Identification of actin-related gene family from *G. barbadense* Giza 88 cultivar using PCR-based positional cloning

(Received: 08.10.2006; Accepted: 18.10.2006)

Osama A. Momtaz*; Ahmed El-Fatih A. El-Doliefy *; Ahmed Bahieldin*,; El-Sayed H. Hassanein****
* Gene Expression Regulation Technology (GERT) Laboratory, Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Ministry of Agriculture, Egypt
** Department of Genetics, Faculty of Agriculture, Ain Shams University, Egypt

ABSTRACT

Plant actins contribute strongly in cell cytoskeleton, microtubule filaments regulation and cellulose deposition orientation during cotton fiber cell development, which directly affects fiber quality. Identification of actin-related gene family from the Egyptian cotton is considered a corner stone in future engineering of fiber cell traits. P1-derived artificial chromosome (PAC) library has been constructed for the Egyptian extra long stable variety Giza88. The Giza88-PAC library comprised 8900 PAC clones with 70 Kb average size; representing 0.3 equivalents to the haploid genome (2118 Mb) of Gossypium barbadense. Randomly selected PAC clones were subjected to actin PCR-based screening using GhACT2 degenerate primers, which resulted in 14 actin positive clones. MPAC94 as one of these positives was purified and subjected to physical mapping and PCR-based positional cloning. The results indicated the recovery of a positive MPAC94/EcoRI actin fragment (16.26 Kb), which was confirmed by RT-PCR and sequence alignment at upland cotton database. This study is the first from its kind to identify one gene fragment of the actin-related gene family in Egyptian cotton Giza88 using PCR-based positional cloning technology.

Key words: *Actin gene family, G. barbadense, PCR positional cloning. restriction fingerprinting, PAC library.*

INTRODUCTION

Cotton fiber is a unicellular epidermal trichome distinct from *Arabidopsis* trichome in which they are derived from the epidermal cells of the reproductive organ (ovule). In addition, its development is a highly regulated with four sequential stages: fiber initiation, primary cell wall formation, secondary cell wall formation, and maturation. Approximately, 30% of cotton ovule epidermal cells develop into fibers (Qin *et al.*, 2005).

Composition of cytoskeleton is a dynamic structure of three fibrous elements, e.g., the microtubules, actin filaments, and intermediate filaments that is involved in many key processes including cell division, organelle movement, and formation of cell wall (Anthony and Hussey, 1999). Plant cytoskeletal genes are generally composed of extended families; its members displayed high sequence homology and partially overlapping expression patterns (Kost *et al.*, 2002). The effective regulation of actin conversion by actin binding proteins such as profilins, actin depolarizing factors such as cofillins, and Rho family GTPase (Clarke *et al.*, 1998; Hussey *et al.*, 2002; Fu *et al.*, 2002) may be critical for pollen tube growth, root hairs, and expansion

of trichome cells (Chen *et al.*, 2003). Genetic studies showed that actin cytoskeleton by interacting with ARP2/ARP3 protein complex, plays a pivotal role in controlling fiber cell shape and several other cell types (Mathur *et al.*, 2003).

In *Arabidopsis thaliana*, disruption of the actin cytoskeleton during trichome development by any of actin interacting drugs resulted in randomly distorted trichomes with un-extended branches (Mathur *et al.*, 1999). Mutations in actin 2 (*ACT2*) and actin 7 (*ACT7*) genes, which are responsible for the development of actin arrays, resulted in dramatic reduction of root hair length (Gilliland *et al.*, 2003).

In cotton fiber, genes (such as *H6*) were identified as fiber-specific transcript (Orford and Timmis, 1998). Others such as *TubB* were identified as fiber-enriched transcripts (Ji *et al.*, 2002). While the predominant forms of actin genes that found functionally expressed in fiber cells are *GhACT1*, *GhACT2*, *GhACT4*, *GhACT5*, and *GhACT11*. Moreover, silencing of the *GhACT1* gene in *G. hirsutum* showed short fibers, sterile ovules, and small bolls, which illustrate the role of *GhACT1* gene in fiber maturation and production. In actin *GhACT1*-knocked out cotton plants, fiber elongation rate was slower by 3 folds, reflecting the role of actin on pollination, seed development, fiber quality and productivity (Li *et al.*, 2005)

Physical mapping and map-based cloning of agronomical important genes combined with sequencing of selected genomic regions require the development of genomic libraries (Martinez and Amemiya, 2002). However, there has been limited progress in the construction of bacterial artificial chromosome (BAC) libraries for species with large genomes like cotton. Recently, genomic libraries were constructed for cotton especially for the Egyptian varieties that need more extensive genomic studies for its great international impact on textile industries (Momtaz *et al.*, 2006).

The vector pPACe4, as one of P1-derived artificial chromosome (PAC), has most of the features found in BAC vectors. Nevertheless, PAC system offers advantages over other large-insert cloning systems (Peterson *et al.*, 2000). It has kanamycin (Kan^R) resistance gene rather than chloramphinicol (Cm^R) in BACs, and a "pUC19-link", containing a high copy number origin of replication, which is used for convenient vector propagation and later removed during vector preparation (Coren and Sternberg, 2001). Moreover, the vector contains *SacB*II gene (encodes sucrose synthase) providing a positive selection for recombinant clones (Momtaz *et al.*, 2006). In addition, *BamH*I cloning site is within *SacB*II gene and thus disruption of *SacB*II gene by insertion of DNA fragment allows for growth of the bacterial cell on media containing sucrose (Ioannou *et al.*, 1994).

Apart from the recent review that proved the functional expression of actin gene (*ACT1*) during cotton fiber elongation (Li *et al.*, 2005), no studies on identification of actin(s) cotton fiber-related gene family (ies) were available in the literature. The lack of actin studies in cotton triggered us to put the main objective of this study as to identify actin-related gene families in the Egyptian cotton. The plan of this work was to construct a genomic library for the Egyptian extra long stable variety (Giza88) by using pPACe4 vector. PCR-based screening was applied to the PAC library using *GhACT2* primers. MPAC94, an identified positive PAC clone, was subjected to restriction fingerprinting and the resulted fragments were then subjected to PCR-based positional cloning using the same primers. While RT-PCR was performed to confirm the actin gene expression.

MATERIALS AND METHODS

Plant material

The seeds of the Egyptian extra long stable variety (Giza88) *Gossypium barbadense* L. were de-linted and planted in greenhouse as described (Momtaz *et al.*, 2006).

Nucleic acid isolation

Genomic DNA was isolated from young leaves of Giza88 using Qiagen DNeasy™ Plant Mini kit (Cat. No. 69104) following the manufacturer manual. Total RNA was isolated from Giza88 young leaves using SV total RNA Promega kit (Cat. No. Z3100) following the manufacturer manual. Nucleic acids samples were stored at -80°C until use.

PAC vector preparation

The pPACe4 (19.5 Kb) vector used in the study was developed by Frengen *et al.* (2000), and delivered from The Children's Hospital Oakland Research Institute (CHORI) (http://bacpac.chori.org/ppac4.htm); a single colony was inoculated into 5 ml of LB medium containing kanamycin (25 µg/ml), and grown overnight at 37°C. One ml of the overnight culture was then inoculated into 5 separate flasks, each containing 1 L of LB medium plus kanamycin and grown overnight. The vector was isolated using Plasmid Qiagen Max kit (Cat. No. 12163) following the manufacturer manual. The vector was digested using *BamH*I (Amersham Pharmacia, USA) at 30°C overnight. Complete digestion was verified by fractionation on 1% TAE agarose gel. Dephosphorylation was performed using calf intestinal alkaline phosphatase (CIAP) enzyme (Biolabs, USA) as modified by Momtaz *et al.* (2006). The large dephosphorelated fragment vector (16.7 Kb) was fractionated on 1% TAE agarose gel, and extracted from the gel using QIAEX® II Gel Extraction kit (Cat. No. 20051). Vector DNA was stored in 5 µl aliquots at -80°C.

Preparation and partial digestion of mega-base DNA

Mega-base DNA was isolated from the nuclei of Giza88 according to Zhang *et al.* (2000). Nuclei were embedded in low melting point (LMP) agarose plugs. Mega-base DNA analyzed on pulsed-field gel electrophoresis (PFGE) using Bio-Rad clamped-contour homogenous electrophoresis field (CHEF) DR® III System as described by Peterson *et al.* (2000). Prior to digestion, nuclei agarose plugs were equilibrated on ice according to Zhang *et al.* (2000). Seven separate digestion reactions were then performed independently using 7 BamHI enzyme concentrations (0, 0.1, 0.4, 0.7, 1.0, 1.3, and 3 units/250 µl reaction), each containing half plug of 450 ng. The optimum partial digestion was 1.3 units of BamHI at 30°C for 30 min. Large-scale partial digestion for 25 plugs were pooled in one large agarose lane and subjected to (PFGE) as described by Peterson *et al.* (2000). The agarose lanes containing the PFGE markers and a small portion of the lane containing the partially-digested DNA were excised from the gel and stained away from the unstained portion in a new container with ethidium bromide. Using the transilluminator, the desired size fraction (50-150 Kb) was marked. The unstained agarose portion containing the partially-digested DNA fragments (50-150 Kb) were remarked, excised, and then eluted in two size fractions (50-100, and 100-150 Kb). The selected sizes were washed several times in TE plus 30 mM NaCl (TEN) buffer and stored as LMP agarose blocks in 70% ethanol at -20°C.

Ligation of HMW DNA fragments into the pPACe4 vector

The LMP agarose block containing the selected fragments (50-100 Kb) was rinsed

twice in TE for 30 min on ice, melted at 65°C for 10 min, transferred to 45°C water bath, and digested with β-agarase enzyme (Biolabs) as specified in the manufacturer manual. When the gel completely digested, DNA solution was spot-dialyzed on VSWP filter membrane 0.025 µM (Millipore, USA) as described by Peterson *et al.* (2000). Two µl (20 ng) dephosphorylated vector (16.7 Kb) and 2 µl (80-100 ng) size selected insert DNA were gently mixed and incubated at 50°C for 1 min. Ligation reaction was performed using 200 units of T4 DNA ligase enzyme in 15 µl total volume in a ratio of 1:5 (vector: insert) for 3 h at room temperature, then overnight at 16°C waterbath.

Transformation and analysis of the recombinant PAC library

Recombinant plasmids were transformed into electro-competent *H101B* cells as the bacterial host for the Giza88 PAC library by electroporation using Bio-Rad Gene Pulser™. Vector was mixed gently with the competent cells in ratio of 1:15-20 (v/v). Electroporation was performed using 1.8 kV pulse, 125 µFD and 100 ohm, using 0.1 pulser cuvette. Recombinant colonies were grown on LB/sucrose agar plates plus kanamycin, picked manually, stored at – 80°C as glycerol stocks, and labeled (APAC1- APAC99, BPAC1-BPAC99.... ZPAC99). About 200 of the recombinant Giza88 PAC clones were isolated by the alkaline lyses method as modified for PACs by David *et al.* (2004). Characterization of undigested recombinant PACs was performed against the dephosphorylated non-recombinant pPACe4 vector as described by Momtaz *et al.* (2006).

PCR-based screening of Giza88 PAC clones

About 500 of recombinant PAC clones were used as templates for PCR-based screening. The *GhACT2* forward primer (5'-TGC CCG GAA GTC CTC TTC CAG-3') and the *GhACT2* reverse primer (5'-ATT TTC CCA GA AGT TTG ACC GCG C-3') degenerate for actin gene family. PCR was performed in 50 µl reaction mixture containing 1 µl of template DNA (50 ng/µl), 2 µl from each of *GhACT2* degenerate primers (10 pmol), 5 µl Taq DNA polymerase buffer (10X), 5 µl $MgCl_2$ (25 mM), 5 µl of dNTPs (2 mM), Taq DNA polymerase (0.5 unit) and H_2O up to 50 µl. PCR conditions included 3 min for first DNA denaturation step at 94°C followed by 35 cycles at 94°C for 30 sec, annealing step at 61°C for 30 sec and extension step at 72°C for 90 sec and the final extension cycle at 72°C for 10 min. Giza88 genomic DNA was used as a positive control to insure results of annealed primers. PCR-based screening reactions were fractionated on 4% TAE agarose gel. Fourteen actin positive PAC clones were identified and stored at -20°C as glycerol stock cells and isolated plasmid DNA.

MPAC94 restriction fingerprinting and PCR-based positional cloning

MPAC94 (one of the identified positive PAC clones) attained fingerprinting using the agarose gel-based restriction fingerprinting method as described by Xu *et al.* (2004). Three restriction enzymes *Hind*III, *Eco*RI, and *Bam*HI (Amersham Pharmacia, USA) were used for digestion. The pPACe4 DNA was digested with the same 3 enzymes as a negative control. Positional cloning started with fractionation of the generated restricted fragments of MPAC94 against the digested pPACe4 negative control on 2% TAE agarose. Restriction fragments, which were found in digestion pattern of MPAC94 and absent in the pPACe4 negative control, were selected. The selected restriction fragments of MPAC94 were labeled, eluted, and purified for PCR-based positional cloning. Elution of the selected fragments was performed using the

QIAEX® II Gel Extraction kit (Cat. No. 20051). PCR-based positional cloning was performed as previously described in PCR-based PAC screening and fractionated on 2% TAE agarose gel.

RT-PCR analysis

Total RNAs (2 µg/reaction) were pre-warmed at 65°C for 10 min and used as template for RT-PCR. The first RT-PCR step was performed using 4 µl of total RNAs (500 ng/µl), 2 µl of both forward and reverse *GhACT2* primers (10 pmol), RNase inhibitor (4 units), 1 µl of dNTPs (10 mM), and H_20 up to 20 µl. The mixture was heated at 65°C for 5 min, then followed by fast chilling on ice for 5 min. The mixture was briefly collected by gentle spin, then returned on ice before adding 2 µl of *M-MLV* transcriptase buffer (10X). RT-PCR reaction was placed at 42°C for 2 min before adding 1 µl of *M-MLV* reverse transcriptase enzyme (200 units), and then incubated at 42°C for 50 min, and inactivated at 75°C for 15 min The second RT-PCR reaction was developed using the actin *GhACT2* primers as described in PCR-based PAC screening but with one exception in which the annealing temperature was performed at 58°C for 30 sec.

RESULTS AND DISCUSSION

HMW DNA preparation from Giza 88 and partial digestion

High yield and quality of HMW DNA is a prerequisite for PAC library construction. Plant rigid cell wall, compared to mammalian and yeast cell membranes, is a constraint in the preparation of HMW DNA from plants (Zhang *et al.*, 2000). HMW DNA is commonly isolated from plants by protoplast and nuclei methods. Nevertheless, protoplast is less preferable because of the high contamination of mitochondria and chloroplast in the nuclei pellet (Peterson *et al.*, 2000). Two different protocols were usually used for nuclei isolation; the sucrose-based (Zhang *et al.*, 2000), and 2-methyle-2,4-pentanediol (MPD) (Peterson *et al.*, 2000). In this study, isolation was performed using sucrose-based method, which provides simple and amenable use of small quantities (10-15 g) of fresh or frozen tissue in contrast to that used by MPD. In addition, liquid nitrogen was used to remove the plant cell wall, TritonX-100 to remove mitochondrial and chloroplast membranes, high pH buffer to inhibit nucleases, β-mercaptoethanol and PVP to counteract with the oxidized polyphenols; finally, nuclei were pelleted using low speed centrifugations (Zhang *et al.*, 2000). This method was applied for the construction of DNA libraries from numerous species including rice, sorghum, wheat, sugarcane, cotton, soybean, barely, and arabidopsis (Peterson *et al.*, 2000). The optimum enzyme concentration for partial digestion was 1.3 units, which makes DNA within the plugs accessible and amenable to restriction enzymes digestion. Partial digestion was scaled up using 64 units of *BamH*I enzyme for 25 nuclei plugs (data not shown), with produced fragments up to 200 Kb by fractionation on PFGE. DNA digested fragments (50-150 Kb) were purified from the gel as first DNA size selection.

Construction of Giza 88 PAC library

The single copy nature of the pPACe4 vector (Fig.1 A); made its purification laborious. Therefore, the isolation started with large scale 5 liters of pPACe4 culture as recommended by Pierce *et al.* (1992) using Plasmid Qiagen Max kit. Vector concentration and restriction preparation were performed by pPACe4/*BamH*I restriction profiling, fractionation on 1% TAE agarose, and elimination of pUC19-link fragment. Self ligation was tested by T4 DNA ligase

treatment to the digested dephosphorylated pPACe4 vector, and fractionated on agarose gel revealed remaining of the linear fragment (16.7 Kb) of pPACe4 to be used in PAC cloning. The dephosphorylated fragment was eluted from the gel using the QIAEX® II Gel Extraction kit and stored in 5 µl aliquots at -80°C. Electroporation was used to introduce the ligated large DNA molecules into the *H101B* competent cells. Transformants were selected on LB agar medium plates containing kanamycin and 5% sucrose. Only clones with inserts within the vector can survive, due to positive selection by the *SacB*II gene in the vector (Frengen *et al.*, 2000).

Because of the relatively large size of the cloning vector (16.7 Kb), the cloning efficiency of the PAC vector was generally lower than most of the BACs (7 Kb) as proved by Shizuya *et al.* (1992). Protocol within this paper, is producing a high cloning efficiency for PACs with about 3500 clones per single electroporation experiment. The most commonly used *E. coli* strains in BAC and PAC libraries is the *DH10ß* (Peterson *et al.*, 2000). This study provides a trial of a new *E. coli* strain (*H101B*) for PAC library construction. Since the bacterial strain affecting the net average size of BAC libraries, transformation using electroporation technology made the electro-competent *E. coli* strain cells able to accept fragments up to 100 Kb (Yu-Ling *et al.*, 1995). This may be due to insert DNA fragments that exceeded the cloning capacity of the *H101B* strain cells which agrees with the results of Sheng *et al.* (1995). More than 200 clones were randomly selected for size fractionation using PFGE, 13 of them were presented (Fig. 1 B). Size fractionation indicated about 10% of recombinants lower than 50 kb in size, 5% were higher than 100 kb, and 85% were the best represented in size 50-70 Kb. When second DNA size selection was used in constructing BAC library, about 10 folds reduction in transformation efficiency were detected as presented by Cai *et al.* (1995). Therefore, first size selection was directly used in Giza88 PAC library to maximize cloning and transformation efficiency. The average size of the randomly selected undigested recombinant PAC clones was about 70 Kb as determined by PFGE (Fig. 1 B). The Giza88 PAC library provides 0.3 haploid genome equivalents with >88% probability of finding any specific sequence.

PCR-based screening for PAC library

The actin *GhACT2* degenerate primers were used to identify the actin positive PAC clones in the PCR-based screening technology. *GhACT2* are conservative primers that matched the *G. hirsutum* actin mRNAs in the NCBI gene bank and designed by Li *et al.* (2005). Hereby, these *GhACT2* degenerate primers were used to amplify and screen the actin conserved regions from *G. barbadense* and Giza88 PAC clones as shown (Fig. 2 A). Depending on the *GhACT2* degenerate primers, 500 of recombinant PACs were isolated and PCR-based screened. PCR screening produced 14 PAC clones comprising some actin conserved fragments (data not shown). After second PCR confirmation, Four clones representing the 14 actin positive PAC clones were comprising the conserved actin fragments (~200 bp, ~450 bp, 550 bp, and ~750 bp) as shown (Fig. 2 A). These data agreed with those of Li *et al.* (2005) using real-time PCR in *G. hirsutum*. These actin bands were not appeared in the pPACe4 vector when used as negative control. The data suggested that these bands refer to some different actin related regions found in the inserted DNA of these positive PAC clones and not related to any sequence of the pPACe4 vector.

MPAC94 restriction mapping and PCR-based positional cloning

MPAC94 was selected from the second PCR screening confirmation, and subjected to physical restriction mapping using simulated agarose gel-based three restriction enzyme fingerprinting method as described by Xu *et al.* (2004). In this method, three restriction enzymes, e.g., *Hind*III lane (H), *Eco*RI lane (E), *BamH*I lane (B) were used in separate digestion reactions and fractionated on 2% TAE agarose gel as shown (Fig. 2 B). The negative control (pPACe4 vector) was digested using *Hind*III, *Eco*RI and *BamH*I enzymes and represented in lanes ph, pe, and pb, respectively. The negative control was fractionated against the digested MPAC94 as shown (Fig. 2 B). Hereby, positional cloning based on PCR was applied to the fragments found in the digestion pattern of MPAC94 and absent in the pPACe4 negative control. The selected MPAC94 restriction fragments were; for *Hind*III (1-5), *Eco*RI (6-8), and *BamH*I (9-11) as shown (Fig. 2 B). Each band was eluted, purified, used individually in separate PCR reactions, and fractionated on 2% TAE agarose gel as shown (Fig. 3 A). Positional cloning was developed in a trial to reach the smallest restricted fragment comprising the actin conserved region. An actin (750 bp) band was developed in PCR when the selected fragment number (8) of lane E was used (Fig. 3 A). This fragment, that generated from MPAC94/*Eco*RI digestion labeled (8) in lane (E), was 16.26 Kb in size as determined by the Gel Doc 2000 System (Bio-Rad) (Fig. 2 B). The conserved actin (750 bp) band was found in the positive genomic control and MPAC94/*Eco*RI digestion in lane (8), while it was absent in the negative control of PAC vector (Fig. 3 A). It means that the insert within MPAC94, which was 70 Kb in size, had reduced to 16.26 Kb and still comprising the actin (750 bp) conserved region.

RT-PCR conformation analysis for actin related gene family

The *GhACT2* degenerate primers were also used in RT-PCR reaction. The actin bands generated from the second step RT-PCR were ~150 bp, ~250 bp, ~450 bp and ~750 bp as shown (lane 1, Fig. 3 B). The positive control Giza88 genomic DNA comprises the bands ~200 bp, 250 bp, ~450 bp, ~550 bp, and ~750 as shown (lane 2, Fig. 3 B). On the other hand, in the second confirmed PCR-screening of the PAC library, the positive MPAC94 in lane (6) comprised the bands 200 bp, 450bp, 550bp, and 750 bp (Fig. 2 A). The actin band (150 bp) in the RT-PCR reaction was absent in both of the MPAC94 confirmed PCR and the positive control genomic DNA. This could be emphasized by mRNA induction in this stage of cotton fiber development. Moreover, the actin bands (550 bp and 200 bp) in the genomic DNA positive control and the MPAC94 confirmed PCR were absent in the RT-PCR reaction (Fig 3 B), which could be emphasized by mRNA splicing occurred for one of the actin genes through mRNA maturation process. The actin bands (~450 bp, ~250 bp and ~750 bp) were found common and expressed in the genomic DNA positive control, MPAC94 confirmed PCR and RT-PCR, which concluded how important these actins to the cotton plant cell. These data proved that the 750 bp fragment was the consensus expressed sequence of actin that was detected using the *GhACT2* primers in the genomic insert of the MPAC94. Besides, it was also shown that these common actin gene members (~450 bp, ~250 bp and ~750 bp) are expressed in more constitutive matter in Giza88 (*G. barbadense* L.). In addition, *GhACT2* degenerate primers can polymerize the conserved region of the actin gene family from *G. barbadense*, in this study, as well as from *G. hirsutum* when it is used for real-time PCR by Li *et al.* (2005). Depending on data of

RT-PCR, *GhACT2* primers can be used to isolate the expressed region of actin gene family and/or many of its family members with its allelic profile existence from the genomic DNA as well as mRNA samples.

This study reports the development of genomic PAC library for Giza88, one of the Egyptian extra long stable varieties, that is high-yielding and has high-fiber quality. Actin is considered one of the effective functionally expressed gene families associated with cotton fiber elongation. Identification of actin related gene family was achieved through the most advanced approaches in biotechnology. Identification revealed 14 actin positive PAC clones and a 16.26 Kb *EcoR*I restricted fragment comprising one of the actin-related gene families. Using such technologies provides a strong proof to the validation of the constructed Giza88 PAC library for further genomics studies. Giza88 PAC library is now available for further screening and identification of any other single genes and/or gene families with economic importance.

As a future prospect, the identified actin (16.26 Kb) *EcoR*I restricted fragment in this study could be used to identify the regulatory elements of actin in *G. barbadense* Egyptian cotton and in clone contig assembly and more physical mapping studies. In conclusion, outputs of this study can help in the identification of other fiber related gene families and ultimately provide novel target genes for the improvement of fiber length and strength as well as other qualities of industrial applications importance.

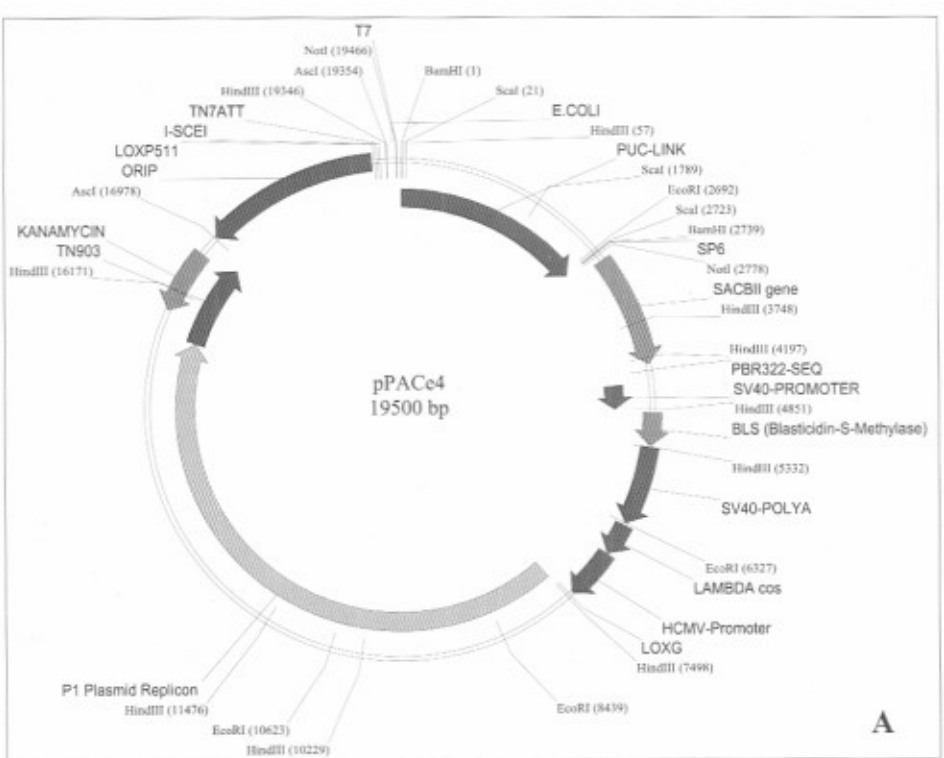

Fig.(1): A). Restriction map of the P1-derived artificial chromosome (pPACe4). As constructed by Frengen et al. (2000).

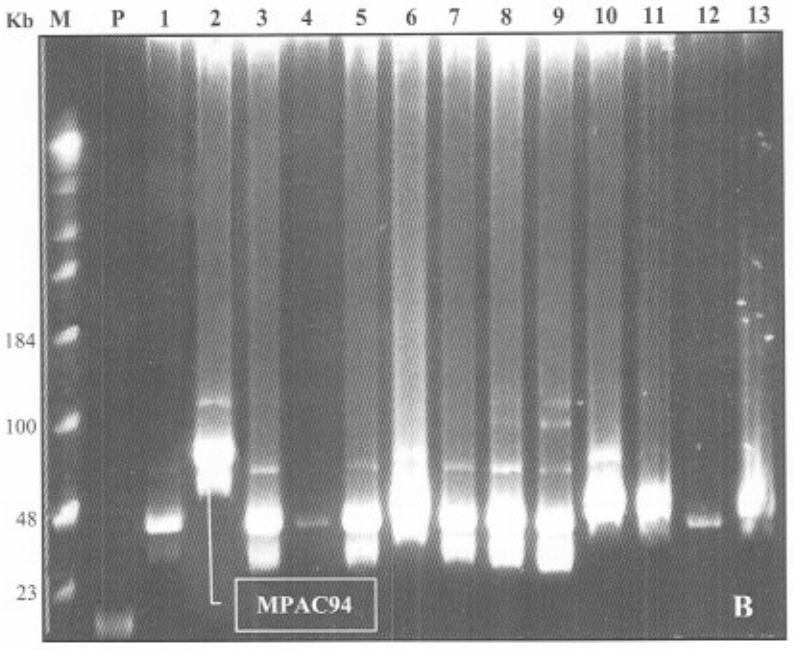

Fig.(1): B). Size fractionation of undigested recombinant PACs using (PFGE). Lane M: the Mid Range (PFGE) lambda ladder (Biolabs). Lane P: the non-recombinant de-phosphorelated pPACe4/BamHI (16.7 Kb) vector. Lanes (1-13): the undigested DNA of recombinant PAC clones. Lane 2: the actin positive MPAC94 clone (~70 Kb).

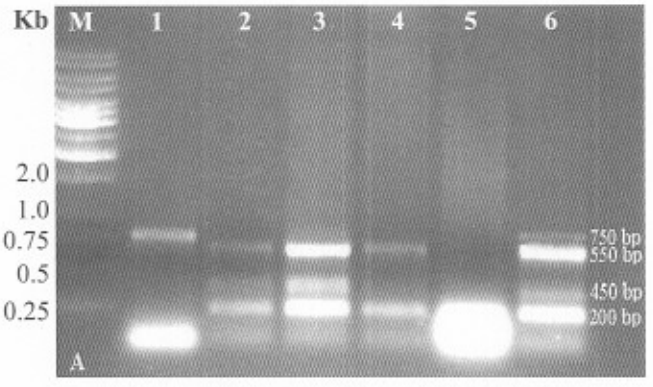

Fig.(2): A). PCR Confirmation for 5 actin positive PAC clones. Lane M: 1Kb DNA ladder (Gibco). Lane 1: PCR reaction of the actin (750 bp) from genomic DNA positive control. Lanes (2-6): (MPAC9, 11, 12, 14, and 94) respectively, the 5 positive PAC clones sharing actin consensus regions (200 bp, 450 bp, 550 bp, and 750 bp). All PCR-based screening reactions used the GhACT2 degenerate primers.

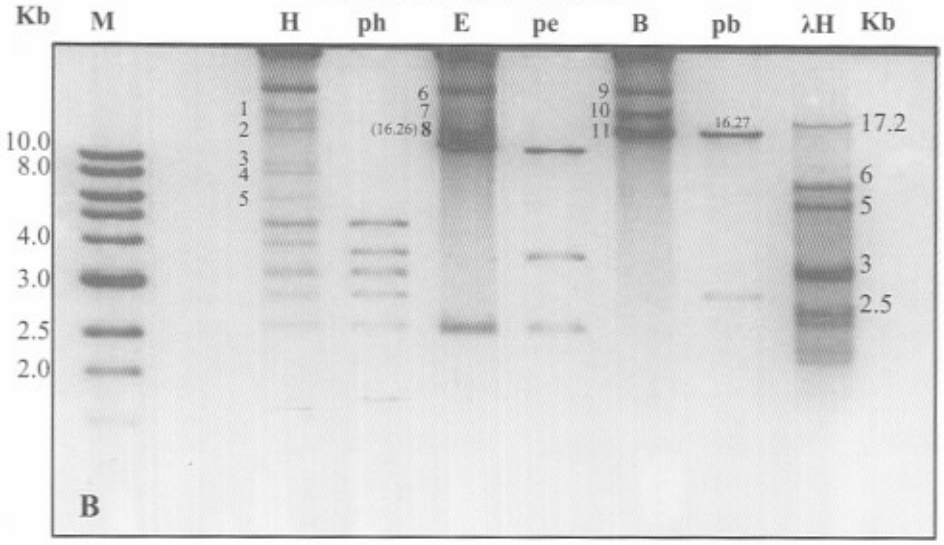

Fig.(2): B). Fragments generated from restriction fingerprinting of MPAC94. Lane M: 1Kb DNA ladder (Biolabs). Lane H: fragments (1-5) generated by MPAC94/HindIII digestion. Lane E: fragments (6-8) generated by MPAC94/EcoRI digestion. Lane B: fragments (9-11) generated by MPAC94/BamHI digestion. Lanes (ph, pe, and pb): the negative control pPACe4 vector digested with HindIII, EcoRI, and BamHI respectively. Lane λH: un-complete HindIII digestion of λ DNA. Fragment (8) in lane (E) shows positive actin (750 bp) conserved region in PCR-based positional cloning

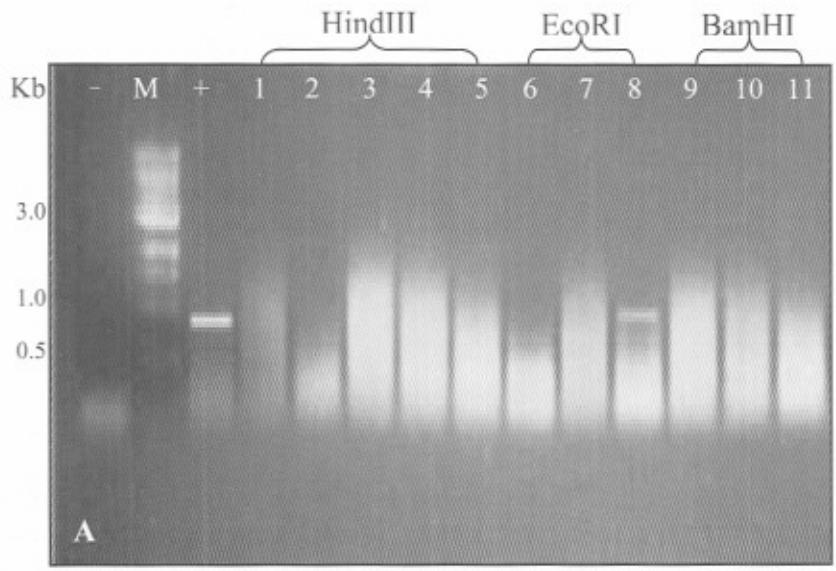

Fig.(3): A). PCR-based positional cloning for fragments generated from MPAC94 restriction fingerprinting. Lane (-): PCR of the pPACe4 vector as a negative control. Lane M: 1Kb DNA ladder (Biolabs). Lane (+): actin PCR fragment (750 bp) of genomic DNA as a positive control. Lanes (1-5): PCR for MPAC94/HindIII digested fragments. Lanes (6-8): PCR for MPAC94/EcoRI digested fragments. Lanes (9-11): PCR for MPAC94/BamHI digested fragments. All PCR-based positional cloning reactions used the GhACT2 degenerate primers.

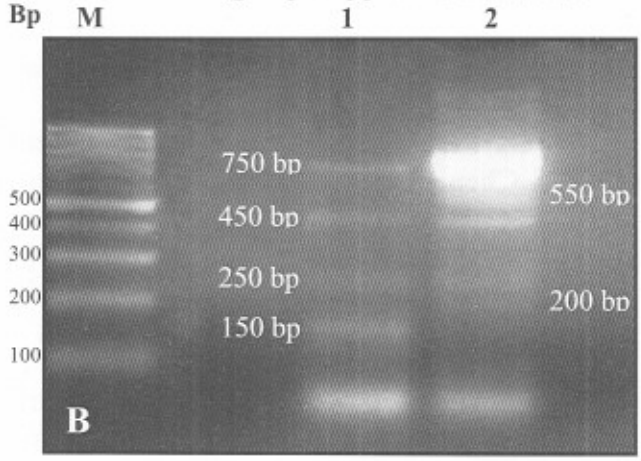

Fig.(3): B). Identification of the RT-PCR (750 bp) actin related gene fragments in Gizaa88. Lane M: 100 bp DNA ladder (Amershame Pharmacia). Lane 1: RT-PCR of conserved actin fragments (~150 bp, 250 bp, 450 bp and ~750 bp). Lane 2: the actin fragments of genomic DNA PCR. Actin GhACT2 degenerate primers were used in PCR for both RT and genomic DNA reactions.

REFERENCES

Anthony, R.G.; and Hussey, P. (1999). Dinitroaniline herbicide resistance and the microtubule cytoskeleton. Trends in the Plant Science. Vol. 4. (3): 112-116.

Cai, L.; Taylor, J. F.; Wing, R. A.; Gallagher, D. S.; Woo, S. S. *et. al.* (1995). Construction and characterization of bovin BAC library. Genomics 29; 413-425.

Chen, C. Y.; Chueng, A. Y. and Wu, H. M. (2003). Actin-depolymerizing factor mediates Rac/Rop GTPase-regulated pollen tube growth. Plant Cell 15; 237–249.

Clarke, S. R.; Staiger, C. J.; Gibbon, B. C.; and Franklin-Tong, V. E. (1998). A potential signaling role for profilin in pollen of Papaver rhoeas. Plant Cell 10; 967–979.

Coren, J. S.; Sternberg, N. (2001). Construction of a PAC vector system for the propagation of genomic DNA in bacterial and mammalian cells and subsequent generation of nested deletions in individual library members. Gene.7264 (1): 11-18.

David, P. V.; Rocio, B.; Francisco, M. C.; and Gonzalo, M. C. (2004). Isolation of Bacterial Artificial Chromosome DNA by means of improved Alkaline lysis and double potassium Acetate precipitation. Plat Mol Biol Reporter 22: 419-425.

Frengen, E.; Weichenhan, D.; Zhao, B.; Osoegawa, K.; Van G. and Jong, P.J. (2000). Modular bacterial artificial chromosome vectors for transfer of large Inserts into mammalian cells. Genomics 58:118-126.

Fu, Y.; Li, H.; and Yang, Z. (2002). The ROP2 GTPase controls the formation of cortical fine F-actin and the early phase of directional cell expansion during Arabidopsis organization. Plant Cell 14; 777–794.

Gilliland, L. U.; Pawloski, L. C.; Kandasamy, M. K.; and Meagher, R. B. (2003). Arabidopsis actin gene ACT7 plays an essential role in germination and root growth. Plant J. 33; 319–328.

Hussey, P. J.; Allwood, E. G.; and Smertenko, A. P. (2002). Actin-binding proteins in the Arabidopsis genome database: propertiesof functionally distinct

plant actin-depolymerizing factors/cofillins. Phil. Tans. R. Soc. 357: 791-798.

Ioannou, P.; Amemiya, C.T.; Garnes, J.; Kroisel, P.M.; Shizuya, H.; Chen, C.; Batzer, M.A.; and Jong, P.J. (1994). A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. Nature Genetics 6:84-89.

Ji, S. J.; Lu, Y. C.; Li, J.; Wei, G.; Liang, X.; and Zhu,Y. X. (2002). A β-tubulin-like cDNA expressed specifically in elongating cotton fibers induces longitudinal growth of fission yeast. Biochem. Biophys. Res. Commun. 296, 1245-1250.

Kost, B.; Bao, Y. Q and Chua, N. H. (2002). Cytoskeleton and plant organogenesis. Philosophical Transactions of the Royal Society, Series B, 357:777-789.

Li, X-B.; Xiao-Ping F.; Xiu Lan W.; Lin C.; and Wei-Cai Y. (2005). The Cotton *ACTIN*1 Gene Is Functionally Expressed in Fibers and Participates in Fiber Elongation .The Plant Cell, Vol. 17; 859–875,

Martinez, P.; and Amemiya, C. T. (2002). Genomics of the HOX gene cluster. Comp. Biochem. Physiol. B Biochem. Mol. Biol.. 133(4):571-580.

Mathur, J.; Mathur, N.; Kirik, V.; Kernebeck, B.; Srinivas, B. P.; and Hulskamp, M. (2003). Arabidopsis CROOKED encodes for the smallest subunit of the ARP2/3 complex and controls cell shape by region specific fine F-actin formation. Development 130, 3137– 3146.

Mathur, J.; Spielhofer, P.; Kost, B.; and Chua, N. H. (1999). The actin cytoskeleton is required to elaborate and maintain spatial patterning during trichome cell morphogenesis in *Arabidopsis thaliana*. Development 126; 5559–5568.

Momtaz, O. A.; Shehata, M. I.; El-Khodary, S.; and Hassan, H. Z. (2006). Idntification and isolation of smHSPs gene family from *Gossypium barbadense* L. c.v. Giza90 Egyptian cotton variety. Arab J. Biotech. 9, (1): 203-215.

Orford, S. J.; Timmis, J. N. (1998). Specific expression of an expansin gene during elongation of cotton fibres, Biochim .Biophys.Acta.1398: 342_/346.

Peterson, D. G; Tomkins, J. P.; Frisch, D. A.; Wing, R. A.; Paterson, A. H. (2000). Construction of plant bacterial artificial chromosome libraries: An illustrated guide. Journal of Agricultural Genomics 5: http://www. cabi-publishing.org/jag/index.html.

Pierce, J. C.; Sauer, B.; Sternberg, N. (1992). A positive selection vector for clning high molecular weight DNA by the bacteriophage P1 system: Improved cloning efficacy. Proc. Natl. Acad. Sci. USA, 89: 2056-2060.

Qin, Y. M.; Pujol, F. M.; Shi, Y. H.; Feng, J. X.; Lui, Y. M. et al. (2005). Cloning and functional characterization of two cDNAs encoding NADPH-dependent 3-ketoacyl-CoA reductased from developing cotton fibers. Cell Res. 15 (6): 465-473.

Sheng Y.; Mancino, V.; and Birren, B. (1995). Transformation of *Eschericia coli* with large DNA molecules by electro-oration. Nucl. Aci. Res. 23: (11): 1990-1996.

Shizuya, H.; Birren, B.; Kim, U. J.; Mancino, V.; Slepak, T.; Tachiiri, Y.; Simon, M. (1992). Cloning and stable maintenance of 300 Kiolbase-pair fragments in Escherichia coli using an F-factor-based vector. Proc. Natl. Acad. Sci. USA 89; 8794-8797.

Xu, Z.; Sun, S.; Covaleda, L.; Ding, K.; Zhang, A.; Wu, C.; Scheuring, C.; Zhang, H-B. (2004). Genome physical mapping with large-insert bacterial clones by finger-printing analysis: Methodlogy, Resource clone genome coverage, and contig map quality. Genomics 84; 941-951.

YuLing S.; Valeria M.; and Bruce B. (1995). Transfromation of Escherichia coli with

large DNA molecule by electroporation. Nucl, Aci, Res. Vol. 23, No. 11; 1990-1996.

Zhang, H.B. (2000). Construction and manipulation of large-insert bacterial clone libraries- manual. Texas A&M University, College Station. Available via http://hbz.tamu.edu

الملخص العربي

تعريف العائلة الجينية للأكتين فى القطن المصرى صنف جيزة ٨٨ بواسطة الكلونة الموضعية باستخدام تقنية ال PCR

***اسامة احمد ممتاز ،* احمد الفاتح الضليفى ،*,**احمد بهى الدين ،**السيد حسن حسين**

*معهد بحوث الهندسة الوراثية الزراعية- مركز البحوث الزراعية- الجيزة.

**قسم الوراثة -كلية الزراعة – جامعة عين شمس – القاهرة.

تشارك الاكتينات النباتية بقوة فى كل من الهيكل الخلوى ، تنظيم خيوط المايكروتيوبيول وتوجيه ترسيبات السيلليولوز اثناء تطورخلية ليفة القطن. ويعتبر تعريف عائلة الجينات المتعلقة بالاكتين من القطن المصرى كحجر الزاوية فى هندسة صفات خلية ليفة القطن فى المستقبل. لذلك فقد تم بناء مكتبة (PAC) اصطناعية الكروموسوم مشتقة من (P1) لصنف مصرى فائق الطول جيزة٨٨. وقد تضمنت المكتبة (Giza88-PAC) على ٨٩٠٠ مستعمرة بكتيرية بحجم ٧٠ كيلو قاعدة فى المتوسط تمثل حوالى ٠,٣ من الجينوم الاحادى (٢١١٨) لاصناف الجوسيبيام باربادينس. كما تم اختيار مستعمرات بكتيرية عشوائيا من المكتبة وعمل كشف للاكتين فيها، اعتمادا على تفاعل البلمرة المتسلسل لتضاعف الـ DNA المسمى (PCR) باستخدام بادئات مخلقة (GhACT2) والتى انتجت ١٤ مستعمرة ايجابية للتفاعل. وكأحد هذه المستعمرات الايجابية، تم عمل خريطة طبيعية و كلونة موضعية بأستخدام تقنية الـ (PCR) على المستمرة MPAC94 . مما انتج قطعة ايجابية للاكتن (MPAC94/EcoRI) بحجم (١٦,٢٦) كيلو قاعدة، والذى تم تأكيدها باستخدام تفاعل البلمرة المتسلسل العكسى (RT-PCR) ومقارنة التتابع على قاعدة البيانات للاقطان الابلند. هذه الدراسة هى الاولى من نوعها فى تعريف احد عائلة الجينات المتعلقة بالاكتن فى القطن المصرى لصنف جيزة٨٨ بأستخدام تقنية الكلونة الموضعية اعتمادا على تفاعل البلمرة المتسلسل.