INTRODUCTION

Transformation is developed to break the boundary of species, widen the germplasm resources, and shorten the breeding courses. In this process, constructing a good acceptor system is a base factor especially for maize. Embryonic callus is a good acceptor and the regeneration efficiency is a key point [1, 2].

After a long-term research in our laboratory, 18599 Hong was screened to be a good acceptor with high efficiency of embryonic callus inducing and regenerating [3, 4]. Significantly, it is still an excellent inbred line, complementary to the shortages in agricultural traits of previous materials such as B73 and A188. Classical genetic analysis of its embryonic callus inductivity and regeneration have been completed [5, 6]. The QTLs have been located but have not been cloned and utilized [7]. Thus, isolation and characterization of genes specifically expressed from embryonic callus regenerating process will help to understand the molecular mechanisms of this process. It is possible to transform the differential expressing genes into other materials with good agricultural traits. Then the scope of acceptor system will be expanded and transgenetic will be advanced.

Suppression subtractive hybridization (SSH) is an efficient method widely used in the study of differential gene expression [8], associating with efficient screening methods such as Northern hybridization, reverse Northern hybridization, microarray, RT–PCR, et al. It has been successfully used to isolate genes in some species such as rice [9], carnation [10], legume Sesbania rostrata [11], barley [12, 13], wheat [14], Pyrenophora teres [15], maize [16–18], et al.

As for maize embryonic callus regenerating process, no genes were identified and isolated till now. In this study, we combined SSH with reverse Northern hybridization to isolate genes expressed during regenerating from embryonic callus. Sequences were submitted to NCBI blast and gene functions were analyzed. The results will provide much genetic information for the embryonic callus regeneration process and advance the engineering development.

MATERIALS AND METHODS

Plant material and culture conditions. Maize inbred line, 18599 Hong, was obtained from Sichuan Agricultural University of China. After 15 days from pollination, fringes were disinfected and seed coats were cut off. The immature embryos were picked out and put onto the inducing medium [3], N6 supplemented with 2 mg l\(^{-1}\) of 2,4-D, 120 mg l\(^{-1}\) of inositol, 500 mg l\(^{-1}\) of hydrolyzed casein, 1.38 g l\(^{-1}\) of proline, 30 g l\(^{-1}\) of sucrose, 5 g l\(^{-1}\) of agar, pH 5.8 at 25°C in darkness, which corresponds to most materials. After 60 days subculture, the same medium was replaced every 20 days, the embryonic calli were transferred into regeneration medium [3], N6 supplemented with 120 mg l\(^{-1}\) of inositol, 1 mg l\(^{-1}\) of kinetin, 100 mg l\(^{-1}\) of hydrolyzed casein, 30 g l\(^{-1}\) of sucrose, 5 g l\(^{-1}\) of agar, pH 5.8 at 25°C in 12 h light/12 h darkness.

After 24 h of culture in regeneration medium, calli could be observed to be green. The green calli, which grew in the regeneration medium for 24, 48, and 72 h, respectively, were mixed as tester. The corresponding
Sequence analysis of ZmECRG cDNA clones

<table>
<thead>
<tr>
<th>ZmECRG cDNA clones</th>
<th>BLASTX results</th>
</tr>
</thead>
<tbody>
<tr>
<td>name</td>
<td>accession, no.</td>
</tr>
<tr>
<td>ZmECRG27</td>
<td>DY843521</td>
</tr>
</tbody>
</table>

Stage calli which not be regenerated, still in the inducing medium for 60 days, were taken as driver.

RNA isolation. Total RNA was extracted from tester and driver calli using TRIzol reagent (Invitrogen, United States), mRNA were separated from total RNA using the PolyATract mRNA Isolation Systems III (Promega, United States).

Suppression subtracting hybridization (SSH) and the subtractive cDNA library construction. According to the manufacturer's instructions, Clontech PCR-Select cDNA subtraction kit (BD Biosciences, United States), 2 µg nRNA of tester and driver were respectively reverse-transcribed to double-cDNAs and digested with RsaI. Adaptors were ligated to tester cDNA. Two rounds of hybridization and PCR amplification were processed to normalize and enrich the differentially expressed cDNAs. Products of the secondary PCR from the subtraction were directly inserted into pGEM-T Vector (Promega) and transformed into Escherichia coli DH5α cells. Positive colonies were picked out to establish the subtracted cDNA library and plasmids were extracted using a Wizard Plus SV Miniprep DNA purification system (Promega).

Reverse Northern and screening. Individual recombinant clones were amplified using nested PCR primer 1 and 2R provided in the PCR-selected cDNA subtraction kit. Thermo-cycling conditions were as follows: an initial denaturation at 94°C for 5 min, 28 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 1.5 min, following a final extension at 72°C for 5 min. The PCR products were denatured in alkaline and blotted onto Hybond XLTM positively charged nylon membranes for two copies (Amersham Pharmacia Biotech, United Kingdom). According to manufacturer's instructions, DIG-High Prime DNA labeling and detection starter kit I (Roche), cDNA from driver and tester were digoxigenin (DIG)-labeled as probes and the labeling efficiency were detected to determine the dosage. Two identical filters were then hybridized with driver and tester labeled probes respectively for 16 h at 65°C.

Labeled blots on the membranes were detected using CSPD. The Reverse Hybridization experiments were repeated three times.

Sequence analysis. Plasmid DNA from selected SSH clones was purified with Qiagen Plasmid Midi kit and sent to Shanghai Sangon Biological Technology and Service Co. in China to sequence with M13 primers. Nucleotide and protein homologies searches were performed using BlastN and BlastX (http://www.ncbi.nlm.nih.gov/blast/bl2seq.cgi).

RESULTS

Subtractive Library Construction and Screening

After 24, 48, and 72 h of culture, RNAs from both un-regeneration culture as driver and regeneration cultures as tester were extracted, reverse transcribed and subjected to the SSH process. Three hundred clones were picked from SSH PCR fragments library and stored in a −80 freezer. Differential screening was performed by reverse Northern hybridization. Seventy clones PCR products were selected to dot-blott onto Hybond XLTM positively charged nylon membranes and hybridize separately with tester and driver DIG-labeled probes. Forty clones, which expressed in regeneration culture while weakly or not expressed in un-regeneration culture, were selected and designated as Zea mays Embryonic Callus Regeneration Genes (ZmECRG).

Analysis of the Genes Sequences

One sequence, ZmECRG27, was submitted to the NCBI database and homologous proteins hits were indicated in table. The nucleotide length of 403 bp encoding a 351 amino acid peptide, shares 80% identity with elongation factor 1 gamma-like protein of Oryza sativa.

DISCUSSION

Elongation factor 1 (EF-1) [19] is a multisubunit nucleotide exchange complex regulating the specific interaction of aminoacyl-tRNA with the ribosome during the translational elongation phase of protein biosynthesis. It is composed of four subunits, α, β, δ, and γ. The α chain binds GTP and aminoacyl-tRNAs. The β and δ chains are highly similar proteins that both stimulate the exchange of GDP bound to the α chain for GTP [20], in which C-terminus seem to be important for the nucleotide exchange activity and N-terminus is probably involved in the interaction with the γ chain. The γ chain was detected in eukaryotes but not in prokaryotes with 410 to 440 residues. It probably plays a role in anchoring the complex to other cellular components. But the