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# Analysis of differentially expressed genes in the precocious line of *Eimeria maxima* and its parent strain using suppression subtractive hybridization and cDNA microarrays

**Hui Dong · Jiaojiao Lin · Hongyu Han · Lianlian Jiang · Qiping Zhao · Shunhai Zhu · Bing Huang**

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**Abstract** The precocious line of *Eimeria spp.*, obtained by repeated passages of oocysts initially collected from feces of previously infected chickens, has unique phenotypes and plays an important role in immunizing chickens against coccidiosis. However, the genetic basis of precocious phenotype in *Eimeria* is still poorly understood. To investigate gene expression changes in sporulated oocysts between the precocious line of *E. maxima* and its parent strain, subtractive cDNA libraries were constructed by suppression subtractive hybridization (SSH). A total of 3,164 cDNA fragments were selected from the SSH cDNA libraries to fabricate cDNA microarrays and further identify the differentially expressed genes. The credibility of the microarray data was verified by real-time PCR. A total of 360 valid expressed sequence tags (ESTs) were obtained, which represented 32 unique sequences. Twenty-one genes were validated as downregulated and 11 genes as upregulated in the precocious line. Homology searching of the public sequence database showed that six genes encoded proteins homologous with previously reported proteins, including rhomboid-like protein and transhydrogenase of *E. tenella*, serpin, and cation-transporting ATPase of *E. acervulina*, a heat-shock protein of *E. maxima*, and a conserved hypothetical protein of *Toxoplasma gondii*. Thus, the remaining 26 ESTs have not been previously reported. Further characterization of these differentially expressed genes will be useful in understanding the genetic basis for the precocious phenotype in *Eimeria spp.*

H. Dong · J. Lin · H. Han · L. Jiang · Q. Zhao · S. Zhu · B. Huang (✉)
Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences,
No. 518 Ziyue Road, Minhang District,
Shanghai 200241, China
e-mail: hb@shvri.ac.cn

## Introduction

Coccidiosis, caused by protozoan parasites of the genus *Eimeria*, has a severe economic impact on commercial poultry production worldwide (Shirley et al. 2005). Current control of *Eimeria* species is based primarily on the use of medication. However, the extensive use of anticoccidial drugs over many years has led to the emergence drug resistance in the field. In addition, there are concerns about drug residues in poultry products (McEvoy 2001) and the strong desire of consumers to ban drugs from animal feeds (Young and Craig 2001). There is, therefore, a pressing need to move away from chemotherapeutic control of coccidiosis towards vaccination.

The life cycle of *Eimeria* species is characterized by an endogenous developmental life cycle that comprises sequential phases of asexual reproduction followed by a terminal phase of sexual reproduction. Each new generation of oocysts is discharged in the feces of the host animal, and these can be collected easily at specific times throughout the patent period (Shirley and Harvey 2000). Serial

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selection for the first oocysts to appear during the patent period has consistently resulted in obtaining precocious lines (Jeffers 1975). Compared with the parent strain, the precocious line has a reduced prepatent period, is markedly less pathogenic, has a lower reproductive potential, but retains its immunogenicity (Shirley and Bedrník 1997). These traits have been shown to be genetically stable and inheritable (Jeffers 1976; Sutton et al. 1986). Therefore, precocious lines are suitable to be used in live vaccines to immunize chickens against coccidiosis, as an alternative to anticoccidial drugs. Immunization with attenuated vaccines developed from precocious lines has been used since 1979 (Johnson et al. 1979) and is increasingly playing an important role in the control of coccidiosis in the poultry industry.

Despite its variety of biological changes and its importance in the control of coccidiosis, little is known about differences in gene or protein expression between the precocious line and its parent strain. Indeed, limited genetic variability might correspond to significant changes in the genome that affect the biological features of the parasite (Shirley and Bumstead 1994). Our research focused on analyzing gene expression changes in sporulated oocysts between the precocious line of *E. maxima* and its parent strain using high-throughput and high-sensitivity approaches. Our results will help to understand the genetic basis of the precocious phenotype in *Eimeria* and contribute to the elucidation of the mechanism that regulates the life cycles of apicomplexan parasites.

## Materials and methods

### Parasites and animals

The Shanghai strain of *E. maxima* was isolated from a litter sample collected in a commercial house in Shanghai, China in 1985 and has been maintained in our laboratory. A precocious line was selected from the Shanghai strain by serial passages through chickens of the first oocysts produced during infection. After 17 passages in chickens, the prepatent period of the parasite was reduced from 142 to 107 h.

Day-old AA broiler chickens, from Huadu Broiler Hatchery, which has a good reputation of producing disease-free chickens, were kept under coccidia-free conditions. Feed and water, containing no anticoccidial drugs or antibiotics, were fed ad libitum. Chickens were orally inoculated with $2\times10^4$ sporulated oocysts of *E. maxima* at 2 weeks of age. Feces were collected from days 6 to 9 postinfection. The methods for handling the parasites, preparation of infective doses, detecting and recovering oocysts from infected chickens, and sporulating them, have been fully described (Long et al. 1976). The sporulated oocysts were purified by centrifugation, salt flotation, and treatment with sodium hypochlorite, as previously described (Wagenbach et al. 1966). They were stored as aliquots at −80°C until use.

### RNA isolation and analysis

Total RNA was isolated from approximately $1\times10^8$ sporulated oocysts of the parent strain or precocious line of *E. maxima* using TRIzol (Invitrogen, Carlsbad, CA). In brief, the sporulated oocysts were combined with approximately 3 g of 1.00 mm glass beads (autoclaved 121°C, 15 min, Fischer Scientific), and 5 ml of TRIzol. Samples were vortexed for 4×1 min alternated with 1-min incubation on ice. The resulting emulsion was pipetted from the glass beads and transferred to a 10-ml round bottom centrifuge tube and 1 ml of chloroform was added. The remainder of the total RNA isolation procedure was carried out according to the manufacturer's recommended protocol. RNA pellets were resuspended in diethylpyrocarbonate-treated water. mRNA was then isolated using a PolyATtract® mRNA Isolation Systems Kit (Promega, Madison, WI), following the manufacturer's recommended protocol. To ensure quality, total RNA and mRNA were each quantified by UV spectrophotometry, and the purities of total RNA and mRNA were assessed on a 1% agarose denaturing, formaldehyde/EtBr gel.

### Construction of subtracted cDNA libraries using SSH technique

The subtractive cDNA libraries were constructed using a PCR-Select cDNA Subtraction Kit (BD Clontech, K1804-1) based on the manufacturers' instructions. Both the precocious line and the parent strain poly(A)+ RNA were used as tester or driver in reciprocal combinations. Poly(A)+ mRNA from the precocious line of *E. maxima* was used as the "tester" while poly(A)+ from the parent strain served as the "driver" (Forward SSH library). Conversely, poly(A)+ mRNA from the parent strain and the precocious line were also used as tester and driver samples, respectively (Reverse SSH library).

After two rounds of PCR amplification of the differentially expressed sequences, the PCR products were purified using the QIAquick®Gel Extraction Kit (Qiagen). Purified PCR products were ligated into the pGEM-T Easy Vector (Promega), transformed into *Escherichia coli* JM109 and cultured on an LB media plate containing ampicillin and X-Gal/IPTG. Randomly selected white colonies were manually transferred to 96-well plates containing LB broth followed by overnight incubation at 37°C. The SSH cDNA clone inserts then, were amplified by PCR

using T7 and SP6 primers. The PCR products were visualized on 2% agarose gels to ensure their quality and quantity.

## cDNA microarray construction

cDNA microarray slide preparation and microarray analysis for the selected SSH cDNA clones were carried out in the United Gene Holdings, Ltd., Shanghai, China. The qualified PCR product were precipitated with isopropanol and 3 mmol/L sodium acetate, washed with 75% ethanol, and dried for 4 h. The dried PCR products were dissolved in a buffer containing 3×standard saline citrate (SSC) solution (1×SSC, 15 mM sodium citrate, 150 mM NaCl) and were spotted onto amino-silanized glass slides using an Omni-Grid Accent Arraying machine (GeneMachines Corp., San Carlos, CA). On average, 200–300 pg of the amplified SSH fragments were spotted. Each cDNA clone was printed in triplicate in 3×SSC printing solution. A total of 3,200 cDNA clones were spotted, including 1,664 clones from the forward library, 1,500 clones from the reverse library, 18 blank controls, and 18 negative controls.

## cDNA probe labeling and hybridization

Total RNA was extracted from the sporulated oocysts of the precocious line and the parent strain, respectively, as described above, and purified using RNeasy® Mini Kit (Qiagen). The purified RNA was used to synthesize Cy3- or Cy5-conjugated dUTP-labeled cDNA probes using an RNA Fluorescence Labeling Core Kit (MMLV Version) Ver.2.0 (Takara, Dalian, China). Two independent biological replicates with same amount of total RNA from different oocysts were applied in each sample, and each replicate was used for independent RNA extraction and labeling reactions. Labeled Cy3 and Cy5 cDNA probes were cleaned using a Qiaquick Nucleotide Removal Kit (Qiagen). The probes were denatured at 95°C for 3 min, dropped onto the center of the array surface and then covered with a coverslip without any bubbles. The slides were placed into a sealed cassette to hybridize in a 65°C water bath for 12–16 h. To increase the reliability of microarray analysis, dye-switch experiments were performed.

After hybridization, the microarray slides were washed once with 2×SSC, 0.1% sodium dodecyl sulfate (SDS) at 42°C for 4 min, once with 0.1×SSC, 0.1% SDS at room temperature for 10 min and three times with 0.1×SSC at room temperature for 1 min. The microarray slides were then washed with distilled water and spin dried. Subsequently, hybridized slides were scanned for Cy3 at 610 nm and Cy5 at 590 nm using a ScanArray 4000 chip Scanner (GSI Lumonics, Billerica, MA). Thus, two separate TIFF images were generated for each slide.

## Data analysis

Raw data analysis was performed with GenePix Pro3.0 software (Axon Instruments, Union City, CA, USA). For each hybridized spot, the background intensity was subtracted and normalized by non-linear Lowess normalization (Workman et al. 2002). The signal intensity was calculated as the mean intensities of the three replicates minus the background signal. To rule out possible artifacts arising from low expression values, only genes with raw intensity values for both Cy3 and Cy5 of more than 200 counts were chosen for differential analysis. The spots that had a Cy5/Cy3 ratio of $\leq 0.5$ or $\geq 2$, or where the absolute value of base 2 logarithm of the ratio was $\geq 1$, were identified as differentially expressed genes.

## DNA sequencing and BLAST identification

The identified differentially expressed genes were sequenced using an ABI 3730 automatic DNA sequencer. The sequences were screened against the UniVec database (http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html) and the *E. coli* whole genome sequence, to remove vector and *E. coli* genome contamination. Poor quality sequences, or sequences less than 100 bp, were discarded. The remaining sequences were assembled into consensus sequences (contigs) using the PHRAP assembly program (http://www.phrap.org). All contigs and singlet sequences were subjected to BLASTX searches against the non-redundant (NR) protein database from NCBI (http://www.ncbi.nlm.nih.gov/BLAST). The criterion for BLASTX was *E* value$\leq 10^{-5}$. Each unique sequence was also compared with all previously sequenced *Eimeria* ESTs deposited in the dbEST database. All the ESTs reported in this study were submitted to the dbEST database at NCBI with GenBank accession numbers GW840269-GW840300.

## Validation of differential expression of genes by real-time RT-PCR

Real-time RT-PCR analysis was employed to validate the relative change in expression of genes identified by the microarray analysis. The RNA samples from the precocious line and the parent strain were isolated using Trizol reagent (Invitrogen). Total RNA from each sample was treated with DNase I (Promega) and used for cDNA synthesis. The first-strand cDNA synthesis was performed with random primers using SuperScript II Reverse Transcriptase (Invitrogen). All PCRs were performed in a Rotor-Gene 3000 (Corbett Robotics, Jacksonville, FL) using a SYBR Green I Real MasterMix reagent (Takara). Gene-specific primers from the identified genes for real-time RT-PCR were designed manually by the Beacon Designer program. The primers

used for each gene are presented in Table 1. To normalize the total amount of cDNA in each reaction, the *E. maxima* 18S ribosomal RNA gene (Accession number: EF122251) was coamplified as the internal control. Each sample was replicated three times, and the experiment was performed twice.

## Results

### Construction of sporulated oocysts subtractive cDNA libraries

To identify the differences and similarities of gene expressions in sporulated oocysts between the precocious line and the parent strain, the SSH technique was used to construct both forward and reverse subtractive cDNA libraries. The forward SSH library used the poly(A)+ mRNA of the precocious line as the "tester" and that of the parent strain as the "driver". Conversely, the reverse SSH library used the poly(A)+ mRNA of the parent strain and precocious line as tester and driver, respectively. PCR amplification revealed that the two subtractive cDNA libraries contained approximately 98% and 97% recombinant clones. The inserted fragments obtained by PCR were mainly distributed between 300 and 1,000 bp. These results indicated that the subtractive cDNA libraries could be used to screen differentially expressed genes.

### Differential screening of subtracted cDNA libraries

To screen the differentially expressed genes from the two subtractive cDNA libraries in large scale, the 1,664 and 1,500 PCR products from the forward and reverse subtract library, respectively, were purified and selected to fabricate cDNA microarrays. The replicates used in this array experiment include independent mRNA extraction from two biologically replicated, dye-switch experiments and three spot duplications, resulting in 12 observations per tentative unique transcript. After hybridization, a total of 426 differentially expressed clones were identified according to the 12 Cy5/Cy3 ratios of each clone.

### Sequence analysis of selected cDNA clones

These 426 clones were selected and subjected to nucleotide sequence analysis. The 426 sequences were trimmed of vector and low-quality sequence and filtered for minimum length (100 bp), allowing the identification of 360 high-quality ESTs with an average length of 430 bp. The PHRAP assembly program was used to identify those ESTs that represent redundant transcripts. The 360 high-quality ESTs were assembled into 29 contigs and three singlets, giving 32 assembled sequences. Of these, 21 sequences were downregulated in the precocious line, and 11 were upregulated.

Each unique consensus sequence was compared with entries in the nr protein database using the BLASTX algorithm. Six (19%) of the sequences shared significant identity (with a score of at least $1\times10^{-5}$) with previously described or hypothetical proteins. Twenty-six (81%) had no significant match in any sequence in the database. As shown in Table 2, six of the ESTs had the highest similarity to apicomplexan sequences and included the rhomboid-like protein and transhydrogenase of *E. tenella*, a serpin, and a cation-transporting ATPase of *E. acervulina*, a heat-shock protein of *E. maxima*, and a conserved hypothetical protein of *Toxoplasma gondii*. These results suggested that these differentially expressed genes are associated with the invasion of the host cell, developmental stage conversion, infectivity, virulence, drug-resistant, and sporulation.

To ensure that these ESTs were novel, all sequences were compared with previously sequenced *Eimeria* ESTs deposited in the dbEST database using the BLASTn algorithm. The results from this comparison are shown in Table 3. Thirteen (40%) of the sequences overlapped with previously isolated *Eimeria* ESTs, of which 7, 5, and 1 were from *E. maxima*, *E. tenella,* and *E. acervulina*, respectively. The remaining 19 ESTs are novel ESTs in *Eimeria*.

### Validation of differential expression of genes by real-time RT-PCR

To examine the quality of the microarray data presented in this report, five genes were selected to quantify their

**Table 1** Primer sequences for real-time PCR

| Clone ID | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|---|---|---|
| SSBR0008 | GCCGCAAGTATGATGAAGTC | GCGTCTTATTGAGCCGATTC |
| SSBR0010 | ATAAGCAACAGCAGCACATTAG | TTATAGCCAACTCGACCAACTC |
| SSBR0012 | AGTTGAACTCACACGAAGG | ATCCAGACGAGGTTTCAGAAG |
| SSBR0018 | CATCTGCGGACAACTTGAAC | CCTCGTCTTCGCCTGTATATC |
| SSBR0030 | GTGCGTCTTGGTCTCATCTC | CCTCATCAATGTTGTTCTGGAAG |
| 18S rRNA | GAGTCTTGGTGATTCATAGTAAC | CCTGCTGCCTTCCTTAGATG |

**Table 2** Homologous proteins of the differentially expressed genes in the precocious line and its parent strain

| ID | Homologous protein, organism (accession no.) | No. of clones | *E* value | Ratio of Cy5/Cy3 | | Predicted function |
|---|---|---|---|---|---|---|
| | | | | Forward | Reverse | |
| SSBR0030 | rhomboid-like protein, *Eimeria tenella* (gb\|ABC50099.1\|) | 23 | 3e−62 | 2.67 | 0.23 | Host cell invasion |
| SSBR0018 | Serpin, *Eimeria acervulina*(gb\|ACA51541.1\|) | 16 | 3e−70 | 0.40 | 2.42 | Cell invasion |
| SSBR0009 | Conserved hypothetical protein, *Toxoplasma gondii* (gb\|EEE19177.1\|) | 38 | 6e−53 | 0.11 | 13.57 | Unknown |
| SSBR0002 | cation-transporting ATPase, *Eimeria acervulina*(gb\|ACB97673.1\|) | 10 | 1e−09 | 0.19 | 5.46 | Drug-resistance development |
| SSBR0027 | Heat-shock 70 protein, *Eimeria maxima* (emb\|CAA87085.1\|) | 57 | 2e−21 | 0.37 | 4.06 | Stage conversion, infectivity, and virulence |
| SSBR0024 | transhydrogenase, *Eimeria tenella* (gb\|AAA29077.1\|) | 4 | 3e−06 | 0.32 | 5.05 | Sporulation |

relative mRNA abundance by real-time PCR. They comprised two upregulated genes in the precocious line (EST numbers SSBR0012 and SSBR0030, whose average Cy5/Cy3 ratios were 4.73 and 2.67, respectively) and three downregulated genes (ESTs numbers: SSBR0008, SSBR0010, and SSBR0018, whose average Cy5/Cy3 ratios were 0.48, 0.11, and 0.40, respectively). As shown in Fig. 1, the results obtained by real-time PCR were consistent with the cDNA microarray-based observation, confirming the accuracy of our data.

## Discussion

*Eimeria* parasites present a variety of phenotypes, which include drug resistance, strain-specific immunity, altered growth rate, and isoenzymes. Previous studies have shown that a biological phenotype is controlled by multiple factors, involving a large number of regulatory genes, while a single-nucleotide mutation can cause various changes in a biological phenotype (Chen et al. 2008). The identification of *Eimeria* genes controlling phenotypes is of

**Table 3** Homologous ESTs of the differentially expressed genes in precocious line and its parent strain

| ID | Homologous *Eimeria* ESTs | No. of clones | *E* value | Ratio of Cy5/Cy3 | |
|---|---|---|---|---|---|
| | | | | Forward | Reverse |
| SSBR0018 | *Eimeria maxima* merozoite cDNA library similar to SERPIN, mRNA sequence (gb\|GO307005.1\|) | 16 | 0.0 | 0.40 | 2.42 |
| SSBR0029 | *Eimeria tenella* Houghton unsporulated oocyst cDNA clone EThOO-04e12.q1k, mRNA sequence (emb\|AM264758.1) | 5 | 2e−90 | 5.31 | 0.37 |
| SSBR0012 | *Eimeria tenella* sporulated oocysts subtracted cDNA clone BW11-F05, mRNA sequence (gb\|ES346904.1\|) | 28 | 3e−173 | 4.73 | 0.24 |
| SSBR0028 | *Eimeria maxima* merozoite cDNA, mRNA sequence (GO304900.1) | 9 | 2e−17 | 2.53 | 0.38 |
| SSBR0004 | *Eimeria maxima* merozoite cDNA, mRNA sequence (gb\|GO305639.1\|) | 8 | 2e−20 | 0.21 | 5.01 |
| SSBR0011 | *Eimeria maxima* merozoite cDNA, mRNA sequence (gb\|GO307307.1\|) | 29 | 3e−132 | 0.40 | 2.09 |
| SSBR0021 | *Eimeria maxima* merozoite cDNA, mRNA sequence (gb\|GO306463.1\|) | 4 | 5e−99 | 0.28 | 4.78 |
| SSBR0016 | *Eimeria maxima* merozoite l cDNA, mRNA sequence (gb\|GO305973.1\|) | 12 | 6e−88 | 0.18 | 6.09 |
| SSBR0027 | *Eimeria maxima* hsp70 gene for heat-shock protein, mRNA sequence (emb\|Z46964.1\|) | 57 | 2e−118 | 0.37 | 4.06 |
| SSBR0031 | *Eimeria acervulina* strain PAPa46 serine rich protein, mRNA sequence (gb\|AF073462.1\|) | 2 | 2.5e−06 | 0.47 | 3.75 |
| SSBR0022 | *Eimeria tenella* transhydrogenase, mRNA sequence (gb\|L08393.1\|) | 7 | 3.8e−09 | 3.17 | 0.42 |
| SSBR0010 | *Eimeria tenella* antigen LPMC61,mRNA sequence (gb\|M30933.1) | 1 | 1.5e−14 | 0.11 | 3.16 |
| SSBR0006 | *Eimeria tenella* mRNA for sporozoite antigen, mRNA sequence (emb\|X15898.1\|) | 3 | 3.3e−11 | 2.8 | 0.38 |

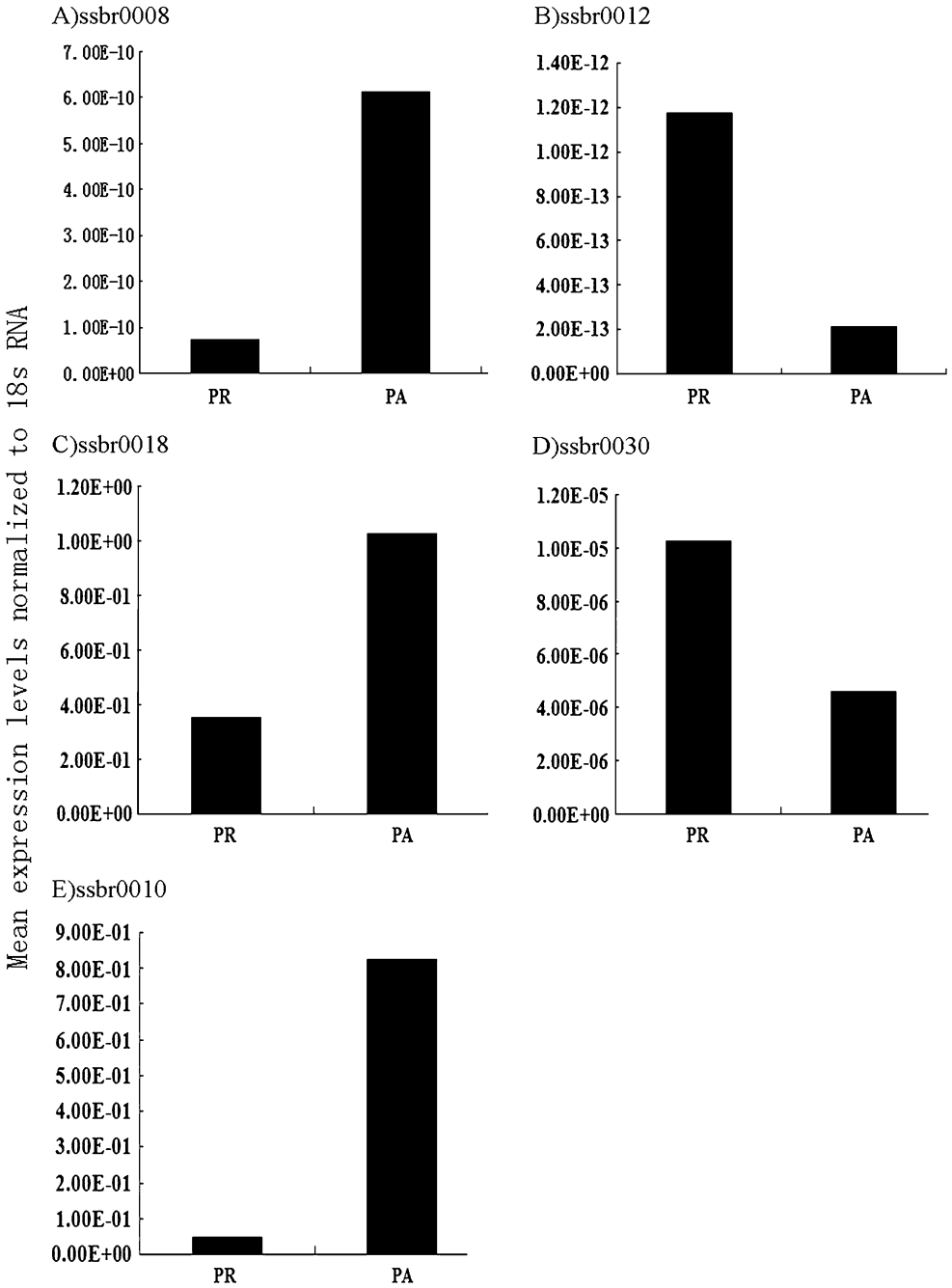


**Fig. 1** Quantitative, real-time PCR analysis of genes differentially expressed in sporulated oocysts between the precocious line of *E. maxima* and its parent strain. *PR* precocious line; *PA* parent strain. SSBR0008 (**A**), SSBR0012 (**B**), and SSBR0010 (**E**) genes encoding unknown proteins. SSBR0018 (**C**) gene encoding protein homologous to SERPIN protein of *E. acervulina*. SSBR0030 (**D**) gene encoding protein homologous to rhomboid-like protein of *E. tenella*

immense importance in the fight against coccidiosis. A precocious line of *Eimeria* was easily selected by serial selection for the first parasites to complete the transition from asexual to sexual reproduction, and it has an abbreviated, but intact, life cycle (Jeffers 1975). The process appears to occur stepwise, and different precocious lines display the same unique biology; however, almost nothing is known about the genes responsible for the precocious trait. In this study, we provided a global perspective on individual and groups of possible genes that might be related to the mechanisms of the precocious trait of *E. maxima*.

The approach of combining SSH and cDNA microarrays for the rapid identification of differentially expressed genes was first described by Yang et al. (1999) and has proved to be very efficient. It has been widely used to study stage- or strain-specific gene expression in parasites (Cottee et al. 2006; Datu et al. 2008). Han et al. (2010) first applied this technology to screen for differentially expressed genes in unsporulated oocysts, sporulated oocysts, and sporozoites of *E. tenella* to identify novel and important genes involved in the development and invasion of this parasite. The results demonstrated that SSH and microarrays can be used together for studying gene expression profiles in the

different developmental stages of *Eimeria*. In the present study, we used this approach to identify the differentially expressed genes of sporulated oocysts between the precocious line and its parent strain of *E. maxima*. More than 3,000 clones from two subtracted libraries were used to construct cDNA microarrays, ultimately yielding 360 differentially expressed transcripts that were isolated and identified between the precocious line and its parent strain of *E. maxima*; however, after eliminating redundant sequences, they only represented 32 unigenes. Some differentially expressed genes were further confirmed by real-time PCR analysis. The results demonstrated that SSH, combined with cDNA microarray analysis, is a suitable method for enriching differentially expressed genes of differential strains of *Eimeria*.

By comparing the ESTs presented in this study with previously isolated *Eimeria* ESTs, we found that the majority of the non-redundant sequences shared no similarity with entries in dbEST. This is not surprising, as *E. tenella* is the most studied member of the species causing poultry coccidiosis and most of the *Eimeria* ESTs deposited in the GenBank database are from *E. tenella* (Schwarz et al. 2010). A previous study had shown that *E. maxima* has some unique features that distinguish it from the other members of the genus, including "unusually large" oocysts, marked immunogenicity, and high genetic variation (Schnitzler and Shirley 1999). The results of the present study indicated that potential molecular differences exist between *E. maxima* and *E. tenella*. It is likely that many more homologs will be identified when the *E. tenella* genome assembly is complete and annotated.

Of the 32 genes, 6 genes (one upregulated and five downregulated in the precocious line of *E. maxima*) shared significant identity with previously described or hypothetical proteins. The upregulated gene encoded a rhomboid-like protein. Rhomboids are a recently discovered family of widely distributed intramembrane serine proteases. They are present in the sequenced genomes of all apicomplexan parasites, including *T. gondii*, *Plasmodium spp.*, *E. tenella*, *Cryptosporidium spp*., and *Theileria spp*. Apicomplexan rhomboids have a potential role in microneme protein cleavage during host cell invasion (Dowse et al. 2005). Recent studies have indicated that a spatially localized *T. gondii* rhomboid protease (TgROM5) cleaves cell surface adhesins that are essential for invasion by *Toxoplasma* (Brossier et al. 2005), thus linking the rhomboid protein with the invasion process. The enhanced expression of this gene in the precocious line might aid the sporozoites or merozoites to invade the host cell and might result in faster schizogonic development of the precocious line than its parent strain.

Another homologous protein that is related to host cell invasion is a serine protease inhibitor (SERPIN), which are essential to many physiological processes by regulating proteolytic cascades (Fetterer et al. 2008). In Apicomplexa, SERPINs can prevent invasion of *T. gondii* tachyzoites into host cells both in vitro and in vivo [Kim 2004] and prevent invasion of *E. tenella* sporozoites (Fuller and McDougald 1990) and *Sarcocystis neurona* merozoites in vitro (Barr and Warner 2003). The low level expression of the SERPIN gene in the precocious line would significantly reduce the inhibition of serine proteases and lead to the accumulation of these proteases, which would help the sporozoites or merozoites to invade the host cell. This presumption was supported by the result that a higher expression of a serine protease, the rhomboid protein, was detected in the precocious line compared with the parent strain.

The most abundant transcript encoded by *E. maxima*, Heat shock protein (Hsp70), was found to be down-regulated in the precocious line. This result was in accordance with previous studies. Heat shock proteins maintain homeostasis inside the cell and are usually constitutively expressed. However, their expression increases under stress, such as heat or nutrient deprivation (Lindquist and Craig 1988). Previous studies have shown that Hsp70 plays important roles in stage conversion, infectivity, and virulence in several protozoan parasites (Coulson and Smith 1990; Lyons and Johnson 1995). Del Cacho et al. (2005) investigated Hsp70 expression levels in the sporozoites of a wild-type parent strain and in two precocious lines of *E. tenella* and found a significant gradual decrease in Hsp70 expression in sporozoites of *E. tenella* as attenuation progressed. This suggested that the Hsp70 expressed in the excysted sporozoites of *E. tenella* might be involved in parasite pathogenicity. The low levels of Hsp70 in precocious lines might correspond to a less pathogenicity found in all precocious lines.

The other downregulated proteins were highly similar to a cation-transporting ATPase of *E. acervulina*, a trans-hydrogenase of *E. tenella*, and a conserved hypothetical protein of *T. gondii*. The transhydrogenase can transduce the proton motive force generated by respiration into the chemistry required to maintain a reducing environment. A transhydrogenase was found to be located in the *Eimeria* refractile body and might play an important role in the sporulation of oocysts (Vermeulen et al. 1993). ATPases can harness the energy from a proton gradient, using the flux of ions across the membrane via the ATPase proton channel to drive the synthesis of ATP. Wang et al. (2006) indicated that after a sensitive strain of *E. tenella* had been exposed to monensin for many passages, the biochemistry and physiology of sporozoite membranes presented certain adaptive changes. These included reduction in the $Na^+$–$K^+$–ATPase activity, cations influx, and membrane fluidity, to confront the effect of monensin, which suggested that $Na^+$–$K^+$–ATPase activity was related to the resistance mechanism

for ionophore compounds. Further research is required to study these genes' roles in the development mechanism of precocious line.

In the present paper, a large number of differentially expressed genes were found to encode proteins of unknown function, indicating the complicated mechanism of the precocious trait. With the initialization and progress of *E. tenella* whole genome sequencing and functional genomics projects (Shirley et al. 2004), more and more information regarding the *Eimeria* genome and its expression will be collected. Further characterization of these genes and their products will provide information useful in elucidating the genes responsible for precocious phenotypes in *Eimeria*.

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