DETECTION OF FIBER QUALITY GENES IN SOME PLANTS BELONGING TO FAMILY *Asclepiadaceae*

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Fiber cells are present in all plants with varied shapes, sizes, and compositions, and they are distributed all over the plant from roots to leaf, stem, fruits, and seeds. Fibers are considered the fabric of civilization and come after the requirement of food. Furthermore, from the dawn of civilization, fiber crops have been a source of economically exploited fibers, and fiber/fabric consumption has been regarded as an evidence of society's civilization.

An increase in population, particularly in developing countries has forced us to utilize new technologies such as biotechnology and genetic engineering to meet the demand for food and clothes. Major families (with several genera) contributing as fiber genetic resources are *Malvaceae, Fabaceae, Arecaceae, Utricaceae, Tiliaceae, Sterculiaceae, and Asclepiadaceae* (Ramawat and Ahuja, 2016).

Three stages are required for the fiber development process that includes initiation, elongation, and maturation (Cheema *et al*., 2010). A large set of genes are required for fiber length, quality differentiation, and development, meanwhile, few genes are known to function about how these genes control and regulate the process of fiber development. The Evolution of fiber quality is a complex procedure and various sets of genes are expressed during each developmental stage. In addition, genes under study (CpExPA3 and CpExPA4) may control fiber elongation and quality benefit approach targeting improvement of fiber crop quality. Ten GhPOX genes (cDNAs) encoding cotton group III peroxidases were identified, of which eight GhPOX genes were the first to be described. GhPoX1 plays an essential role during the development of fiber cell elongation and quality (Mei *et al*., 2009). In advance, numerous studies have been led to determine the qualitative and quantitative expression of fiber genes but knowing the exact timing and amount of expression of most fiber genes is still unclear (lqbal *et al*., 2016). Real-Time or qRT-PCR is the most suitable technique for gene expression analysis because it

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allows specific detection of each gene (Abd El-Twab *et al*., 2018). The technique of Quantitative Real-Time PCR (qRT-PCR) can be used to determine the copy number of low gene (Complete quantitative real-time PCR), differences in a great number of a template and low gene expression levels among samples (Lu *et al*., 2012). Furthermore, qRT-PCR is necessary and has become a high-throughput method for accurate gene expression describing the technique used to analyze gene expression levels and the increasing importance of gene expression analysis in the biological research field (Zhang *et al*., 2016).

Family *Asclepiadaceae*, is a relatively large family of angiosperms comprising some 348 genera and about 2900 species (APG IV, 2016), distributed mainly in the tropical and subtropical regions of the world. It is commonly known as the "Milkweed family" (Sinha and Mondal, 2017). The plant species are annual or perennial herbs (*Asclepias*), shrubs (*Calotropis* and *Leptadenia*), woody climbers (*Daemia*, *Tylophora*), *scramblers*, or *xerophytic succulents* (Stapelia) with copious milky latex. *Calotropis procera* and *Pergularia daemia* (Forssk.) Chiov. were chosen for the present study.

Calotropis procera is a species of the *Asclepiadaceae* family, it appears as a spreading shrub or medium-sized tree that grows to 2.5 to 6 meters. When the plant is damaged, it has deep roots that reach 3-4 m in-depth and a secondary root system with woody lateral roots that may quickly produce adventitious shoots. Additionally, the stems are twisted, and the corky bark is fissured. The grey-green leaves are 15-30 cm long and 2.5-10 cm wide, with a succulent and waxy look, hence the name *procera,* which refer to wax in Latin (ECOCROP, 2011 and Orwa *et al*., 2009). The stem of *Calotropis procera* has been characterized as a source of natural cellulosic best fibers wherein the commercially valuable properties (physical, chemical, and tensile) such as good staple length, fiber strength, fiber uniformity ratio, fiber fineness, and moisture absorption. Fiber elongation is found to be intermediate between that of cotton and flax (Maji *et al*., 2013).

Furthermore, it is well acclimatized to salinity and drought. It is a multiuse tree whose stems provide a valuable fabric that may be used to make ropes, bags, nets, and paper (Orwa *et al*., 2009). The seeds contain silky white floss, a potential silk replacer (Batello *et al*., 2004). Wood is useful as a source of fuel and lumber (Orwa *et al*., 2009; Kiew, 2001). The ethnomedicinal effects of milky sap (latex) are well-known (Batello *et al*., 2004; Iqbal *et al*., 2005) and as a food, particularly as a thickening clotting agent for cheese making in West Africa (O'Connor, 1993). *Calotropis* yields 90 tons of biomass twice a year and is a potential source of renewable energy (Parsons and Cuthbertson, 2001). *Calotropis* is also used as fodder. In times of shortage, young pods, senescing leaves, and blossoms can be given to main animals (rarely cattle). The latex includes hazardous ingredients that might

damage cattle. The species is highly cross-pollinated and has 2n=22 chromosomes (Cheema *et al*., 2010; Eisikowitch, 1986 and Raghavan, 1957).

Pergularia daemia is a perennial herbaceous climber that scrambles above the ground or twines itself nearby other plants for support. It is extensively spread throughout the world's tropical and subtropical areas. At the base, the stems are a little woody. The plant has a range of traditional medicinal uses, as well as supplying food and fiber. In African marketplaces, the roots and leafy branches are marketed for medical purposes. Because of its sweet-scented flowers and its climbing habit, the plant is cultivated as an ornamental pergola in tropical gardens. The stems yield a strong fiber, used in many places to make rope and fishing lines. The fiber is said to resist fire longer than other species. In India, the stems fibers are used as a flax substitute and makes fishing lines (Lewis, 1986). It has also been used in folklore medicine to treat infantile diarrhea and intermittent malarial overheat, as a laxative, and antipyretic expect. Various phytochemical components including terpenoid, flavonoids, sterols and cardenolides have been isolated and identified from the various parts of the plant (leaves, stems, shoots, roots, seeds, and fruits). Various tribal communities widely use it in Western Ghats of India to treat various ailments, while predominantly the roots of the plant have been used to treat liver disease and jaundice (Karthik and Murugan, 2013).

Therefore, the present investigation was conducted to detect and investigate the expression levels of two fiber quality-related genes (CpExpA3 and CpExPA4) in *C. procera* and *P. daemia* in different tissues (leaves and stems) compared to flax, *Linum usitatissimum* (Egyptian cultivar: Giza 10) using qRT-PCR. In addition, the plants under study have genetic potentialities that enable them to be good genetic resources and alternatives to economically important fiber crops, moreover, for Guidance to domestication and cultivation of inexpensive fiber resources that are tolerant to various environmental stresses and do not exhaust the farmlands. Finally, as a basis for genetic improvement of fiber crops missing these properties in future studies.

MATERIAL AND METHODS

Plant samples (leaves and stems) of *Calotropis procera* and *Pergularia daemia* were collected from the western North coast and North Sinai. In addition, flax (cv. Giza 10) was obtained from the Fiber Crops Research Department, Field Crops Res. Institute (FCRI), Agricultural Research Center (ARC), Giza, Egypt. Samples were frozen immediately in liquid nitrogen and kept at -80ºC until use.

Total RNA extraction and purification

Total RNA was extracted from leaves and stems of *C. procera* and *P. daemia* and flax (Giza 10) using Direct-zol™ RNA MiniPrep (Zymo Research, USA; catalog Nos. R2050) according to the following main steps:-

A- Sample preparation

A 100 mg from both leaf tissue and stem were ground in liquid nitrogen then transferred immediately to a tube containing 600µl TRI Reagent® and samples were homogenized. Centrifugation was carried out at 10000 xg for 1 minute to remove the particulate debris and the supernatant was transferred into an RNase-free tube.

B- RNA purification

Equal volume ethanol (95-100%) was added to a sample lysed in TRI Reagent® and mix thoroughly. The mixture was transferred into a Zymo-Spin™ IICR Column in a collection Tube and centrifuge. The columns were Transfer into a new collection tube and discard the flow-through. *DNase* digestion step: Eliminating genomic DNA contamination by DNase I with DNase/RNase-Free Water (Cat. # E1009-A, ZYMO Research Crop. Set 250 U). About 400µl Direct-zol™ RNA Pre-Wash was added to the column and centrifuged. The flow-through was discarded and repeats this step. About 700µl RNA Wash Buffer was add to the column and centrifuge for 1 minute to ensure complete removal of the wash buffer. The column was transfer carefully into an RNase-free tube. To elute RNA, 50µl of DNase/RNase-Free Water was added directly to the column matrix and centrifuge. The eluted RNA was used immediately or stored frozen until use.

Estimation for the RNA concentration

The purified total RNA was quantified and the yield of total RNA was measured by absorbance at 260 nm (A_{260}/A_{230} and A_{260}/A_{280} ratios) using NanoDrop (ND-1000) spectrophotometer (NanoDrop, Technologies Inc.). The integrity of total RNA was verified by electrophoreses separate analysis on 1.2% agarose gel.

Oligonucleotide primers designing

Specific primers pair was designed to amplify the nucleotide sequences of full- length CpEXPA3 gene from *Calotropis procera*. One degenerate primer pair was designed from CpEXPA4 genes at the conserved nucleotide sequences region that was determined based on the multiple sequence alignment of other EXPAs gene families sequence from selected plant species in the universal database (Table 1). Alignments between related species to plants under this study showed a homology at some regions of the gene (ORF) that were species-specific. *Calotropis procera* (EXPA4) (EF434784.2), *Carthamus tinctorius* (EXP1) (MK103359.1), *Prunus avium* (EXPA1) (XM_021951753.1), *Prunus dulcis* (EXPA1) (XM_034354222.1), *Prunus mume* (EXPA1) (XM_008223377.2), *Prunus salicina* (EXPA3) (JN675713.1), *Prunus persica* (PpExp2) (AB047518.1), *Jatropha curcas* (EXP1) (XM_012212277.3), *Fragaria vesca* subsp. *vesca* (EXP1) (XM_004297244.2), *Olea europaea* var. *sylvestris* (EXPA1) (XM_023026059.1), *Glycine soja*

(EXPA10) (XM_028366801.1), *Gossypium hirsutum* (EXPA9A) (MG000836.1), *Gossypium hirsutum* (EXPA10) (XM_016818728.2) and *Daucus carota* subsp. *sativus* (EXPA1) (XM_017376064.1) were used for multiple sequences alignment of nucleotide (BLSTn) to design degenerate primer for the amplification of the partial middle fragment of ORF.

Reverse transcription (RT-PCR) analysis

The reverse transcription reactions were conducted by almost 2 microgram total RNA as template and with the kit from Takara, which synthesizes first- and second-strand cDNAs using random hexamer and oligodT primers.

Real-time PCR

Real-time PCR was performed with the SensiFAST™ Probe SYBR Lo-Rox kit. A real-time PCR (qRT-PCR) reaction was applied according to Schmittgen and Livak (2008) as follows: Fast SYBR Green master mix 10 µl, forward primer (5 pmol/µl) 0.8µl, Reverse primer (10 pmol/µl) 0.8µl, template cDNA (25ng/ µl) 2µl and ddH_2O up to 20µl were added. Reactions were run on Strata gene Mx3000p (Agilent Technologies, Santa Clara, CA). Conditions were: 10 min at 95ºC, 40 cycles of 5sec at 95ºC, 45sec at 60ºC, 20sec at 72ºC and 4ºC (infinite). PCR products were examined by melt curve analysis. Each reaction was repeated three times for each cDNA sample (Schmittgen and Livak, 2008). Beta Tubulin (TBB4) was used as a reference gene selection from *Calotropis procera* for data normalization (Coêlho *et al*., 2019). The Ct values were obtained using the MxPro Mx3000P v3.00 software (Agilent Technologies, Santa Clara, CA).

Real-time PCR data analysis

Derivation and examples of this method have been described by (Livak and Schmittgen, 2001 and Schmittgen and Livak, 2008). The relative difference in gene expression using the $2^{-\Delta\Delta Ct}$ method was calculated as follows: Relative fold change in gene expression = $2^{-\Delta\Delta Ct}$, where: $\Delta\Delta_{Ct} = \Delta_{Ct}$ control – Δ_{Ct} treatment, and Δ_{Ct} = (Ct genes – Ct Beta Tubulin gene).

Thermo-cycling PCR program

PCR amplification was performed in a Perkin-Elmer/Gene Amp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 40sec., an annealing step at 50ºC for 50sec and an elongation step at 72ºC for 60sec. The primer extension segment was extended to 7 min at 72ºC in the final cycle.

Detection of the PCR Products

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in a 1X TBE buffer at 95 volts.

A 50 bp DNA ladder was used as a molecular size standard. PCR products were visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000).

Purification of PCR Products

Amplified products for all PCR were purified using the EZ-10 spin column Kit for PCR products purification (Cat. # 9K-006-0007 Bio Basic Inc.). PCR reaction mixture was transferred to 1.5 ml microfuge tube and three volumes were added of binding buffer II after that the mixture solution was transferred to the EZ-10 column and let stand at room temperature for two minutes after that centrifuge, 750 µl of wash solution was added to the column and centrifuge at 10.000rpm for two minutes, repeated washing, 10.000 rpm was spin for an additional minute to remove any residual wash solution. The columns were transferred into a clean 1.5 ml microfuge tube and add 30-50 µl of elution buffer, incubated at room temperature for two minutes, and store purified PCR at -20ºC.

PCR sequencing and computational analysis

Sequencing chromatograms were analyzed by Macrogen Company, Seoul, and South Korea. Sequences were aligned using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) and phylogenetic trees were constructed using MEGA 7.0 software program.

RESULTS AND DISCUSSION

Expression profiling of target genes through qRT-PCR amplification

To investigate the genetic factors controlling the quality of fibers in *Calotropis procera* and *Pergularia daemia*, Quantitative real-time PCR (qRT-PCR) was conducted to demonstrate the expression levels of some fiber-related genes compared with flax (cultivar Giza10) as control (EL-Shimy *et al*., 2015 and Rashwan *et al*., 2016). The results of expression patterns (Fig.1) revealed that the expression of gene CpExpA4 in *C. procera* was 2.29 and 3.48 folds in leaves and stems, respectively, compared to control. While, it showed less expression in *P. daemia*; 1.2 folds in leaves and 1.86 folds in stems relative to control. Moreover, the CpExpA3 gene exhibited expression 2.46 and 4.28 folds in leaves and stems, respectively, compared to control. In contrast, it displayed less expression in *P. daemia* 1.4 folds in leaves and 2.1 folds in stems. It is evident from the results that the expression of the fiber genes in *C. procera* was higher than that in *P. daemia* overall plant parts under study. Furthermore, the expression of the studied fiber genes was higher in stems than leaves for *C. procera* and *P. daemia*. This result is in agreement with Cheema *et al*., (2010) who reported that CpEXPA3 showed significant expression in *C. procera* fibers but the maximum transcripts were detected in the stem.

Detection of CpExpA4 and CpExpA3 genes using PCR

According to the EXPAs family homologous sequence conservative region; one degenerate primer pair was used to amplify a partial middle fragment of cDNA CpEXPA4 gene in plant species (*C. procera* and *P. daemia*) which gave one fragment with a size of about 450 base pairs. PCR amplification product of full-length CpExpA3 gene in stem samples of *C. procera* revealed that, that CpExpA3 gene produced a band of size 850 bp as shown in Fig. (2).

Analysis of the CpExpA4 and CpEXPA3 protein Sequence Alignment

Sequencing chromatograms of EXPA3 and EXPA4 were analyzed by Macrogen Company, Seoul, South of Korea and translated into amino acid sequences by the *ExPASy* online program (http://web.expasy.org/translate) (Figs. 3 and 4). Homology searches were conducted using Basic Local Alignment Search Tool (BLAST) with data recorded in The National Center for Biotechnology Information GenBank database (NCBI), (http://www.ncbi.nlm.nih.gov) (Altschul *et al*., 1990). Alignment was conducted based on nucleotides sequence and amino acids sequence (BLASTn/BLASTp program). BLASTp for EXPA4 gene showed high similarity (100%) to EXPA4 gene in *C. procera* (ABO30978.2) with 1e-106 E-value. In contrast, it revealed a low similarity value (78.57) with EXPA1 in *Carthamus tinctorius* (QFZ95618.1) with E-value 4e-88 (Table 2). BLASTp for EXPA3 showed a maximum similarity level (100%) to EXPA3 in *C. procera* (ABO30977.1) with a 3e-179 E-value (Table 3). On the other hand, the lowest similarity was recorded (69.75%) with EXP-A8 in *Hibiscus syriacus* (XP_039066816.1) with E-value (4e-57). In addition, it revealed a similarity of 76.54% with EXPA4 in *C. procera* (ABO30978.2) with E-value (6e-144).

Phylogenetic Analysis of CpExpA4 Gene Sequence in *C. procera* species:

The phylogenetic analysis was conducted to investigate similarity and evolutionary relationships among species. In the current study, a phylogenetic tree was constructed using the MEGA 7.0 software program by maximum Likelihood (ML) with the highest log likelihood (-1728054) (Kumar *et al*., 2016) (Fig. 5). It was structured based on 15 amino acids sequences encoded by expansin genes recorded in the NCBI database and the CpEXPA4 gene under study. The dendrogram revealed a high similarity between isolated EXPA4 from *C. procera* (current study) and EXPA4 of *C. procera* (EF434784.2) (grouped in the same cluster).

Phylogenetic Analysis of CpExpA3 Gene Sequence in *C. procera* species:

The phylogenetic tree was constructed using the MEGA 7.0 software program by maximum Likelihood (ML) with the highest log likelihood (-1439.94) (Kumar *et al*., 2016). The dendrogram was initiated on the of basis 24 amino acids

sequences encoded by expansin genes recorded in the NCBI database and *C. procera* under investigation (Fig. 6). The phylogenetic tree involved two major clades divided into subgroups. The first clade is belonging different expansin genes such as; EXPA, EXPA5, EXPA2, EXPA4, EXPA1, and EXPA10. The isolated CpEXPA3 (Seq.1 and Seq.2, current study) were involved in clade I fall within the same group with *C. procera* (ABO30977.1).

The stem of *C. procera* is a source of natural bast fibres where in the commercially important features for example cellulose content, fiber strength and fiber elongations are found to be intermediate between that of cotton and flax. Furthermore, fibers from *C. procera* possess more weight per square meter than the cotton fibers Maji *et al*., (2013). A great number of genes are involved in the complex procedure of fiber expansion (Arpat *et al*., 2004). GhPoX1 from ten GhPOX genes (cDNAs) encoding cotton class III peroxidases were played an essential role during the development of fiber cell elongation and quality (Mei *et al*., 2009). Many fiber quality genes are expressed in fiber and are thought to have an essential role in fiber morphogenesis. Meanwhile, fiber-related genes are expressed clearly during a particular developmental stage which reflects the specificity of the genes to this stage (Iqbal *et al*., 2016). In this regard, the relative gene expression of CpEXPA1, CpEXPA2, CpEXPA3, and CpEXPA4 in root, stem, fiber and leaves tissues of *C. procera* indicated that the four genes expressed in all tissues but in variable levels. In addition, the lowest transcription of the four genes was apparent in elongating roots indicating that root tissue may have specific expansins other than those limited to air-grown organs (Cheema *et al*., 2010). Moreover, Bajwa *et al.*, (2013) transformed the CpEXPA3 gene from *C. procera* into the local cotton variety of Pakistan (NIAB-846) by *Agrobacterium* strain LBA 4404. The results showed that the fiber strength of the transformed cotton plants was significantly improved as compared to control. Additionally, the over gene expression of CpExpA3 (having the highest expression in *C. procera* fibers) may result in an increase in fiber length in cotton by enhancing the primary cell wall relaxing. Whilst, the expression of four genes CpExpA1, CpExpA2, CpExpA3, and CpExpA4 in *C. procera* under water stress showed significant differences only when this expression was evaluated in the leaves. Meanwhile, CpEXPA3 and CpEXPA4 isoforms exhibited the highest levels of expression due to water stress (Londe and Pinho, 2017). In addition, Khatoon *et al*., (2018) reported that CpEXPA3 was the closest homolog of GhEXP15 (76% amino acid identity) and the relative gene expression level was 80% higher in *C. procera* fibers. Expression of sucrose synthase was at its high level in 5–15 DPA cotton plant fibers, as well as, CpPiP2, CpEXPA3 genes

and sucrose synthase are the best candidate genes for fiber modification in *G. hirsutum* and other fiber plants. Transcription profiling and qRT-PCR analysis indicated that the expansin genes have feature expression patterns between different stages of cotton and other fiber plants in both quality and elongation. For convenience, three genes (GhEXPA4o, GhEXPA1A, and GhEXPA8h) were highly expressed in the initiation stage, nine genes (GhEXPA4a, GhEXPA13a, GhEXPA4f, GhEXPA4q, GhEXPA8f, GhEXPA2, GhEXPA8g, GhEXPA8a, and GhEXPA4n) had high expression during the fast elongation stage, and GhEXLA1c and GhEXLA1f were specially expressed in the evolution stage of fiber development (Lv *et al*., 2020). In addition, Yaqoob *et al*. (2020) observed that overexpression of the gene CpEXPA1 under the GhSCFP and CaMV35S promoters improved fiber cellulose content, fiber quality, and growth after being compared to the control set. Moreover, our findings were in agreement with Khatoon *et al*., (2018) who reported that the real-time transcriptome profiling of the expansin homolog CpEXPA3 was highly observed in *C. procera* fibers and might have a major involvement in fiber elongation. Furthermore, the CpEXPA3 and CpEXPA4 had 80% amino acid identity and were observed in the same clade indicating that those are paralogues too but evolved a long time ago. This indicated that *C. procera* fiber elongation requires two highly divergent expansins (CpEXPA3 and CpEXPA1), while CpEXPA2 and CpEXPA4 might play a minor role in elongating *C. procera* fibers.

SUMMARY

Two fiber plant species belonging to the family *Asclipidaceae*; *Calotropis procera* and *pergularia daemia* were chosen for the present study. Gene expression analysis of fiber quality genes (EXPA3 and EXPA4) was achieved using quantitative real-time PCR (qRT-PCR) in two plant parts (leaf and stem) in *C. procera* showing a high expression level of EXPA3 gene compared to the EXPA4 gene. Moreover, the expression level in stems is higher than that in leaves regardless of plant species. Detection of genes (EXPA3) was carried out by PCR reaction producing band in *Calotropis procera* with sizes 850bp but for EXPA4 gene PCR product in *C. procera* and *Pergularia daemia* with sizes 450bp. The obtained fragments were sequenced and phylogenetic trees based on amino acid sequences using Mega 7.0 software were accomplished. The results revealed the close relatedness of CpEXPA3 understudy to *C. procera* (ABO30977.1) was recorded in the National Center for Biotechnology Information (NCBI) database. In addition, the close relatedness of CpEXPA4 understudy to CpEXPA4 in *C. procera* (EF434784.2) was recorded in the NCBI database. The present study recommends conducting more researches on fiber crops due to their economic importance. Furthermore, their genetic potentialities

which enable them to be good genetic resources and alternatives to economically important fiber crops. Moreover, For Guidance to domestication and cultivation of inexpensive fiber resources that are tolerant to various environmental stresses and do not exhaust the farmlands.

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Table (1): Primers sequence used for CpEXPA3 gene and CpEXPA4 gene.

Gene Name	Primer Sequence	Size PCR
I. Specific primer for CpEXPA3 gene (Cheema *et al*., 2010)		
alpha expansin protein 3 (EXPA3)	**Fwd_1:** 5´ATGGCTAATATTCTTGCATTCTC ´3	850bp contained ORF=759bp
	Rev_{850}: 5´CAAGTCAACTGAAACACAAACGACCC ´3	
II. Degenerate primer for CpEXPA4 gene		
alpha expansin protein 4 (EXPA4)	**Fwd_{33}:** 5´ GGS ACA AAT GGG TGG KGC TTG TGG ´3	459bp
	Rev_{491}: 5´ TG CCA ACC MGT YCT BGA MCC TTT ´3	
III Primer sequences used for real-time PCR analysis (Cheema *et al*., 2010)		
alpha expansin protein 3 (EXPA3)	**Fwd**: 5' TCTGTCCACCTAATCCTTCC 3'	181bp
	Rev: 5' CCGTTGATTGTAACCTTATTCC 3	
alpha expansin protein 4 (EXPA4)	**Fwd:** 5' GCCACTCTTACTTCAACTTG 3'	195bp
	Rev: 5' CTACACTCCGACCATCAC 3'	
Beta-Tubulin (Housekeeping gene) for *Calotropis procera* (Coêlho *et al*., 2019)		
Beta-Tubulin (TBB4)	**Fwd:** 5´ CTTGCACCCTAACTCCACAAA ´3	99bp
	Rev: 5´ CAACTTCCCAGAACTTTGATCC ´3	
Notes: S=C/G, K=G/T, M=A/C, and B=C/G/T		

Table (2): NCBI- BLAST analysis of amino acids sequence homology encoded by EXPA4 partial gene in *C. procera* understudy.

No.	Scientific name	Accession No.	Per. Identity	E-value
1	**Seq1 current study C. *procera* (EXPA4)**	----------	---------	--------
2	***Calotropis procera* (EXPA4)**	**ABO30978.2**	**100.00**	**1e-106**
3	***Carthamus tinctorius* (EXP1)**	**QFZ95618.1**	**78.57**	**4e-88**
4	***Prunus avium* (exp-A1)**	**XP_021807445.1**	**91.43**	**1e-99**
5	***Prunus dulcis* (exp-A1)**	**XP_034210113.1**	**91.43**	**9e-100**
6	***Prunus mume* (exp-A1)**	**XP_008221599.1**	**90.00**	**6e-98**
7	***Prunus salicina* (exp-3)**	**AEQ28765.1**	**90.71**	**3e-99**
8	***Prunus persica* (Exp2)**	**BAC66786.1**	**91.43**	**7e-100**
9	***Jatropha curcas* (exp-A1)**	**XP_012067667.1**	**92.14**	**1e-99**
10	***Fragaria vesca* subsp. *Vesca* (exp-A1)**	**XP_004297292.1**	**90.00**	**8e-100**
11	***Olea europaea* var. *sylvestris* (exp-A1)**	**XP_022881827.1**	**89.29**	**7e-98**
12	***Glycine soja* (exp-A10)**	**XP_028222602.1**	**89.29**	**5e-88**
13	***Gossypium hirsutum* (EXPA9A)**	**AXQ39562.1**	**88.57**	**5e-97**
14	***Gossypium hirsutum* (exp-A10)**	**XP_016674217.1**	**88.57**	**5e-97**
15	***Daucus carota* subsp. *Sativus* (exp-A1)**	**XP_017231553.1**	**87.86**	**2e-96**

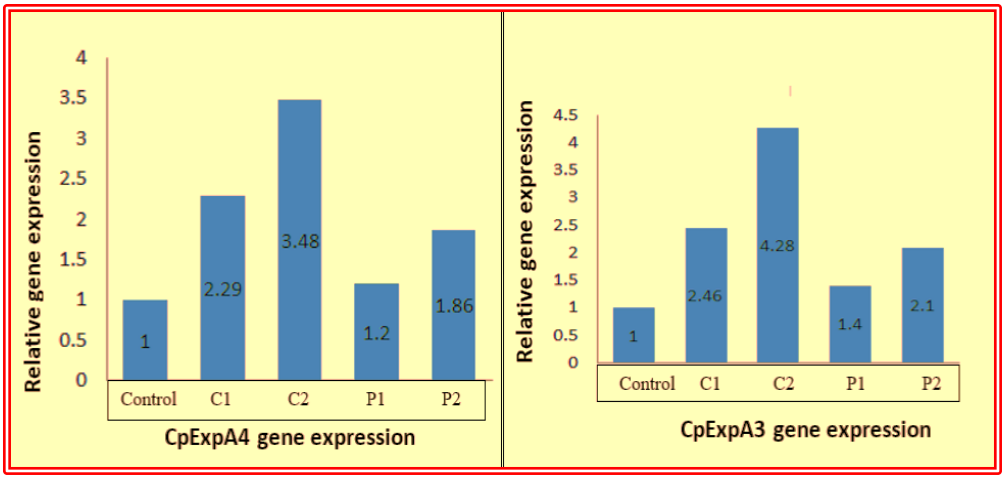
Table (3): NCBI- BLAST analysis of amino acids sequence homology encoded by EXPA3 gene in *C. procera* understudy.

Amino acid sequences alignment				
No.	**Scientific name**	**Accession No.**	**Per. Identity**	**E-value**
1	**Seq1 current study *C. procera* (EXPA3)**	----------	----------	--------
2	**Seq2 current study *C. procera* (EXPA3)**	----------	----------	--------
3	***Calotropis procera* (EXPA1)**	**ABO30976.2**	70.87	9e-118
4	***Calotropis procera* (EXPA4)**	**ABO30978.2**	76.54	6e-144
5	***Calotropis procera* (EXPA2)**	**ABP48816.1**	77.29	7e-118
6	***Calotropis procera* (EXPA3)**	**ABO30977.1**	100.0	3e-179
7	***Manihot esculenta* (EXP-A2)**	**XP_021602659.1**	85.31	4e-143
8	***Olea europaea* var. *sylvestris* (EXP-A8)**	**XP_022864754.1**	83.27	3e-146
9	***Vigna unguiculata* (EXP-A4)**	**XP_027939541.1**	82.13	3e-145
10	***Nicotiana attenuata* (EXP-A8)**	**XP_019246696.1**	81.22	3e-142
11	***Nicotiana attenuata* (EXP-A8)**	**XP_019260691.1**	81.22	4e-146
12	***Hevea brasiliensis* (EXP-A8)**	**XP_021687828.1**	77.46	1e-135
13	***Hibiscus syriacus* (EXP-A4)**	**XP_039045835.1**	79.04	2e-133
14	***Nicotiana tabacum* (EXP-A1)**	**XP_016485935.1**	76.89	9e-121
15	***Hibiscus syriacus* (EXP-A4)**	**XP_039045458.1**	77.97	2e-130
16	***Nicotiana sylvestris* (EXP-A1)**	**XP_009794018.1**	76.89	5e-121
17	***Lupinus angustifolius* (EXP-A1)**	**XP_019420232.1**	75.21	4e-134
18	***Olea europaea* var. *sylvestris* (EXP-A10)**	**XP_022856554.1**	73.20	6e-129
19	***Hibiscus syriacus* (EXP-A8)**	**XP_039066816.1**	69.75	4e-57

Table (3): Cont''

20	***Ananas comosus* (EXP-A4)**	**XP_020102234.1**	80.77	2e-124
21	***Gossypium hirsutum* (EXP)**	**AXQ39557.1**	73.13	5e-124
22	***Gossypium hirsutum* (EXP-A5)**	**XP_016729704.1**	73.13	1e-118
23	***Gossypium raimondii* (EXP-A5)**	**XP_012441175.1**	7357	3e-125
24	***Ananas comosus* (EXP-A10)**	**XP_020086668.1**	72.17	4e-119

Fig. (1): Expression profiles of CpExpA4 and CpExpA3 fiber-related genes in



leaves (C1 and P1) and stems (C2 and P2) of *C. procera* and *P. daemia* using real-time PCR analysis (qRT- PCR).

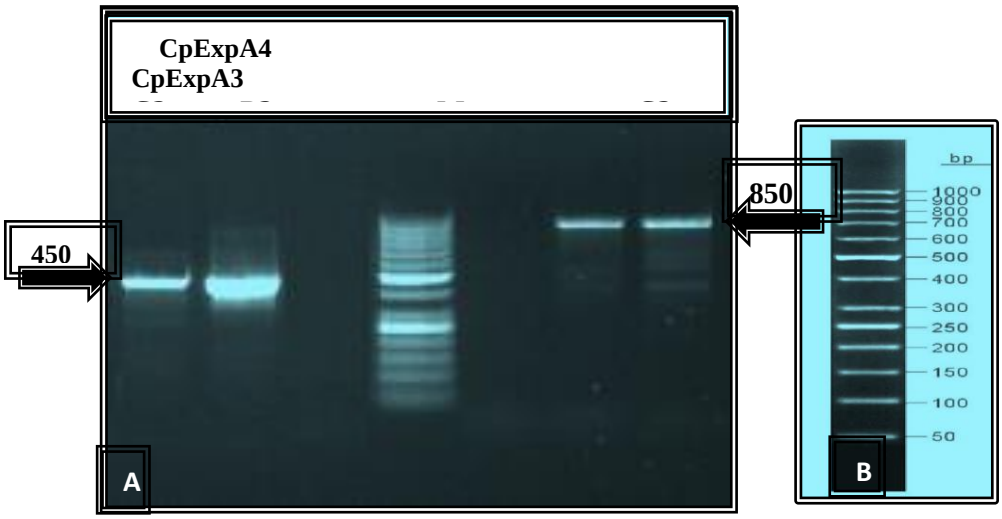


Fig. (2): A: PCR product of fiber quality-related genes full-length gene (CpExpA3) in *C. procera* and partial gene (CpExpA4) in *C. procera* (C2) and *P. daemia* (P2) regading size marker (M). B: Size marker 50-1000 bp DNA ladder.

Fig.(3): Nucleotide sequence of partial CpEpxpA4 gene and deduced amino acid sequence expressed by single letters from *Calotropis procera*.

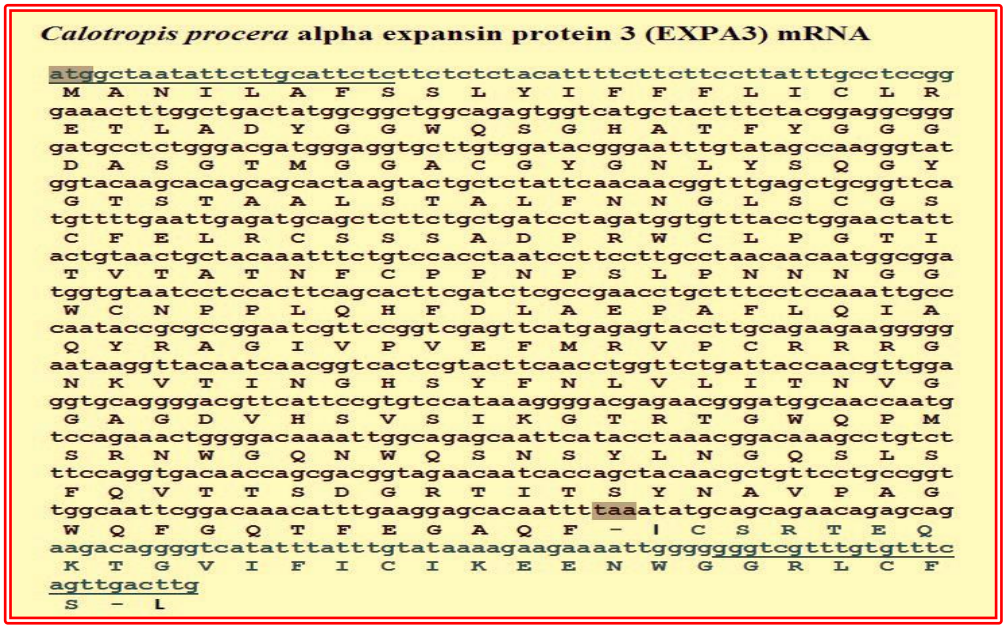


Fig. (4): Nucleotide sequence of full-length CpEXPA3 gene and deduced amino acid sequence expressed by single letters from *Calotropis procera*.

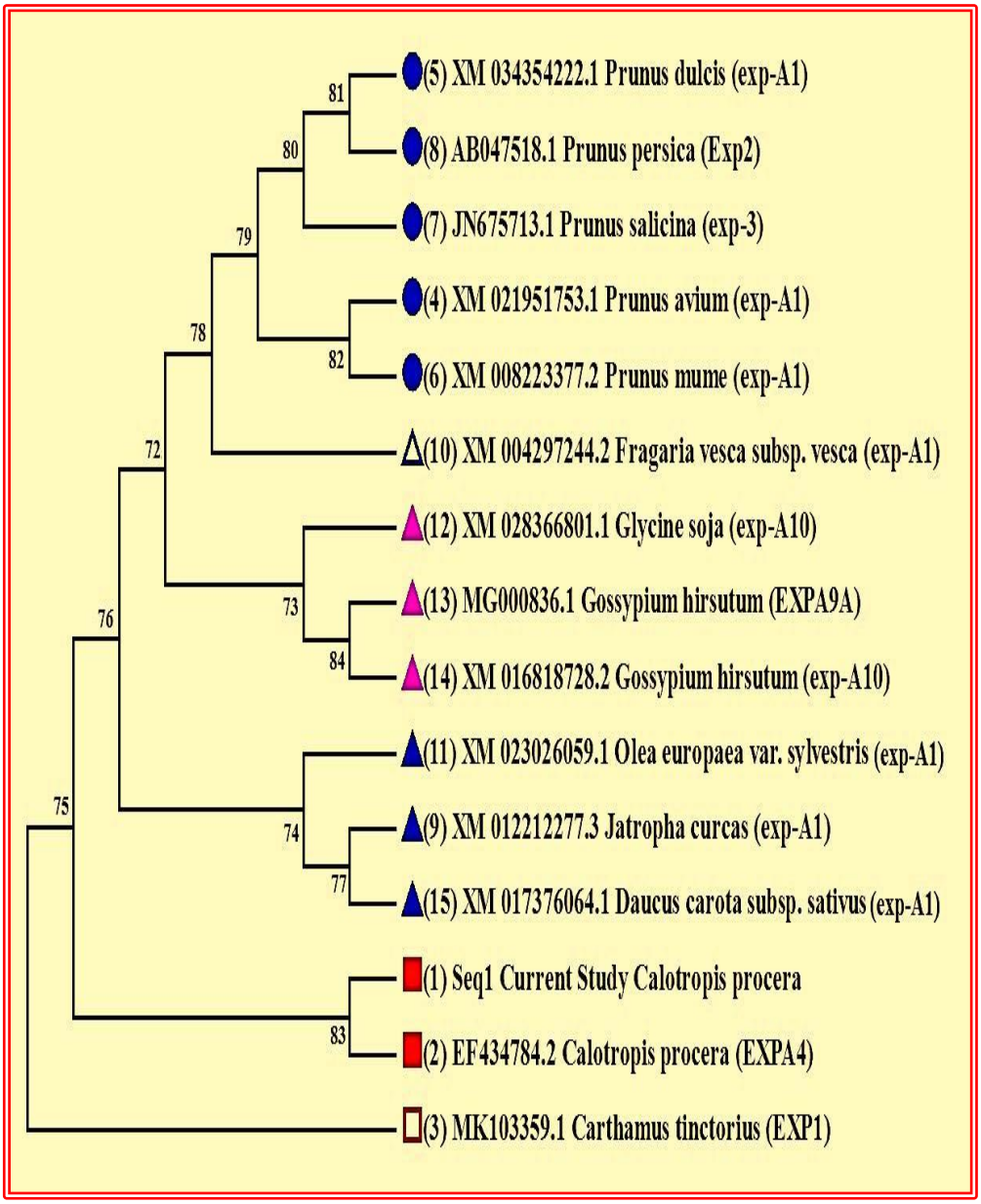


Fig. (5): Molecular phylogenetic analysis involved 15 amino acid sequences EXPAs genes from different species included one sample current study (CpEXPA4 gene from *Calotropis procera*) were conducted in MEGA 7.0 software program by Maximum Likelihood method. The tree with the highest log likelihood (-1728.54) is shown (Kumar *et al*., 2016).

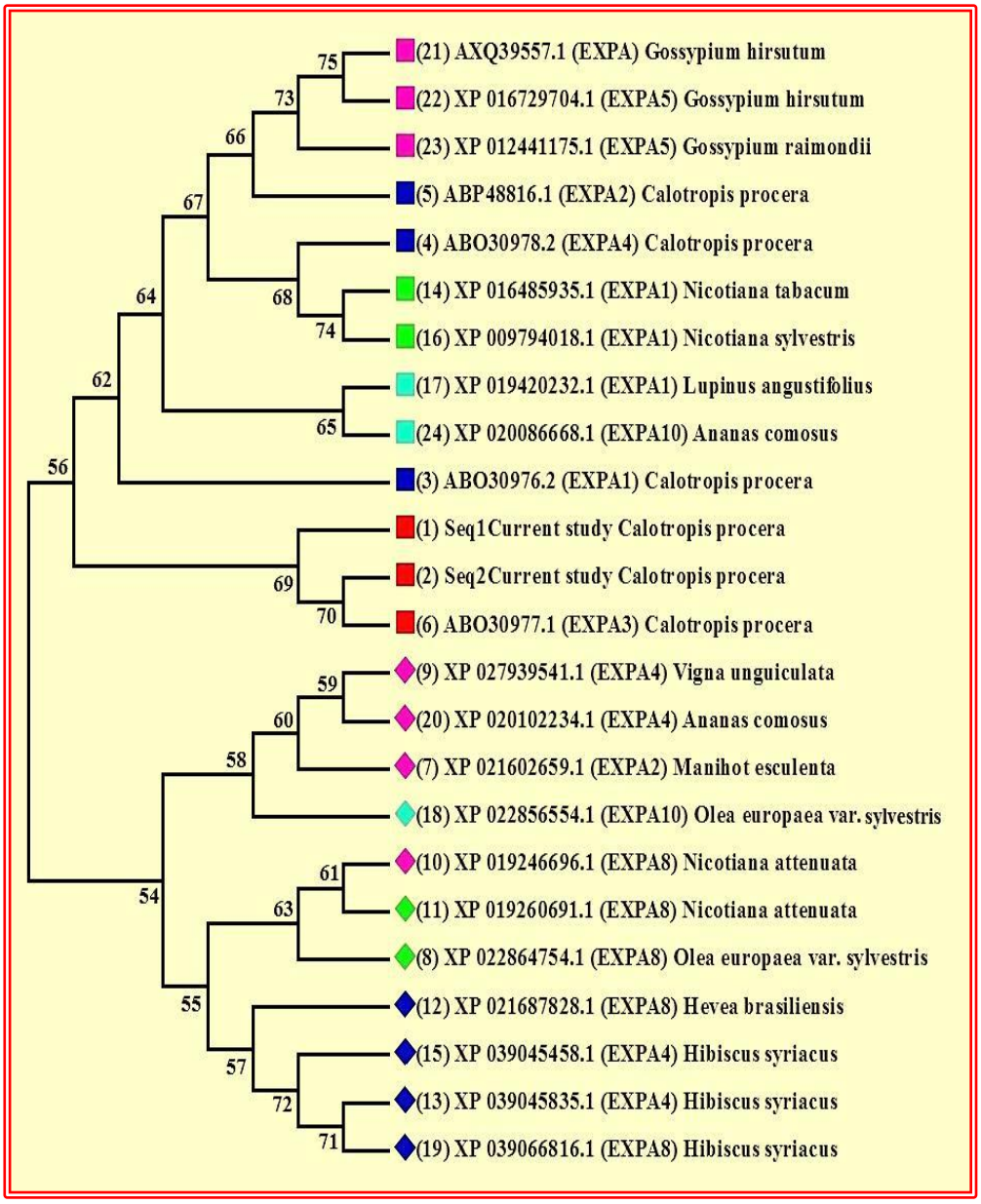


Fig. (6): Molecular phylogenetic analysis involved 24 amino acid sequences EXPAs genes from different species included two samples current study (CpEXPA3 gene from *Calotropis procera*) were conducted in MEGA 7.0 software program by Maximum Likelihood method. The tree with the highest log likelihood (-1439.94) is shown (Kumar *et al*., 2016).