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Research Note

Isolation and expression of antimicrobial Camel lactoferrin (*cLf*) gene in tobacco

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Abstract

In this study, cDNA encoding the *Camelus dromedarius* lactoferrin (*cLf*) was isolated from mammary gland by reverse transcription polymerase chain reaction and cloned to the pART27 expression vector. Its predicted amino acid sequence was analyzed and compared with lactoferrin of other mammalian species. Secondary and tertiary structures were predicted and modeled by online tools. Then *cLf* cDNA was expressed in *Nicotiana tobaccum* cv. Xanthi using *Agrobacterium* mediate transformation. Analysis of transgenic plants by PCR, RT-PCR and Real-time PCR showed the recombinant camel lactoferrin gene was expressed in transgenic plants. Tests on protein extract from transgenic tobacco leaves demonstrated antimicrobial activity against microbial strains. Evaluations for inhibition efficiency against *Staphylococcus aureus* PTCC1112 (ATCC 6538), *Escherichia coli* PTCC 1330 (ATCC 8739), *Bacillus subtilis* PTCC1023 (ATCC 6633) and *Candida albicans* PTCC5027 (ATCC 10231) were 48.8%, 52.7%, 34.6% and 41.6%, respectively. However, tests on protein extract from non-transformed plants did not display any sign of antimicrobial activity. Lactoferrin is a valuable protein that takes part in a large number of important physiological processes. Lf expression in plants provides a suitable system for large-scale production of this protein. Furthermore, it has been demonstrated that Lf expression in plants confers resistance to plant diseases, so this antimicrobial protein could also be used to improve crop quality.

Keywords: Antimicrobial activity; Lactoferrin; Molecular farming; Transgenic tobacco.
Abbreviations: *cLf*_camel lactoferrin; Lf_ lactoferrin; LPS_ Lipopolysaccharide; LTA_ Lipoteichoic acid; nptII_ Neomycin phosphotransferase; TA_ Teichoic acid.

Introduction

Plant molecular farming is the expression and production of valuable proteins in plants. The first recombinant pharmaceutical protein (the human growth hormone) was produced in transgenic plants in 1986 (Hiatt et al., 1989). Research has demonstrated that plants have the ability to produce mammalian functional proteins with therapeutic activity, for example serum proteins, hormones, vaccines, cytokines, enzymes and antibodies (Lienard et al., 2007). Tobacco (*Nicotiana tabacum*) is the main plant system applied for green bioreactors. Tobacco is a leafy plant with high leaf biomass and high levels of soluble proteins compared with other plant expression systems. Tobacco has also demonstrated transformation capability for transient and stable transformations (Menassa et al., 2001). Lactoferrin (Lf) is a non-hemic iron-binding glycoprotein of the transferrin family. Lf exists in most biological fluids such as milk and colostrum, saliva, tears, nasal and bronchial fluids, bile and gastrointestinal secretions (Rodriguez et al., 2005; Oztas-yesim et al., 2005). Lf is involved in various physiological functions, including those of iron transfer, immune response, anti-oxidant, anti-carcinogenic and anti-inflammatory properties and protection against microbial invasion. The Lf gene present in different chromosomes according to species and genomic size is estimated from 23 to 35 kb (Teng et al., 1997). The Lf genomic sequence is arranged into 17 exons and exon/intron junction and the number of amino acids in each exon is highly conserved, however intron size varies according to species (Teng 2002). Lf genes with a mRNA of about 1900–2600 bp are highly conserved among distinct species (Wally et al., 2007). The Lf is a glycoprotein containing 700-720 amino acids, with high homology among mammalian species. This protein is part of a polypeptide chain that is folded in the form of two regular lobes (N and C lobes), which are highly homologous (between 33–41% homology) (Oztas-yesim et al., 2005). The Lf polypeptide contains amino acids 1–332 that comprise the N-lobe, and 344–703 that comprise the C-lobe (Mazurier et al., 1980). These lobes can bind to iron, copper, zinc, manganese and carbonate ions (Baker et al., 2004). The major physiological activities of Lf are due to binding to these ions. The structural properties of Lf provide strong antimicrobial activity against gram positive and gram negative bacteria, fungi, yeasts and viruses (Rodriguez et al., 2005). The antimicrobial effect of Lf is generally caused by two mechanisms. One of these mechanisms is iron absorption in sites of infection, which deprives microbes of the nutrient. The other mechanism is that of direct antipathenogenic activity. Positive amino acids in the Lf structure can interact with negatively charged molecules on some bacterial, fungal and parasite surfaces, causing cell lysis (Baker and Baker, 2005). Lf glycoprotein has multiple important roles so, there is an expanding interest in establishing a system of expression that produces it in large amounts. Natural Lf was first purified from mammalian milk and colostrum. Then recombinant Lf was produced from animal, plant, bacterial and fungal expression systems (Furmanski et al., 1990; Furmanski et al., 1989). The first expression systems exploited baby hamster kidney cells to express human Lf (Stowell et al., 1991). Recombinant human and murine Lf were successfully produced in filamentous fungi *Aspergillus*

(Ward et al., 1992; Ward et al., 1995). Expression systems were developed in yeast, and these have produced human (Ying et al., 2004; Jiang et al., 2008) caprine, porcine (Wang et al., 2002) equine (Paramasivam et al., 2002) goat (Chen et al., 2007) and yak (Dong et al.,2006) recombinant Lf. Bacterial expression platforms were established to produce bovine (Tian et al., 2007) Kumin (Wang et al., 2010) and human (Chen et al., 2010) recombinant Lf. Viral plasmids have been used to enable production of Lf by insect expression systems. Recombinant human (Zhang et al., 1998; Liu et al., 2005; Liu et al., 2006) and porcine (Wang et al., 2005) Lf has been successfully expressed in this system. Using the technique of microinjection and direct infection with viral plasmids, transgenic animals have been generated that produce recombinant Lf. So far, human Lf has been expressed in transgenic goats, mice, rabbits and cows (Zhang et al., 2008; Nuijens et al., 1997; Han et al., 2008; Van-Berkel et al., 2002). Plants can also provide a suitable expression system for the production of important proteins such as Lf. This expression system is advantageous because there is no contamination in the final protein product. In addition, plants are able to synthesize proteins with the appropriate glycosylation, folding and functional activity. Human Lf has already been produced successfully in several plants such as tobacco, potato, tomato, rice, maize and Alfalfa; while bovine Lf was expressed in pear, tobacco, Arabidopsis and wheat plants (Mitra and Zhang 1994; Zhang et al., 1998; Rachmawati et al., 2004; Nandi et al., 2005; Suzuki et al., 2003; Chong and Langridge 2000; Lee et al., 2002; Samyn-Petit et al., 2001; Vlahova et al., 2005; Nguyen et al., 2011; Malnoy et al., 2003; Han et al., 2012). To the extent of our knowledge, this present research is the first report on cloning Lf from Persian breeds of *Camelus dromedaries*. It is also the first report of expression of this protein in plants. In this study, the cDNA of camel Lf was cloned and evaluated by bioinformatics tools. The cloned cDNA was expressed in tobacco (*Nicotiana tobaccum* cv xanthi) plants.

Results

Isolation, cloning and bioinformatics analysis of cLf

The *cLf* cDNA was isolated from the mammary gland of a Persian breed *Camelus dromedaries* (Fig 1). Fragments produced by digestion from pTZ57R-*cLf* and pART27-*cLf* (*Xho*I and *Hind*III) were about 2.15kb and this was consistent with the size of *cLf* cDNA (Fig 2). Accuracy of the sequence construct was further determined by DNA sequencing and the isolated sequence was registered to GenBank with the accession number KF915308. Sequence analysis demonstrated presence of full length cDNA of *cLf*. The full-length cDNA sequence of *cLf* comprises a 2127 bp open reading frame (ORF) encoding a mature protein of 689 amino acids with a signal peptide of 19 amino acids. The ORF of *cLf* started with an ATG codon and ended with a TAA codon (Fig 3). BLASTn in NCBI demonstrated that *cLf* was closely identifiable to Lf reported in other species. The *cLf* showed similarity with 99% identity to that of Arabian Camel (AJ131674.1 and AF165879.1), 99% identity to *Camelus ferus* (XM_006194472.1), 88% identity to *Ovis aries* (FJ541507.1) and *Capra hircus* (U53857.1), 85% identity to *Equus caballus* (NM_001163974.1), 84% identity to *Sus scrofa* (M81327.1), 83% identity to *Bos taurus* (L19981.1), 82% identity to *Homo sapiens* (X53961.1), 80% identity to *Macaca cyclopis* (EU523857.1), 76% identity to *Mus musculus* (FJ538998.1).

BLAST protein search results exhibited 99%, 98%, 77%, 76%, 78%, 75%, 76%, 72%, 74% and 67% identity with those of Arabian Camel, *Camelus ferus*, *Ovis aries*, *Capra hircus*, *Equus caballus*, *Sus scrofa*, *Bos taurus*, *Homo sapiens*, *Macaca cyclopis* and *Mus musculus*, respectively. In addition, the putative amino acid sequence had all the Lf conserved transferrin domains. Signal P and PSORT servers predicted secretion signal with 19 amino acids in the *cLf* structure (Fig 3). Multiple sequence alignment showed eight completely conserved amino acids (Asp60, Tyr92, Tyr192, His253, Asp395, Tyr433, Tyr526 and His595) in *cLf* structure. The scan prosite tool indicated that these amino acids were iron binding sites in all Lf structures. By using the online tools at http://web.expasy.org/cgi-bin/protparam/protparam, the theoretical molecular weight (MW) and the isoelectric point (pI) of *cLf*, proteins were predicted as 77.301 kDa and 8.61, respectively, which were very close to Lf from other species. Furthermore, the server Proteus was used to predict the secondary structure of *cLf*. Results indicated that *cLf* mainly consisted of random coils (59%), alpha helices (24%) and beta sheet (17%). A comparative 3D-structure model was made in order to better describe the *cLf* protein using Phyre2 protein fold recognition server. Data indicated that *cLf* structure was similar to the known structure of Lf from other species, which comprises two homologous globular lobes. Both lobes have the same fold, and these lobes are connected by a short a-helix. Each lobe is further divided into two α/β domains; the iron-binding site is located between these two domains. This structural arrangement is common among all Lfs (Fig 4).

Generation of transgenic plants and molecular analysis

The pART27expression vector with the kanamycin resistance gene was used for selection of transformed plants. Forty-five individual plants were regenerated under kanamycin selection medium. These plants were investigated with various different molecular techniques such as PCR, RT-PCR and quantitative real-time PCR (Fig 5). Detection of the 2127 bp *cLf* sequence in the genomic DNA and cDNA of transformed plants was performed by polymerase chain reaction and *cLf* specific primers. The *cLf* gene was detected in 29 of the 45 regenerated plants, some of these PCR products are shown in (Fig 6). The *cLf* sequence was not detected in the non-transformed, control plants. These results indicate that the *cLf* gene was correctly integrated into the genomic DNA of transformed plants. The PCR positive plants were subjected to quantitative real-time PCR analysis. Expression of *cLf* in tobacco plants was performed by the pART27 plant expression vector. RT-PCR analysis indicated presence of 2.3 kb *cLf* mRNA (Fig 6). Accumulation of *cLf* in transformed plants was studied in order to select plants with high levels of *clf*. Results of real-time PCR indicated that amounts of *cLf* mRNA were variable among transformed plants. The *cLf* gene was highly expressed in the two transgenic tobacco plants of Lf28 and Lf33, and lower levels were detected in the three transgenic tobacco plants of Lf24, Lf32 and Lf39. A trace amount of *cLf* was detected in other transgenic tobacco plants and the expression of *cLf* mRNA was not detectable in non-transformed tobacco plants (Fig 7). The two transgenic tobacco plants with higher levels of *cLf* were selected and used for antibacterial assay.

Table 1. Antibacterial activity of protein extracts from transgenic tobacco expressing cLf.

Strain	Growth inhibition
Staphylococcus aureus PTCC 1112	48.8*±3.6
Escherchia coli PTCC 1330	52.71*±3.2
Bacillus subtilis PTCC 1023	34.6*±2.9
Candida albicans PTCC 5027	41.6*±2.5

Asterisks (∗) indicate significant differences from plant extracts ($p < 0.05$) as determined by t-test. Data are average of three replicates.

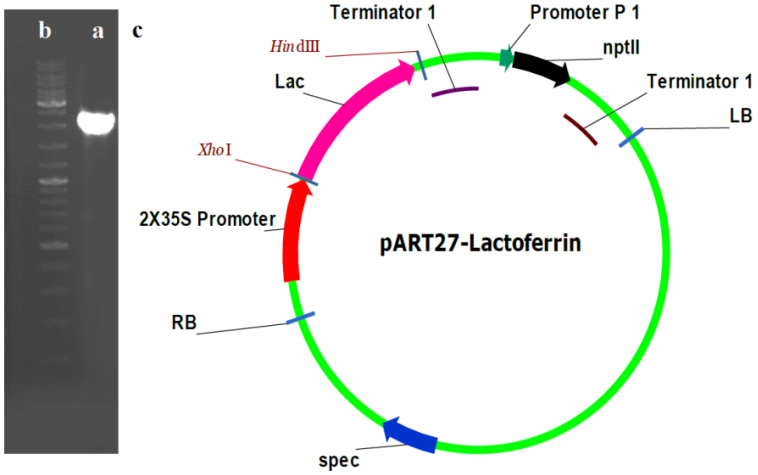

Fig 1. Electrophoresis of isolated fragment of cLf and pART27-cLf construct. (A) 2127 bpcLfcoding sequence. (B) 100 bp DNA Ladder. (C) Schematic diagram of plant expression vector pART27-cLf for tobacco transformation, 2X35S, double 35S promoter of cauliflower mosaic virus; Lac, cLf gene; Terminator 1, nopaline synthase (NOS) terminator and octopine synthase (OCS) terminator; Promoter P1, nopaline synthase (NOS) promoter; nptII, neomycin phosphotransferase II selectable marker; Spec, spectinomycin resistance gene; LB and RB, T-DNA left and right borders.

Antibacterial assay of cLf producing tobacco plants

To determine antibacterial activity of transgenic tobacco containing *cLf*, total protein extracts (without concentration or purification) were prepared from tobacco leaf (Lf33) that had the highest level of *cLf* and CFU counts were determined from that plant. The ability of *cLf* to inhibit growth of bacterial and fungal pathogens was evaluated in NA medium. As shown in (Table 1) and (Fig 8), protein extract from transgenic plants inhibited growth of tested bacterial and fungal strains. Growth rates were determined for *Staphylococcus aureus* PTCC 1112, *Escherchia coli* PTCC 1330, *Bacillus subtilis* PTCC 1023 and *Candida albicans* PTCC 5027 and estimated at 51.2 %, 47.3 %, 65.4 % and 58.4 % respectively. The tested bacterial and fungal strains exhibited different susceptibility to *cLf* and showed a reduction in colony growth mediated by the leaf protein extract of transgenic tobacco plants. This reduction in colony growth was not observed in the control protein extracts.

Discussion

Lactoferrin is a multifunctional iron-binding protein of the transferrin family (Baker and Baker, 2005). Current sequence databases contain Lf sequences from mammalian species such as human, mouse, cow, horse, pig, goat, sheep, buffalo and camel. Total nucleotide sequence of isolated *cLf* fragments was analyzed and translated with CLC, BLASRn and BLASTp software. BLAST analysis of the sequence data indicated that *cLf* was highly similar to other members of the Lf family. In the present study, *cLf* cDNA was cloned and the encoded polypeptide was shown to contain the characteristic transferrin domains shared by all Lf proteins. The 3-D modeling in phyre2 showed a structure similar to that of other mammalian Lf. In each lobe, alpha helix and beta sheet refer to N1, N2, C1 and C2 domain, enclosed in a deep cleft where it serves as the site for iron binding, similar to that in other species Lf (Fig 4). The proteins in this family have been characterized as acting to transfer iron to tissues and thus controlling levels of free iron in blood and body secretions. These tests have shown that the protein encoded by isolated *cLf* sequence has essential domains and as such is an appropriate structure for this activity. The pART27 binary vector with a kanamycin resistance selectable marker (*npt*II) was used to isolate of transformed plants in the selection medium. *Nicotiana tabacum* cv. xanthi plants were transformed via leaf discs by recombinant *Agrobacterium tumefaciens* carrying the pART27-*clf* construct. The transformed plants had usual appearance and similar growth characteristics to the non-transformed plants. Furthermore, transformed plants did not show phenotypic abnormality. For high *cLf* expression in plants, its gene was located under the control of 2X CaMV 35S strong promoter. High expression levels were determined and this revealed stability of the *cLf* gene in transgenic plants. Antimicrobial effect is one of the numerous biological activities of Lf; its mode of action and

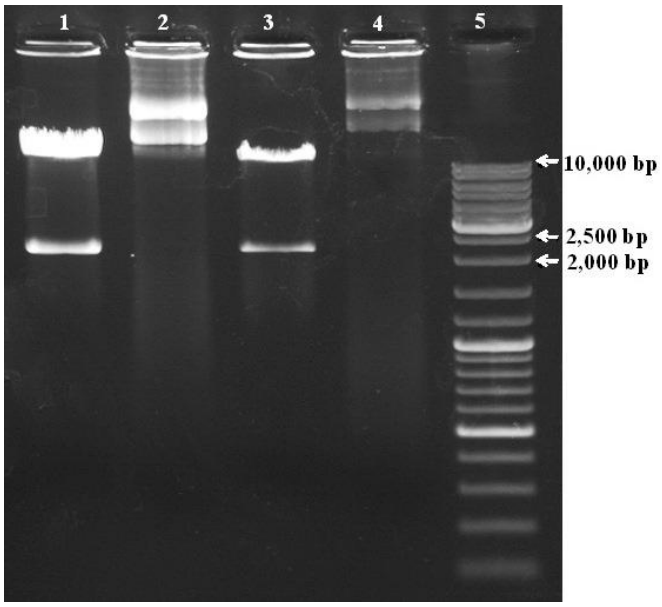

Fig 2. Electrophoresis of restriction digest of pART27-*cLf* plasmid DNA. (2), (4) undigested pART27-*cLf*plasmid. (1), (3) pART27-*cLf* plasmid double-digested with *Hind*III and *Xho*Ito obtain 2127 bp*cLf* fragment. (5)100 bp DNA Ladder.

a

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ATGAAGCTCTTCTTCCCCGCCCTGCTGTCCCTCGGGGCCCTTGGACTGTGTCTGGCTGCCTCTAAGAAAAGTGTTC
GATGGTGCACCACATCACCAGCAGAGTCGTCAAAATGTGCCCAATGGCAACGGAGGATGAAAAAAGTGCGTGGTCC
CTCTGTCACCTGCGTAAAGAAGACATCTCGCTTTGAATGCATCCAGGCCATCTCGACAGAAAAGGCAGATGCTGTG
ACCCTTGACGGTGGTTTGGTGTATGACGCAGGCCTGGACCCCTACAAGCTGCGGCCGATAGCGGCAGAGGTCTATG
GGACAGAAAACAATCCCCAAACCCACTATTATGCCGTTGCCATTGCCAAAAAGGGCACCAACTTTCAGCTGAACCA
GCTACAAGGCCTGAAGTCCTGCCATACCGGCCTTGGCAGGTCCGCTGGGTGGAACATCCCTATGGGGCTACTTCGT
CCATTCTTGGACTGGACAGGGCCTCCTGAGCCCCTCCAGAAAGCTGTGGCCAAATTCTTCTCTGCCAGCTGTGTTC
CCTGCGTGGATGGAAAAGAGTACCCCAACCTGTGTCAGCTGTGTGCAGGGACGGGGGAAAATAAATGTGCCTGCTC
CTCCCAGGAACCATATTTTGGCTACTCTGGTGCCTTCAAGTGTCTGCAAGATGGGGCTGGAGATGTGGCTTTTGTC
AAGGACAGTACAGTGTTTGAGAGCCTGCCAGCGAAGGCGGACAGGGACCAGTATGAGCTGCTCTGCCCAAACAATA
CTCGGAAACCAGTGGATGCATTCCAGGAGTGTCATCTAGCCCGGGTCCCTTCTCATGCTGTTGTGGCCCGAAGTGT
GAATGGCAAGGAGGACTTGATCTGGAAACTTCTCGTCAAGGCACAGGAAAAGTTTGGAAGAGGCAAGCCATCAGCA
TTCCAGCTCTTTGGCTCTCCTGCTGGGCAGAAGGACCTGCTGTTCAAAGACTCTGCCCTTGGGTTGTTGAGGATCC
CCTCAAAGATAGATTCTGGGCTGTACCTGGGCTCCAACTACATCACTGCCATCCGAGGCCTGAGGGAAACGGCGGC
GGAGGTGGAGTTGAGGCGCGCGCAGGTCGTGTGGTGCGCCGTGGGCTCCGACGAGCAGCTCAAGTGCCAGGAGTGG
AGCCGCCAGAGCAACCAAAGCGTGGTCTGTGCCACGGCCTCCACCACCGAGGACTGCATCGCCCTGGTGCTGAAAG
GAGAAGCTGATGCTTTGAGCTTGGATGGAGGATATATCTACATTGCGGGCAAGTGTGGCTTGGTGCCTGTCTTGGC
GGAGAGCCAACAATCCCCCGAAAGCAGTGGCTTAGATTGTGTGCATCGACCGGTAAAAGGGTATCTTGCCGTGGCG
GTTGTCAGGAAAGCAAATGACAAGATCACCTGGAATTCTCTGAGAGGCAAGAAGTCCTGCCACACCGCCGTGGACA
GGACCGCAGGCTGGAACATCCCCATGGGCCTGCTCTTCAAAAATACAGACTCCTGCAGATTTGATGAATTCTTCAG
TCAAAGCTGTGCCCCTGGGTCTGACCCAAGATCCAAGCTCTGTGCTCTGTGTGCAGGCAACGAGGAGGGCCAGAAC
AAGTGTGTGCCCAACAGCAGCGAGAGATACTATGGCTACACTGGGGCTTTCAGGTGCCTGGCTGAGAATGTTGGGG
ATGTTGCGTTTGTGAAAGATGTCACCGTCTTAGACAACACTGATGGAAAGAACACTGAGCAGTGGGCTAAGGATTT
GAAGCTGGGAGACTTTGAGCTGCTGTGCCTCAATGGCACCAGGAAGCCTGTGACTGAGGCTGAGAGCTGCCACCTG
GCCGTGGCCCCAAATCATGCTGTGGTATCTCGGATTGATAAGGTAGCACACCTGGAACAGGTGCTGCTCCGCCAAC
AGGCTCATTTTGGAAGAAATGGACAAGACTGCCCAGGCAAGTTTTGCTTGTTCCAGTCCAAAACCAAAAACCTCCT
GTTCAATGACAACACTGAGTGTCTGGCCAAACTCCAAGGCAAAACAACATATGAAGAGTATTTGGGACCACAGTAT
GTCACGGCCATTGCTAAGCTGAGACGATGCTCCACCTCCCCGCTTCTGGAAGCCTGCGCCTTCCTGATGAGGTAA
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b

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MKLFFPALLSLGALGLCLAASKKSVRWCTTSPAESSKCAQWQRRMKKVRGPSVTCVKKTSRFECIQAISTEKADAV
TLDGGLVYDAGLDPYKLRPIAAEVYGTENNPQTHYYAVAIAKKGTNFQLNQLQGLKSCHTGLGRSAGWNIPMGLLR
PFLDWTGPPEPLQKAVAKFFSASCVPCVDGKEYPNLCQLCAGTGENKCACSSQEPYFGYSGAFKCLQDGAGDVAFV
KDSTVFESLPAKADRDQYELLCPNNTRKPVDAFQECHLARVPSHAVVARSVNGKEDLIWKLLVKAQEKFGRGKPSA
FQLFGSPAGQKDLLFKDSALGLLRIPSKIDSGLYLGSNYITAIRGLRETAAEVELRRAQVVWCAVGSDEQLKCQEW
SRQSNQSVVCATASTTEDCIALVLKGEADALSLDGGYIYIAGKCGLVPVLAESQQSPESSGLDCVHRPVKGYLAVA
VVRKANDKITWNSLRGKKSCHTAVDRTAGWNIPMGLLFKNTDSCRFDEFFSQSCAPGSDPRSKLCALCAGNEEGQN
KCVPNSSERYYGYTGAFRCLAENVGDVAFVKDVTVLDNTDGKNTEQWAKDLKLGDFELLCLNGTRKPVTEAESCHL
AVAPNHAVVSRIDKVAHLEQVLLRQQAHFGRNGQDCPGKFCLFQSKTKNLLFNDNTECLAKLQGKTTYEEYLGPQY
VTAIAKLRRCSTSPLLEACAFLMR*
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Fig 3. Nucleotide sequence and the predicted amino acid sequence of *cLf*. (a) A full length cds (2127 bp) of *cLf*, which started with ATG and finished with TAA codons. (b) Predicted amino acid sequence (708aa) of *cLf*, the signal peptide and transferrin domains have been shown in red, green and yellow, respectively.

other activities are remarkable. Previous research has suggested that iron chelation and the effect of limiting iron absorption are reasons for antimicrobial activity of Lf (Caraher et al., 2007; Zarember et al., 2007). Furthermore, other reports have demonstrated the ability of Lf to bind to other macromolecules (proteins, DNA) (Baker and Baker, 2009; Nevinskii et al., 2009) as well as direct interaction with bacterial and fungal membranes (Ellison III et al., 1988; Orsi, 2004). This study presents successful expression of active *cLf* in *Nicotiana tabacum* cv xanthi. To our knowledge, this study is the first report of Camel lactoferrin expression in a plant system. It was observed that plants with higher lactoferrin expression showed stronger antimicrobial activity. For example, the transgenic plants Lf28 (data not shown) and Lf33 exhibited a significant reduction in colony formation in tested bacterial and fungal pathogens. Greater reduction in colony growth was observed in comparison to results previously reported (Mitra and Zhang, 1994; Chong and Langridge, 2000; Choi et al., 2003). High levels of recombinant *clf* in leaf tissue and stronger activity of camel lactoferrin may have contributed to strong antimicrobial activity that was observed in these tests. The silico study (data not shown) for structural properties of Lf showed that, compared to other species, the *cLf* had an additional transferrin motif. This additional motif probably caused stronger iron metabolism and antimicrobial activity. The general antimicrobial activity of Lf functions to restrict iron metabolism and this activity is common for both gram negative and gram positive bacteria. Chelation of iron away from the bacterial growth medium inhibits bacterial growth and reduces expression of factors affecting its virulence Arnold et al., 1977; Reyes et al., 2005). Another Lf mode of action is that of direct interaction with lipopoly saccharide (LPS) molecules and alteration to outer membrane permeability in gram-negative bacteria (Ellison, 1988; Rossi et al., 2002). In gram-positive bacteria, Lf interacts with teichoic acid (TA) or lipoteichoic (LTA) acid. In this study, tested bacterial strains to *cLf* had different levels of susceptibility and this was probably determined by cell wall structure (Table 1) and (Fig. 7). It seems that thickness of the peptidoglycan layer in gram positive bacteria prevented Lf activity. Lf in direct activity against gram positive bacteria needs other protein such as lysozyme. Lf interacts with LTA on the surface of gram positive bacteria that causes a decrease of the negative charge on its surface, thus allowing lysozyme to reach the peptidoglycan (Leitch and Willcox, 1999). Molecular farming, which includes the use of plants as systems for protein production, is a valuable tool for the production of important therapeutic and industrial proteins. One advantage of this method is the lack of contamination from pathogens and toxins in the recombinant protein. In addition, higher plants produce proteins with the appropriate post translational modification and function. *cLf* was expressed in tobacco plants because the biochemical properties of recombinant protein purified from plants were very similar to those of native proteins. The expression of Lf in a plant system is safe and efficient for large-scale production.

Materials and Methods

cLf cDNA isolation

Total RNA was extracted from mammary gland tissue of camel using RNX-Plus buffer (Cinnagen, Iran). According to buffer protocol, 100 mg of tissue was homogenized in liquid nitrogen. The homogenized powder was transferred to an RNase-free microtube then1 ml of RNX-Plus buffer was added, mixed gently, and stored at room temperature for 5 min. 0.2 ml of chloroform was then added to the microtube and contents were mixed gently. The mixture was centrifuged at 12,000xg at 4^{o} C for 15 min, the supernatant was transferred to a new tube and precipitated with an equal volume of isopropanol for 15 min on ice. The pellet was washed using 75% ethanol and dried and resuspended in 50 µl of RNase-free water. Quality and quantity measurements were taken for purified total RNA by 1.0 % agarose gel and Nano-Drop ND 1000 Spectrophotometer (Wilmington,USA). Five µg of purified RNA was used for cDNA synthesis. According to protocol, using15 pmol dNTPs, 20 U RNase inhibitor, 100 pmol 18 mer oligo-dT and 200 U M-Mulv reverse transcriptase in a 20µl final volume (cDNA synthesis Kit, Fermentas); the cDNA encoding of *cLf* was amplified by PCR using forward primer with *Xho*I site, 5′-ATACTCGAGCGCCTCAGGACCCCAGACAT-3′ and reverse primer with *Hind*III site, 5′-GAAAGCTTGGGAGAGGAAGGTGCAGCACA-3′. These primers were designed according to conserved regions of Lf sequences in Genbank (M81327, L19981, M83202, AJ010930, D88510, X78902, EU523857, AJ311874, AJ131674). According to protocols, 1µl of cDNA was used as a template for amplification by *Pfu* DNA polymerase (Fermentas).

Cloning of the cLf cDNA and sequence analysis

Blunt ends of *cLf* amplified fragments first ligated to the pTZ57R cloning vector (Fermentas) in *sma*I site. The prepared constructs were introduced to the DH5α strain of *E.coli* by electroporation. The *cLf* fragment then cut and ligated to pART27 binary vector into *Xho*I and *Hind*III sites. The pART27 expression vector comprising 2X CaMV 35S promoter for the inserted gene and neomycin phosphotransferse (*npt*II) was the selectable marker. The final construct was transformed into *Agrobacterium tumefaciens* strain C58 for plant transformation. Nucleotide and amino acid sequences of *cLf* were analyzed with BLAST at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignment analysis was performed by CLC (www.clcbio.com) and Vector NTI Suite 10. Subcellular localization and signal peptide prediction were performed using Signal P (http://www.cbs.dtu.dk/services/SignalP) and PSORT servers (http://psort.hgc.jp/form.html). Scan prosite tool (http://prosite.expasy.org/scanprosite/) and expasy protparam (http://web.expasy.org/cgi-bin/protparam/protparam) were used for protein analysis. Proteus2 server (http://www.proteus2.ca/proteus2/index.jsp) and CLC protein workbench tool were employed to predict secondary structures. The software Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was used to present a 3-D structure model.

Transformation of tobacco plants

Leaf discs of the tobacco plant (*Nicotiana tabacum cv xanthi*) were transformed by the *Agrobacterium tumefaciens* strain C58 comprising the *cLf* gene following the method described by (Horsch et al, 1985). Inoculated leaf discs were transferred to solid MS (Murashige and Skoog, 1962) co-cultivation medium containing 0.1mg/l NAA and 2mg/l BAP. Leaf discs were then placed on a selection medium1 containing0.1mg/l NAA, 2mg/l BAP, 400mg/l Cefotaxime and 50mg/l Kanamycin for shoot formation. Regenerated shoots were transferred to a selection medium 2 containing 0.1mg/l NAA,

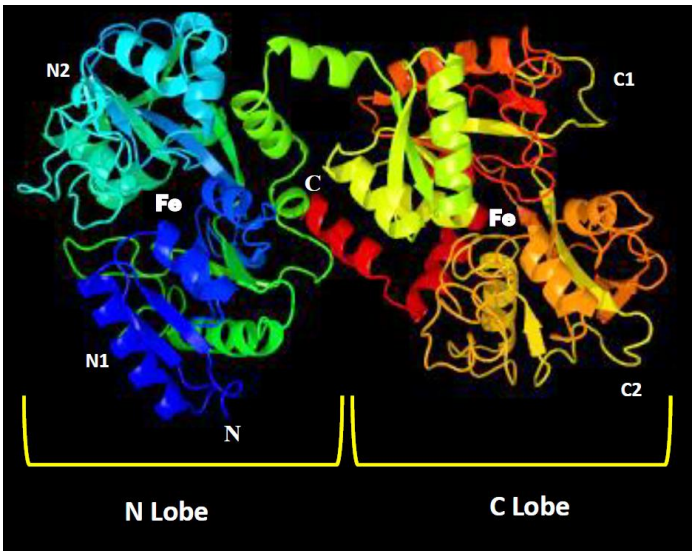

Fig 4. The 3D structure of *cLf*predicated by phyre2.The *clf*protein is folded into two lobes (N and C lobes) andeach lobe containstwo domains (N1, N2 and C1, C2). The iron binding site is located between the two domains in each lobe (Fe).

Fig 5. Generation of transgenic tobacco plants. A Transformed leaf disks. B Regenerated seedling in growth chamber. C Regenerated plants in green house.

2mg/l BAP, 400mg/l Cefotaxime and 100mg/l Kanamycin. Shoots that survived were then placed on a rooting medium containing 2mg/l IBA, 400mg/l Cefotaxime and 100mg/l Kanamycin. After 10 days, regenerated roots and small seedlings were transferred to sterile soil for growth in the greenhouse.

Detection of the cLf gene in transformed plants

Plant genomic DNA was isolated from leaf tissue of regenerated tobacco plants using the method of CTAB (Doyle and Doyle, 1987). The *cLf* gene was detected in the genomic DNA of the putative transgenic plants by PCR with specific primers of the *cLf* gene.

Quantitative real-time PCR

Total RNA was extracted from transformed plants using RNX-Plus buffer (CINNAGEN, Iran) and used for cDNA synthesis. Real-Time PCR reactions were prepared in 20µl total volume containing 5 µl cDNA (diluted) of transformed plant, 10 µl 2x Sybr Green buffer, 1 µl of 100 pmol primers (forward and reverse), and 0.5 U of Taq DNA polymerase (5 U/µl). Amplification was carried out in a lineGeneK thermal cycler (Bioer, China) under the following conditions: 94^{o} C for 2 min, 40 cycles of 94^{o}C for 10 s, 55.4^{o} C (Ta) for 15 s and 30 s of extension at 72^{o}C. The forward 5′-GCTGCTGTGCCTCAATGG-3′ and reverse 5′-GTGTG-CTACCTTATCAATCCG-3′ real-time PCR specific primers were designed based on 3′ region of *cLf* mRNA sequence.

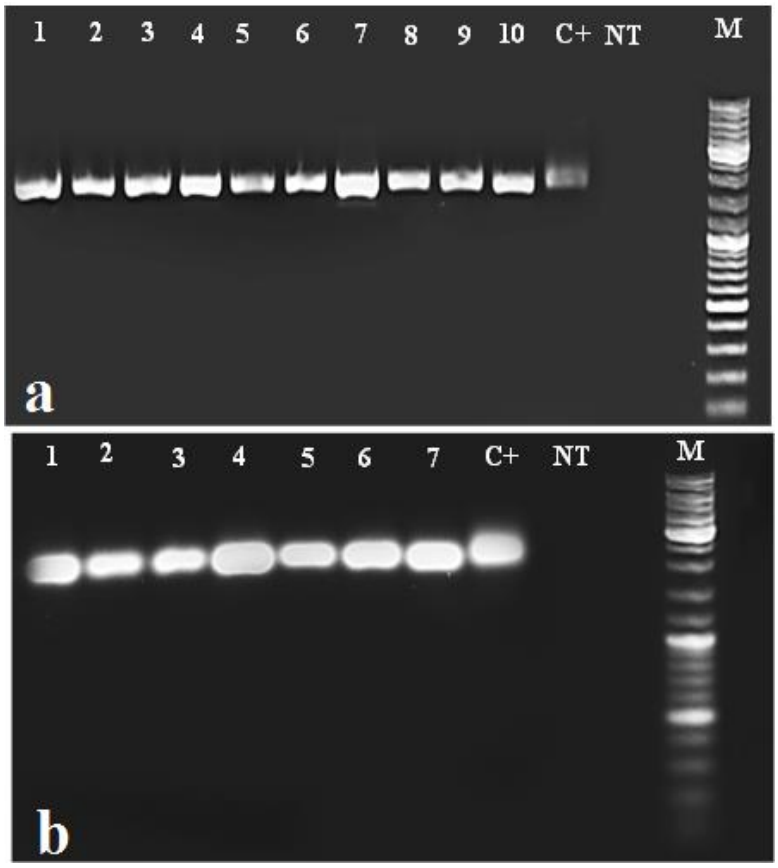

Fig 6. Electrophoresis of genomic and cDNA PCR products.(A) PCR.(B) RT-PCR. The predicted PCR product fragment is 2127 bp comprising the entire *cLf* coding sequence. (C+) positive control. (NT) non-transformed plant. (1-10) and (1-7) transgenic plants. (M)100 bp DNA Ladder.

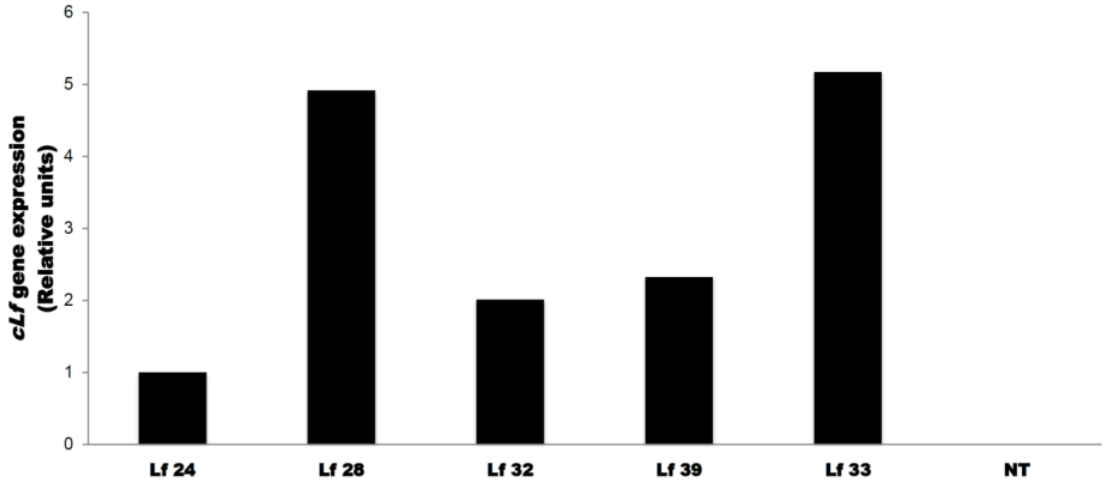

Fig 7. The expression levels of *clf* mRNA in transgenic tobacco plants were calculated using the comparative threshold cycle (Ct) method and were indicated as fold-changes compared with the reference plant NT. Independent transgenic tobacco plants (Lf 24 to Lf 33) are showed on the diagram. Non-transformed plants were used as the reference.

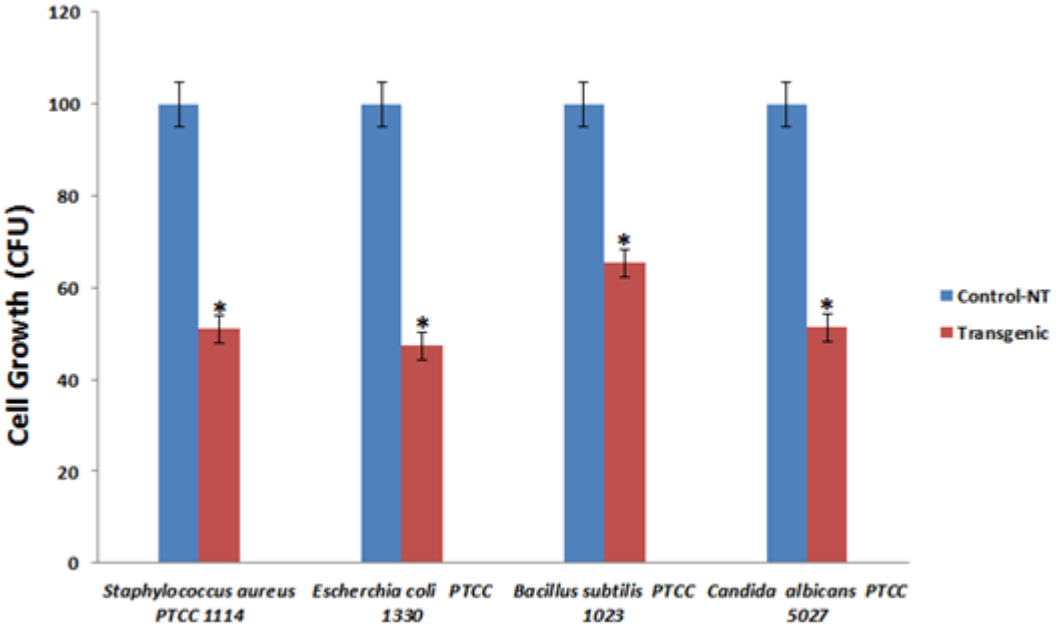

Fig 8. Antimicrobial assay of *cLf*. Bacterial and fungal strains were treated with 500 µg of total protein from transgenic and non-transgenic plants. Asterisks (*) indicate statistically significantdifference between transgenic and non-transgenic protein extracts ($p < 0.05$) that determined by t-test. Error bars represent SE. Data are average of three experiments

Real-time PCR data were analyzed using the comparative Ct method.

Protein extraction

Three g of fresh weight transformed tobacco leaves were homogenized in liquid nitrogen. An equal volume of freeze extraction buffer (50 mM potassium phosphate, pH 7) was added and centrifuged at 12000 xg for 20 min at 4 ◦C. Total soluble protein concentration was determined using the Bradford method (Bradford, 1976).

Antimicrobial activity Assay

Total soluble proteins of transgenic tobacco leaves were extracted to make assesments for antimicrobial activity. Three bacterial strains, *Staphylococcus aureus* PTCC 1112 (ATCC 6538), *Escherichia coli* PTCC 1330 (ATCC 8739) and *Bacillus subtilis* PTCC 1023 (ATCC 6633) and one fungal strain, *Candida albicans* PTCC 5027 (ATCC 10231) were selected for assay. These strains were grown in liquid LB to an optical density of 0.4 in A_{600} and 1 ml amounts of each culture were each transferred to distinct tubes. Then, an amount of 500 µg of total soluble proteins was added to each tube and incubated at 37°C on a shaker set at 270 rpm until the A_{600} of the control plant reached 1.0. Extracts from non-transformed tobacco plants were used as a negative control. After incubation, CFU counting was determined by serial dilution on NA plates and cell growth was computed (Chong and Langridge, 2000). For each strain, CFU tests were carried out in three replications and data comparison was made between protein extracts from transgenic and non-transformed plants. Statistical analysis was performed using Minitab 14 and Microsoft Excel software. Analysis of variance and the Student's T-test ($p < 0.05$) were employed to compute significant difference between transgenic and non-transformed protein extracts.

Conclusion

Using plants as bioreactors is a new approach to recombinant Lf production. Accumulation of large amounts of recombinant Lf in plants requires further research to identify stronger promoters and other methods of transformation. Up to now, most studies conducted on Lf have been limited to particular species. Thus, advantages of other types of Lf remain unexamined. Further study is required to utilize the advantages of Lf from other species. It has been shown that expression of Lf in plants confers resistance to plant diseases, which means that this system could be applied to improve crop resistance. Lactoferrin is a natural antimicrobial protein and it is expected that it will be applied as an antimicrobial drug and supplement.

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