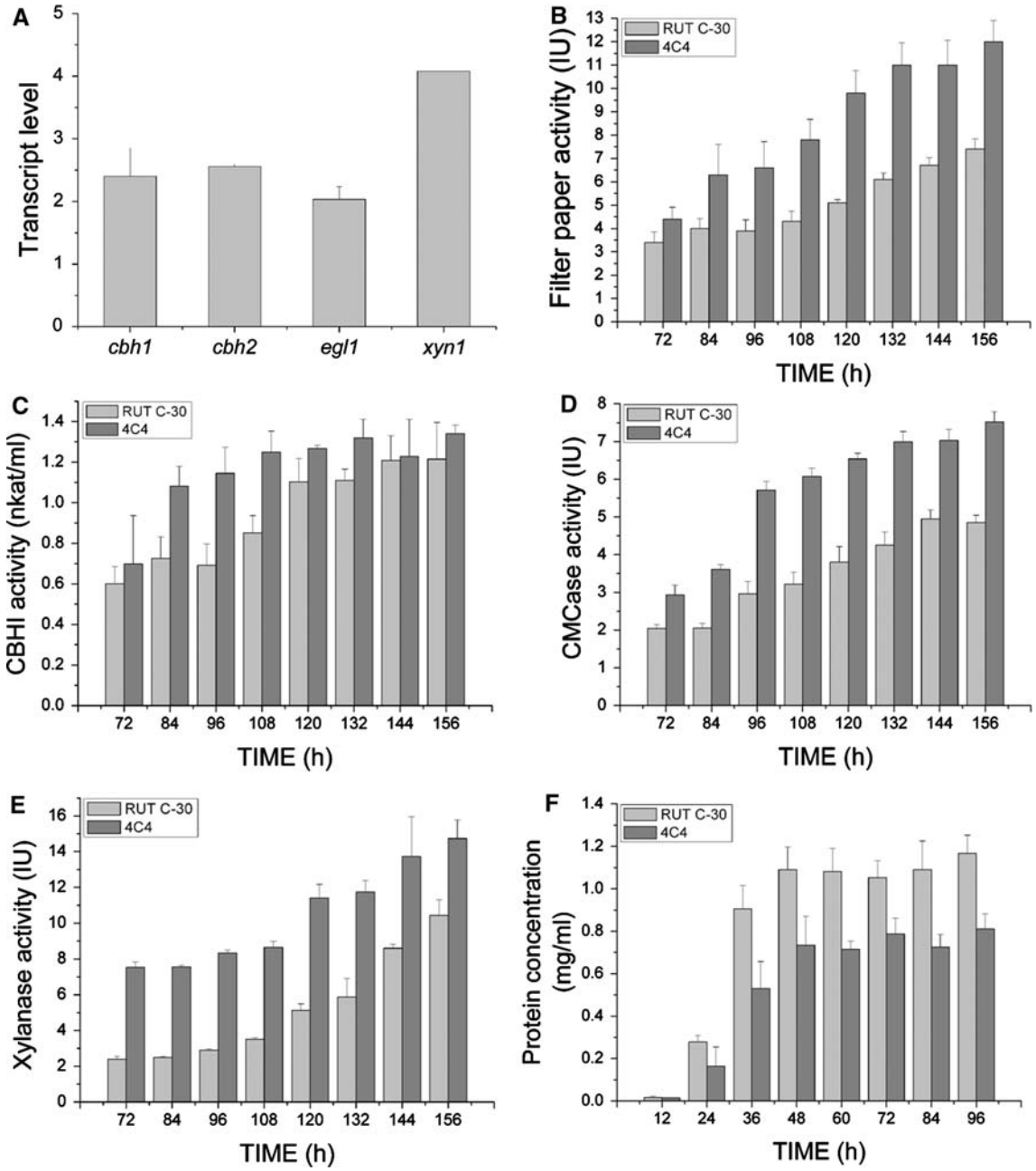

Fig. 3 CAA enhanced the expression of cellulase and xylanase genes in vivo and altered the transformant's growth characteristics. **a** qRT-PCR measurement of the transcript levels of CBHI, CBHII, EGI and Xylanase 1 at 96 h after induction. The relative transcript levels in the transformant 4C4 were in comparison with those of RUT C-30, which were all set as 1.0. The error bars indicate the standard deviations from three independent experiments. **b–e** The comparison of enzyme activities of the filter paper activity (general cellulase activity), CBHI, CMCase and xylanase of RUT C-30 and 4C4. The *x* axis indicates the time after cellulose induction. The error bars indicate standard deviations from three independent experiments. **f** 4C4 exhibited slower growth in liquid cellulose medium. The *x* axis indicates the time after cellulose induction. The *y* axis indicates the NaOH-extracted mycelial protein concentrations of RUT C-30 and 4C4

in vivo and could promote cellulase and xylanase gene transcription (Fig. 3a). The general cellulase activity, CBHI activity, CMCase activity, and xylanase activity in the culture fluid all increased (Fig. 3b–e). Furthermore, 4C4 grew more slowly in cellulose liquid medium, suggesting that CAA might affect some unknown genes besides cellulase and xylanase genes (Fig. 3f).

Identification of the elevated transcripts in the CAA transformant by SSH

As CAA was proved to be functional in vivo, we proceeded to discover what other genes were affected. Using SSH, 166 differentially expressed sequence tags were identified. Nineteen cDNA clones were selected for further investigation, based on the clearly stronger signal intensity (≥2 fold) on the reverse Northern blot hybridized with the probe prepared from the transformant 4C4 (Fig. 4). Their transcription levels were elevated in 4C4 in cellulose culture against RUT C-30. These cDNA clones were sequenced and denoted as *caa* (*cre1*- or *ace1*- associated) genes (Table 2).

Sequence analysis revealed that these genes were involved in multiple cellular pathways including respiration, sporulation, protein synthesis and secretion, stress response, sugar transporting and degradation, lipid biosynthesis, ubiquitin-mediated protein degradation, cation transport and unknown pathways (Table 2).

At first sight it was puzzling that none of the cellulase genes were identified. Similar result was observed in a subtraction hybridization experiment in *T. reesei* QM 9414/QM 9978 (Schmoll et al. 2004). Two reasons could account for this: first, at the time when we collected mycelia for preparing RNA, the difference of the transcription levels of cellulase genes in the two strains was moderate, which could not be detected by SSH. Secondly, the screening method in differential expression analysis can only reveal a subset of genes (Huang et al. 1999; Jiang et al. 2000).

Binding of Cre1 or ACEI to the promoters of the identified genes

The putative translation start sites of the SSH-identified genes were manually affirmed by homology based alignment. All of the 19 SSH-identified genes had the consensus binding motifs for Cre1 (5′-SYGGRG-3′) and ACEI (5′-AGGCA-3′) within their 1,000-bp promoters (Fig. 5). The binding sequences of ACEI are very variable because the nucleotides flanking the consensus sequences 5′-AGGCA-3′ largely affect the binding (Saloheimo et al. 2000). However, for the *cbh1* promoter, ACEI could bind to all

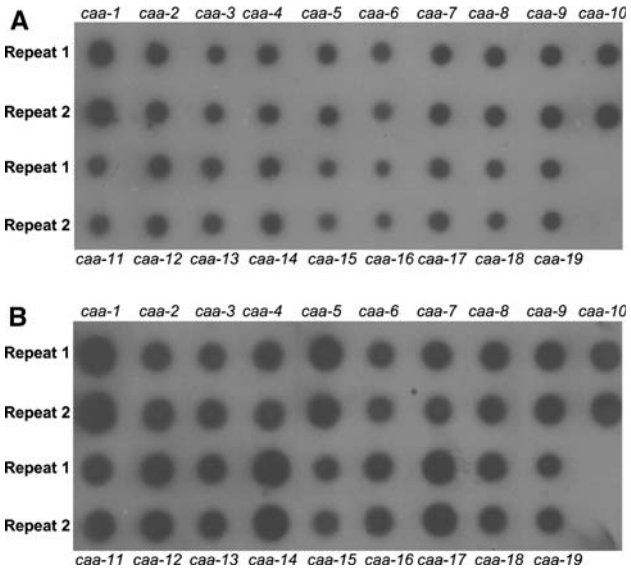

Fig. 4 The 19 differentially expressed genes analyzed by reverse Northern blot. **a** Membrane hybridized with the DIG-labeled RUT C-30 cDNA (driver); **b** membrane hybridized with the DIG-labeled 4C4 cDNA (tester). For each strain, the cDNAs were prepared from the combination of the RNA from the mycelia induced in cellulose for 24 h and 48 h

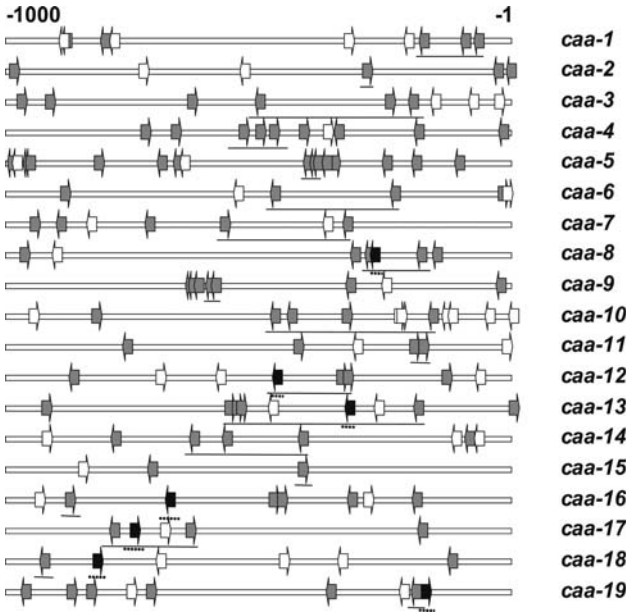

Fig. 5 Schematic map of the putative Cre1 and ACEI binding motifs in the 1,000-bp promoters of the identified genes showing the relative positions and orientations of the motifs. The 5′-SYGGRG-3′ sequence was used for screening the promoters for putative Cre1 binding sites (*gray arrow*). For ACEI, the 5′-AGGCA-3′ motif was used for screening (*white arrow*). And the *black arrows* indicate the most representative 5′-AGGCAAA-3′ motif for it. The direction of the arrows indicates the orientation of the binding sites in the promoters. The *solid and dotted underlines* indicate the relative positions of the DNA fragments used in the EMSA assays for Cre1 and ACEI binding, respectively

the 5′-AGGCAAA-3′ sequences (Saloheimo et al. 2000). Therefore, the most representative sequence 5′-AGGCAAA-3′ for ACEI binding was used to further screen these promoters. Not surprisingly, seven genes were found to have this sequence in their promoters. DNA fragments containing this sequence in these promoters were used in the in vitro binding assays.

EMSA was carried out to measure if Cre1 or ACEI could directly bind to the promoters of the identified genes. Cre1 could bind to all (Fig. 6a), but ACEI could bind six out of the seven tested genes with the corresponding binding motifs in their promoters (Fig. 6b). This fact suggests that the SSH-identified genes could not be over-expressed due to putative stress induced by introducing a novel chimeric transcription factor into the cell, and an existing binding motif in the promoter does not necessarily mean that the transcription factor will bind to it (*caa-8* for ACEI). This is important for ACEI, since ACEI was shown to be able to bind to all the 5′-AGGCAAA-3′ sequences in the *cbh1* promoter in vitro.

It was shown that two closely spaced 5′-SYGGRG-3′ repeat were present in in vivo functional Cre1 binding sites in the *xyn1* promoter in *T. reesei* (Mach et al. 1996). Closely spaced 5′-SYGGRG-3′ sites were observed in the 1,000-bp promoters of *caa-5*, *caa-9*, *caa-12*, *caa-13* and *caa-16*. However, unlike the cases for *xyn1* or *cbh1* promoter, the motifs did not exist as inverted or tandem repeats. In the in vitro EMSA assays, the DNA fragments containing these sites could be bound by Cre1 (Fig. 6a). And though no closely spaced 5′-SYGGRG-3′ were found in the promoters of the rest *caa-* clones, binding of Cre1 to the DNA fragments containing single or multiple 5′-SYGGRG-3′ was observed, as well (Fig. 6a). Similar result was reported for the in vitro binding of Cre1 to the *cbh1* promoter containing only one single site (Takashima et al. 1996).

Cre1 is responsible for glucose repression in *T. reesei*. The expression of *hfb2* and *hex1* has been reported to be repressed by glucose in *T. reesei* (Nakari-Setala et al. 1997; Curach et al. 2004). The EMSA results gave further evidence that they might be under direct control of Cre1. Glucose repression is also found for the yeast polyubiquitin gene highly homologous to *caa-2*. In *A. nidulans*, the transcript level of polyubiquitin was higher in stationary phase in maltose and sorbitol medium than that in glucose (Noventa-Jordão et al. 2000). The corresponding promoter of *caa-2* could be bound by Cre1. Further, Cre1 may be involved in regulation of mitochondrial respiration (*caa-8*, a putative ubiquinol cytochrome C reductase subunit). Interestingly, the promoters of some genes involved in stress response were shown to be direct targets of Cre1 (*caa-2*, *caa-4*, *caa-6* and *caa-9*). The four gene products are protective against various stresses such as starvation (polyubiquitin), heat stress (polyubiquitin, small heat shock protein), oxidation stress (non-classical protein transporter) and cell damage stress (Hex1). One attractive viewpoint is that glucose starvation is sensed as an unfavorable condition which can trigger activation of protective responses to many types of stress (Tamai et al. 1994; Alepuz et al. 1997). And Cre1 could bind to the promoters of *caa-11* (ribosome biogenesis) and *caa-6* (non-classical protein secretion).

A transcription analysis of *A. nidulans* wild-type and *creA* mutant during growth on glucose or ethanol using

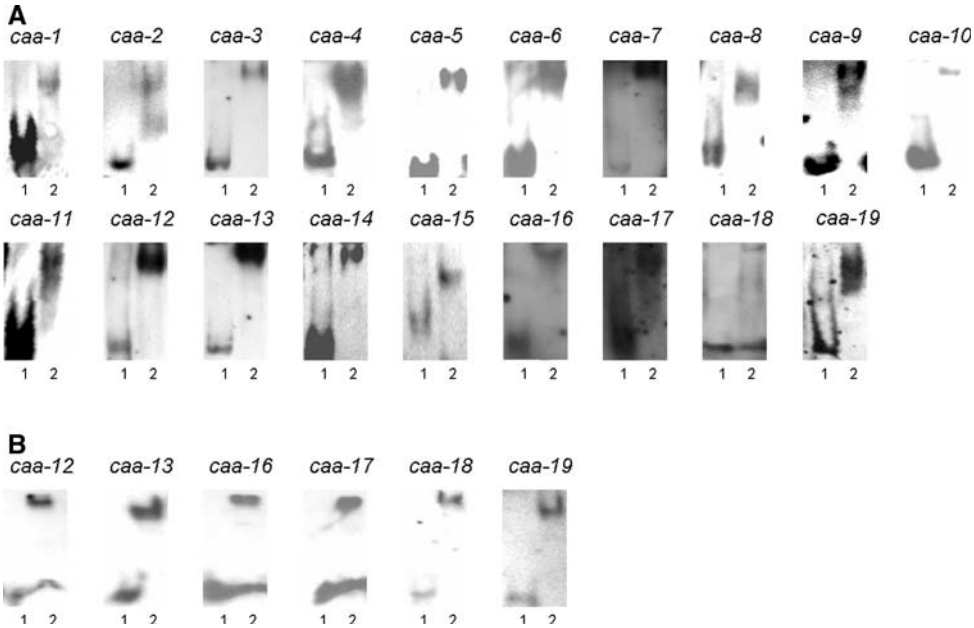

Fig. 6 Cre1 could bind to the promoters of all the SSH-selected genes with the corresponding binding motifs in their promoters in vitro, while ACEI could bind to six out of the seven tested genes. **a** Electrophoresis mobility shift assay of Cre1DB to digoxigenin-labeled promoter fragments of *caa-1* to *caa-19*. **b** Electrophoresis mobility shift assay of ACEIDB to digoxigenin-labeled promoter fragments of *caa-12*, *caa-13* and *caa-16* to *caa-19*. For all the probes, lane 1 is the free probe; lane 2 is the probe mixed with Cre1DB or ACEIDB. The amounts of Cre1DB and ACEIDB were 3 μg and 5 μg for each probe, respectively

high-density micro-arrays has been reported (Mogensen et al. 2006). Among the SSH-identified genes, only six (*caa-3*, *caa-4*, *caa-8*, *caa-9*, *caa-14* and *caa-17*) were found to have homologues (AN0250.2, AN4695.2, AN4388.2, AN7892.2, AN2436.2 and AN0170.2, respectively) analyzed in their data. These *A. nidulans* homologues were not classified into those genes most significantly regulated by Cre1. However, this did not rule out the possibility that they might be under Cre1 regulation since, as pointed out by the authors, the significance level of 1% was rather conservative. In fact, the transcript levels of all of them were more or less up- or down-regulated in the *creA* mutant in either glucose or ethanol medium. The reason for this complex expression pattern was suggested by them that Cre1/CreA has a rather complicated regulation role, characterized by its ability to both repress and activate the downstream genes in a direct or indirect way. Moreover, Cre1/CreA can also interact with the culture conditions when regulating, suggesting that other unidentified proteins might be involved in the regulation (Cubero and Scazzocchio 1994; Gómes et al. 2002).

ACEI could bind to the promoters of six out of the seven tested genes. *caa-17* encodes a thioredoxin-like protein. Thus, ACEI may also be involved in the cell's response to stress. The *caa-16* encodes a glycyl-tRNA synthetase. The promoters of other aminoacyl-tRNA synthetase genes were surveyed for the binding motif of ACEI. The motif exists in seven of them (data not shown). The role of ACEI in expression of aminoacyl-tRNA synthetases remains to be elucidated. Since no *ace1* mutant of *A. nidulans* has been reported to date, the comparison is not applicable for the six genes as the case for Cre1.

Among the nineteen promoters, *caa-5* had the maximal number of fourteen 5′-SYGGRG-3′ patterns of Cre1, while the *caa-15* and *caa-18* promoters had the minimal number of two (Fig. 5). The average number of this pattern was 5.3. This was a little bigger than the average number of 4.7 of this pattern found in the 1,000-bp promoters of the genes most possible to be repressed by CreA in *A. nidulans* (Mogensen et al. 2006). Interestingly, there seemed to be a trend showing more 5′-SYGGRG-3′ patterns in promoter regions of genes under CreA repression (Mogensen et al. 2006). For the nineteen promoters, the distribution of the different 5′-SYGGRG-3′ patterns in percentages was as follows: (C/G-C/T-G-G-A/G-G): (56/44-41/59-100-100-50/50-100).

The promoter of *caa-10* had the maximal number of seven 5′-AGGCA-3′ patterns of ACEI, while those of *caa-4, caa-9* and *caa-15* had the minimal number of one (Fig. 5). The average was 2.7, much smaller than that of 5.3 of Cre1 pattern. In the nineteen promoters, both the putative Cre1 and ACEI binding sites occurred more frequently in reverse direction (60% and 63%, respectively).

The promoters of the nineteen genes had fewer putative binding sites for ACEI (51) than Cre1 (101). In theory the occurrence frequency of the ACEI's binding motif is smaller than that of Cre1's 5′-SYGGRG-3′. And unlike Cre1 which is defective in the CAA transformant, ACEI is intact and can compete with the transformed CAA to bind to the same site.

In summary, we introduced a plasmid encoding a chimeric transcription activator which retained the DNA binding capability and specificity of Cre1 and ACEI into *T. reesei*. Nineteen elevated transcripts in the transformant were identified using suppression subtractive hybridization. The functions of the identified genes are diversified. Their roles in cellulase expression and other pathways remain unclear in *T. reesei* and need further investigation. In EMSA assays Cre1 could bind to the promoters of all the nineteen genes and ACEI could bind to the promoters of six out of the seven tested genes. Although these in vitro bindings cannot fully represent the in vivo situation, the results given here provide informative knowledge for Cre1 and ACEI regulation. The identified genes were good candidates for further investigation into their relation to Cre1 or ACEI. Little is known about how Cre1 and ACEI regulate their downstream genes besides cellulases. This research was the first step towards an understanding of the regulatory roles of the two transcription repressors in cellular pathways, which would be helpful in strain improvement of *T. reesei*.

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