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Expression of recombinant human lactoferrin in transgenic alfalfa plants

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Abstract

In order to produce human lactoferrin (Lf) in alfalfa (*Medicago sativa* L.), a construct containing human Lf cDNA under the control of cauliflower mosaic virus 35S promoter was engineered. As a selectable marker *bar* gene whose expression in plant cells confers tolerance to *L*-phosphinothricin (ppt) was used. Plants from a highly embryogenic alfalfa clone from the Bulgarian cultivar Obnova 10 were transformed using *Agrobacterium tumefaciens* mediated leaf disc method. Transgenic alfalfa plants were established from ppt-resistant calli *via* indirect somatic embryogenesis. The presence of human Lf cDNA in the genome of the selected regenerants was confirmed by polymerase chain reaction (PCR). Reverse transcriptase (RT)-PCR and Western blot showed expression of human Lf in leaf tissue. Studies on antibacterial effect of the recombinant glycoprotein were conducted and resistance of the transgenic alfalfa plants to two phytopathogens, *Pseudomonas syringae pv. syringae* and *Clavibacter michiganensis*, was demonstrated. The obtained results suggest that the expression of human Lf in alfalfa could be beneficial not only for producing recombinant protein for clinical application but also for crop quality improvement.

Additional key words: *Agrobacterium tumefaciens, Clavibacter michiganensis*, disease resistance, gene expression, *Medicago sativa*, *Pseudomonas syringae.*

Introduction

Lactoferrin (Lf) is an iron-binding glycoprotein, found in large amount in human milk and normally detected in most of exocrine secretions. Massive presence of Lf in the organism shows that it plays a role in numerous important physiological processes as iron absorption, natural protection against pathogens, anti-inflammatory activities, and modulation of the immune system.

Recombinant human Lf has been produced in yeast (Liang *et al*. 1993), fungi (Ward *et al*. 1992, 1995), mammalian cell cultures (Van Berkel *et al*. 1995), as well as transgenic animals (Nuijens *et al.* 1997, Zhang *et al*. 2008). A novel approach is provided by molecular farming which uses plants for production of recombinant proteins. Plants have a lot of advantages over expression systems mentioned above, the most important are: *1*) low production costs, *2*) lack of human or animal pathogens, oncogenic DNA sequences, and endotoxins, and *3*) ability to synthesize proteins from eukaryotes with correct folding, glycosylation, and activity (Horn *et al*. 2004). Human Lf has been successfully produced in tobacco (Salmon *et al*. 1998, Zhang *et al*. 1998), rice (Anzai *et al.* 2000, Nandi *et al.* 2002, Takase *et al.* 2005), tomato (Anzai *et al.* 2000, Lee *et al.* 2002), potato (Chong and Langridge 2000), sweet potato (Min *et al.* 2006), maize (Samyn-Petit *et al.* 2001), ginseng (Kwon *et al*. 2003), and barley (Kamenarova *et al.* 2007).

A suitable candidate for development of a new and efficient system for recombinant human Lf production is alfalfa (Stefanova *et al.* 2008). The advantages of this forage crop include a high biomass production at low cost, reduced need of fertilization, lack of toxic compounds, high vitamin, mineral, and protein content, as well as relatively ease of genetic transformation and *in vitro* clonal propagation. Although accumulation of recombinant proteins in plant leaves is considered as disadvantage because of their lower storage capacity, using dried leaf material seems to be easy and cheap alternative for some proteins. It has been shown that

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Abbreviations: CFU - colony forming unit; Lf - lactoferrin, ppt - *L*-phosphinothricin.
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recombinant phytase A, produced in *Medicago truncatula,* retains its enzymatic activity and plant crude extracts contain less impurities from non-desirable proteins after drying process which supposes easier subsequent isolation and purification (Abranches *et al.* 2005). Another research shows that recombinant antibodies produced in alfalfa have been highly stable in protein extracts and could be stored for a long time without detectable degradation because of weak proteolytic activity (Busse *et al.* 2001). Precise selection of plant species for molecular farming is very important for authenticity and quality of the final product. An important advantage of alfalfa is its ability to generate homogenous N-glycan structures (Bardor *et al.* 2003, D'Aoust *et al.* 2004) which ensures batch-to-batch reproducibility and makes it suitable for *in vitro* N-glycan modification. It means that using alfalfa expression system for recombinant protein production could solve some problems relevant to plant N-glycosylation pattern and potential allergenicity of the product.

In this paper, we report expression of recombinant human Lf in transgenic alfalfa plants. The aim of our study is development a new and reliable plant production system for valuable proteins. On the other hand, taking into account the antimicrobial activity of Lf, we assume that the expression of human Lf cDNA in alfalfa plants leads to increased resistance to some plant pathogens of significant economic importance and could be used as a strategy for crop quality improvement.

Materials and methods

Vector construction: The original plasmid pBI-LF containing human Lf cDNA was kindly provided by Dr. Hiroyuki Anzai (Ibaraki University, Ami, Ibaraki, Japan). The human Lf cDNA was subcloned into the plant binary expression vector pCB 302-3 (Xiang *et al*. 1999) using Xba I restriction sites. The resultant expression vector was named pCB-LF and transferred into *Agrobacterium tumefaciens* strain LBA4404. It contained human Lf cDNA under control of the constitutive *cauliflower mosaic virus* (*CaMV*) *35S* promoter and *bar* selectable marker gene, encoding phosphinothricine acetyltransferase (PAT), under control of the nopaline synthase promoter. This allowed selection of transgenic alfalfa plants using *L*-phosphino-thricine (ppt), the active compound of herbicide *BASTA*. Schematic representation of the binary pCB-LF vector is given in Fig. 1.

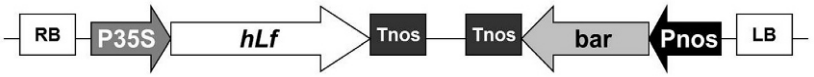

Fig. 1. Binary vector pCB-LF containing human Lf cDNA for alfalfa transformation: P35S - *cauliflower mosaic virus 35S* promoter; *hLF* - human lactoferrin cDNA; Tnos - nopaline synthase terminator; *bar* - phosphinotricin acetyltransferase gene; Pnos - nopaline synthase promoter.

Genetic transformation and plant regeneration: *In vitro* alfalfa (*Medicago sativa* L.) plantlets from highly embryogenic line R4 of cv. Obnova 10 (Barbulova *et al*. 2002) were developed from sterilized seeds on hormone free Murashige and Skoog (1962; MS) culture medium. Plantlets were cultivated under a 16-h photoperiod, irradiance of 40 μmol m^{-2} s^{-1}, and temperature of 24 °C. *Agrobacterium tumefaciens* mediated leaf-disk transformation (Horsch *et al.* 1985) with a few modifications was applied. Explants (leaves and petioles) were pre-cultivated on solid B5II medium (Barbulova *et al.* 2002) in the dark for 24 h. Bacterial cells were grown at 28 °C for 24 h in Luria-Bertani (Bertani 1951; LB) medium, containing 100 mg dm^{-3} rifampicin and 100 mg dm^{-3} kanamycin. The overnight cultures were centrifuged at 4 000 *g* and 4 °C for 10 min and resuspended in B5II liquid medium without 2,4-dichlorophenoxyacetic acid (2,4-D) to final absorbance (A_{600}) = 0.6. Explants were submerged in bacterial suspension for 15 min at room temperature and then transferred to solid B5II medium in the dark for 48 h. After co-cultivation, they were washed with sterile distilled water, blotted dry on a sterile filter paper, and placed, for callus induction, on B_5II medium containing 2 mg dm^{-3} ppt (*Duchefa Biochemie*, Harleem, The Netherlands) and 500 mg dm^{-3} abricef (*Actavis*, Razgrad, Bulgaria) against bacterial contamination. Selective concentration of ppt was determined according to previous sensitivity test. Individual ppt-resistant cell clusters were transferred to fresh selective medium every 3 weeks. Plants were obtained from ppt-resistant calli using the established protocol for regeneration *via* indirect somatic embryogenesis of alfalfa cv. Obnova 10 (Barbulova *et al.* 2002) (Fig. 2*A-D*).

In order to test regenerated putative transgenic plants for the expression of *bar* gene, an enzyme assay based on chlorophenol red pH indicator was carried out. Plants were placed for root formation on selective MS medium containing 2 mg dm^{-3} ppt and 50 mg dm^{-3} chlorophenol red, pH 6.0, having a red colour. Untransformed alfalfa plants cultivated under the same conditions were used as a control. Observation was performed after 3 weeks of cultivation. Change of the medium colour from red to

yellow/orange was considered as indicative for transgenic plants (Fig. 2*E*).

Polymerase chain reaction (PCR): Genomic DNA was isolated using *Nucleon*™ *Phytopure*™ genomic DNA extraction kit (*GE Healthcare*, Buckinghamshire, UK). Presence of the insert in the plant genome was verified by amplification of 356 bp fragment of the human Lf cDNA. A pair of primers LTF8 forward (5'-TCACTGCCA TCCAG AACTTG-3') and LTF9 reverse (5'-TCTGA TCTCCTAACCACCGC-3') was used. Amplification was carried out in thermocycler (*Quanta Biotech*, Surrey, UK). Reaction mixtures contained 1× Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 μM of each primer, 1 U of Taq DNA polymerase (*Fermentas*, Burlington, Canada) and 100 ng of genomic DNA. Ten nanograms of pCB-LF were used in positive PCR control mixture. Amplification was performed under following conditions: 5 min initial denaturation at 94 °C, 35 cycles of 45 s denaturation at 94 °C, 45 s annealing at 58 °C and 1 min elongation at 72 °C; final elongation at 72 °C for 8 min. The amplification products were separated in 0.8 % (m/v) agarose gel and visualized under UV-radiation after staining with ethidium bromide.

Karyotype analysis: In order to identify the chromosome constitution of *in vitro* alfalfa transgenic plants, a cytological technique according to Gecheff (1989) was applied with slight modification. Fresh roots were incubated in 0.025 % (m/v) colchicine solution saturated with α-bromonaphtalene for 2 h prior to fixation in Clarke solution (ethanol and glacial acetic acid, 3:1). Feulgen method was performed for staining the chromosomes after hydrolysis in 1 M HCl at 60 °C (water bath) for 7 min and treatment with Shiff's reagent for 1 h. Then the root tips were additionally macerated in 4 % (m/v) pectinase solution for 12 min, briefly rinsed in distilled water, and gently squashed on slide glasses in a drop of 0.8 % (m/v) acetocarmine. The karyotype analysis of the metaphase meristematic cells was done with microscope (*Olympus BX 41*, Tokyo, Japan).

Reverse transcriptase-polymerase chain reaction (RT-PCR): Total RNA was isolated using *Illustra* RNAspin mini isolation kit (*GE Healthcare*). Concentration and sample purity were determined by *Nanodrop 2000* spectrophotometer (*Thermo Fisher Scientific*, Wilmington, DE, USA). Absence of DNA contamination was proven by control PCR reaction of RNA using a pair of primers for amplification of a fragment from 25S ribosomal genes. Oligo $(dT)_{18}$ primer was employed for synthesis of first strand cDNA from RNA templates using *RevertAid*™ first strand cDNA synthesis kit (*Fermentas*). Each reaction contained 3 μg of total RNA. Reverse transcription was performed at 42 °C for 60 min and the reaction was terminated by heating at 70 °C for 5 min. The product of first strand cDNA synthesis was then amplified by PCR using forward LTF8 and reverse LTF9 primers under conditions described above. The amplification products were separated in 1 % (m/v) agarose gel and visualized under UV-radiation after staining with ethidium bromide.

Enzyme-linked immunosorbent assay (ELISA): Indirect ELISA was performed according to the standard procedure with total protein extracts from transgenic and non-transgenic leaf tissue ground in carbonate buffer (pH 9.6) in a 1:10 dilution. Extracts were bound in 96-well microtiter plate overnight. Commercially available Lf from human milk (*Sigma-Aldrich*, St. Louis, MI, USA), diluted in carbonate buffer at concentrations of 5, 10, 50, and 100 ng per well, was used as positive control. The plate was blocked with 1 % (m/v) BSA in PBST buffer, pH 7.4, containing 136 mM NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.6 mM KCl, and 0.05 % (v/v) *Tween 20*. It was incubated with rabbit anti-human Lf antibody (*Sigma-Aldrich*) in PBST (1:10000) and then with goat anti-rabbit IgG alkaline phosphatase conjugate (*Sigma-Aldrich*) in the same dilution. The absorbance was measured at 405 nm in an ELISA *Humareader* (Human, Egypt) after incubation with a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) solution.

Immunoblot detection of human lactoferrin in transgenic plants: Leaf material from transformed and untransformed alfalfa plants was ground in a mortar and a pestle in liquid nitrogen and homogenized in 50 mM Tris-HCl (pH 7.4) extraction buffer. Homogenates were centrifuged at 14 500 *g* and 4 °C for 10 min. Total soluble protein content in the supernatant was determined (Bradford 1976). Approximately 30 μg of total protein from each sample was heated at 85 °C for 3 min (Suzuki *et al.* 2003) and separated on 10 % (m/v) SDS-PAGE. Commercially available Lf (100 ng) from human milk (*Sigma-Aldrich*) was used as a positive control. Protein bands were transferred onto a nitrocellulose membrane (*GE Healthcare*). The membrane was dried overnight at room temperature and blocked with 3 % BSA in PBST buffer, pH 7.4, at room temperature for 1 h. Rabbit anti-human Lf antibody in PBST (1:2000) was used as primary antibody and goat anti-rabbit IgG alkaline phosphatase conjugate (1:5000) was used for immunodetection. The recombinant protein was visualized after incubation of membrane in BCIP/NBT solution at room temperature in the dark for 10 min.

Bioassay for resistance of transgenic alfalfa plants to pathogenic bacteria: Strains of bacteria *Pseudomonas syringae* pv. *syringae* (causing stem blight) and *Clavibacter michiganensis* (causing wilting), both alfalfa pathogens, were used. The bacteria were cultivated in Petri dishes on a solid potato dextrose agar medium in the dark at 25 °C for 24 h. The bacterial cells from both

species were diluted with sterile water to concentration 1×10^6 CFU cm^{-3}, referred as a work concentration.

On the upper part of each segment of the detached leaves of alfalfa 5 mm^3 of *Pseudomonas syringae* pv. *syringae* suspension was placed . The leaves were kept on a moist filter paper in high humidity (95 %) at 25 ^oC, a 16-h photoperiod, and irradiance of 40 μmol m^{-2} s^{-1}. The occurrence of necrosis and chlorosis was scored 5 d after inoculation.

Fresh branches (approximately of the same age and about 15 cm in length) of alfalfa plants grown in a greenhouse were dipped in 50 cm^3 suspension of *Clavibacter michiganensis* in 100 cm^3 volume flasks and kept under natural irradiance at room temperature. The wilting of the branches was evaluated after 5 d by the following scale: without symptoms (-), beginning of wilting (+), visible wilting and chlorosis (++) , progressed wilting and withered zones (+++), and complete wilting (++++) (Fig. 2*H- I*).

Results

Transformed alfalfa plants were regenerated *via* indirect somatic embryogenesis on selective media containing 2 mg dm^{-3} ppt (Barbulova *et al.* 2002) (Fig. 2*A-D*). Including chlorofenol red in the selective medium for root formation allowed rapid screening of transgenic plantlets expressing *bar* gene. Cell proliferation in the presence of ppt caused medium colour shift from red to yellow/orange in 3 weeks of cultivation due to pH decrease (Fig. 2*E*). Five independent transformants (35, 43, 49, 53 and 79) were selected and *in vitro* multiplied for further analyses.

The presence of human Lf cDNA sequence in plant genomic DNA isolated from leaf tissue was confirmed by PCR analysis in the five selected clones. The products of PCR amplification (Fig. 3*A*) corresponded to the expected 356 bp PCR product when plasmid pCB-LF was used as a template. No amplification was observed in the DNA controls from non-transformed plants.

Twenty micropropagated plants from the five transgenic clones confirmed by PCR analysis as well as the non-transformed control were involved in cytological analysis in order to determine their chromosome number and structure. All analyzed plants except one, which was aneuploid (2n=4x=31), had a chromosome number and karyotype structure characteristic for the starting alfalfa tetraploid line (2n=4x=32). Thus, it was confirmed that the ploidy level of the experimental plant material was not affected by transformation and regeneration procedures.

To confirm the expression of human Lf in alfalfa plants, RT-PCR, ELISA, and Western blot analyses were performed. Accumulation of human Lf mRNAs was detected only in the clone 53 using gene-specific primers, amplifying a fragment corresponding to the expected 356 bp PCR product when positive control was used as a template (Fig. 3*B*). Although presence of human Lf transcripts was not detected in the other clones, they were included in further experiments.

Transgenic plants were screened for presence of the recombinant protein in leaf tissue and Lf expression levels were determined by indirect ELISA method. The amount of plant produced human Lf was calculated by comparison of the absorbance values obtained for extracts from transgenic plants with absorbance values of purified Lf from human milk with a known protein concentration. Quantitative analysis revealed that human Lf content was 0.0047 % of total soluble protein (TSP) in the clone 53. Transgenic clone 35, in which human Lf transcripts were not detected by RT-PCR, expressed the recombinant protein but in lower level 0.0035 % of TSP. In the other clones tested, the recombinant protein was not detected. To confirm expression and the size of the recombinant human Lf, Western blot analysis was performed. The protein detected in alfalfa leaf tissue from the clone 53 had a molecular mass of approximately 80 kDa, similar to that of the full-length native human Lf. A band of the same size was also detected in leaf extracts from the clone 35 but the signal was too weak (not shown). Extracts of non-transformed alfalfa plants did not react with the anti-human Lf antibody.

In order to evaluate disease resistance due to expression of human Lf cDNA, two bioassays using strains of bacteria *Pseudomonas syringae* pv. *syringae*, causative agent of bacterial stem blight, and *Clavibacter michiganensis*, causing bacterial wilt, were performed. For the experiment, 6 alfalfa plants from the transgenic clone 53, for which expression of human Lf was confirmed, and 1 plant from the clone 79, for which the recombinant protein was not detected, were used. Non-transformed alfalfa plants were used as controls.

After *Pseudomonas syringae* pv. *syringae* inoculation on detached alfalfa leaves, symptom progress was followed for 5 d. Four plants expressing human Lf cDNA showed less severe *Pseudomonas syringae* pv. *syringae* infection than the plant not expressing human Lf and the non-transformed controls (Fig. 2*I*). Inoculation of fresh branches of alfalfa with *Clavibacter michiganensis* caused different level of symptom development. Four transgenic plants of the clone 53 showed increased resistance to the pathogen, one developed no symptoms (-) and the rest showed reduction of symptoms evaluated by the scale as (+), compared to the non-transformed control (++) and the plant from the clone 79 (+++). As a result from these bioassays, four transgenic plants were determined as less susceptible to both the pathogens presumably due to expression of human Lf.

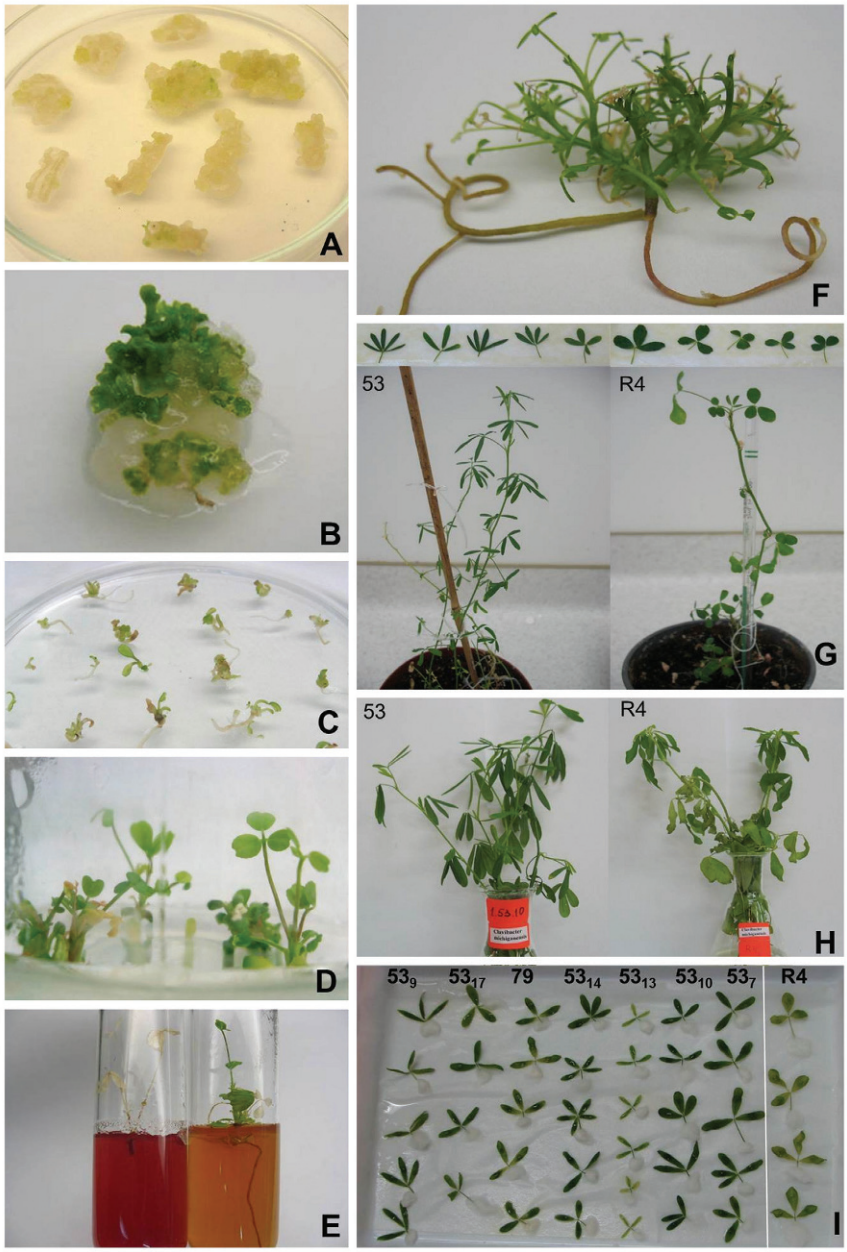

Fig. 2. Callus development at cut-ends of explants on selection medium (*A*), embryo formation (*B*), plantlets established *in vitro* (*C,D*), chlorophenol red assay: a control non-transformed plant (*left*) and a transformed plant (*right*) (*E*), an *in vitro* cultivated transgenic plant from clone 35 which possessed branched stem, shortened internodes, and small leaves (*F*), phenotype of a non-transformed plant (R4) and transgenic alfalfa (clone 53): plants of clone 53 were predominantly multifoliate with elongated leaflets compared to the controls which possesed threefoliate leaves (*G*), bioassay for resistance of transgenic alfalfa to pathogenic bacteria: alfalfa branches from the transgenic clone 53 and the non-transformed plant (R4) dipped in a suspension of *Clavibacter michiganensis* after 5 d of inoculation (*H*), and detached leaves of transformed and non-transformed alfalfa 5 d after inoculation with *Pseudomonas syringae* pv. *syringae* (*I*).

Discussion

In this report, we described for the first time the expression of recombinant human Lf in transgenic alfalfa plants. The introduction of Lf cDNA under control of the constitutive *35S CaMV* promoter into the alfalfa genome was appropriate strategy for development a new model system for investigation the opportunity for obtaining

high quality recombinant protein as well as for quality improvement of this important forage crop. As the production of recombinant protein with high authenticity is of significant importance for its application as therapeutic agent as well as for achieving crop quality improvement, we aimed obtaining full length Lf in alfalfa plants. Our results have shown that transformation of alfalfa with human Lf cDNA under control of the *35S CaMV* promoter using *Agrobacterium tumefciens*-mediated gene transfer leads to production of recombinant human Lf with molecular mass of approximately 80 kDa in leaf alfalfa extracts which corresponds to the size of the native protein. Maximum expression level of the

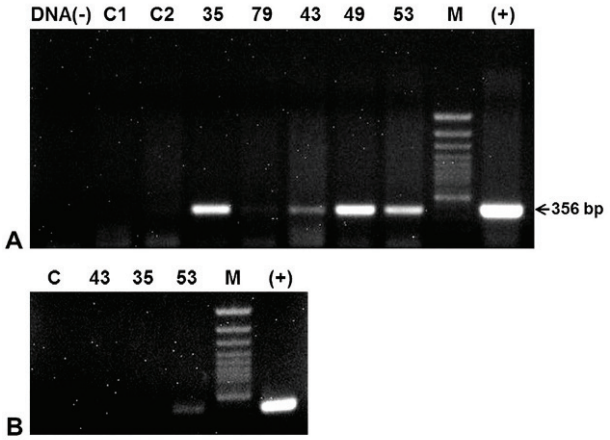

Fig. 3 Transgenic status confirmation of transgenic alfalfa. PCR analysis for detection human Lf cDNA in the plant genome (*A*): non-template (DNA -), DNA from non-transformed plants (C1 and C2), DNA from transformed clones (35, 79, 43, 49, and 53), molecular mass marker (M), and pCB-LF plasmid as a positive control (+). RT-PCR analysis confirming presence of human Lf mRNA in plant cells (*B*): cDNA from non-transformed controls (C), cDNA from transformed clones (43, 35, and 53), marker (M), and a positive control (+).

recombinant human Lf, determined by ELISA assay, was 0.0047 % of TSP (clone 53). It is comparable to previously reported expression levels in transgenic alfalfa plants bearing a gene encoding the avian reovirus σC protein under control of the *35S CaMV* promoter (Huang *et al*. 2006) and could be explained with the relative low efficiency of this constitutive promoter in alfalfa compared to other dicotyledoneus species (D'Aoust *et al*. 2004). Different expression of recombinant human Lf in leaf tissue are reported in other systems – tobacco (0.1 - 0.8 % of TSP) (Zhang *et al*. 1998, Salmon *et al*. 1998) and potato (0.01 - 0.1 % of TSP) (Chong and Langridge 2000). A very low signal (Western blot experiments) was observed in the leaf extracts from the clone 35 probably due to low expression of the protein and changes in growth performance and phenotype of the plants (Fig. *2F*). It is known that stress under cultivation leads to catabolic changes and increased synthesis of stress gene products which influences plant growth and development and affects recombinant protein expression in transgenic plants (Jamal *et al*. 2009). That could be a possible explanation of the low amounts of recombinant human Lf in the transgenic alfalfa regenerants which possess poor growth performance and variation in morphology. Although the obtained protein expression was unsatisfactory, we assume that the alfalfa plants provide a potential for high quality recombinant protein production and further analyses on glycosylation pattern, characteristics, and biological activity could confirm its effectiveness as expression system for the use in pharmacy.

On the other hand, expression of recombinant human Lf could be beneficial for achieving crop quality improvement. It has been shown that expression of antimicrobial proteins in plants is a successful strategy for increasing resistance to plant pathogens (Mitra and Zhang 1994, Zhang *et al.* 1998, Lee *et al.* 2002, Takase *et al.* 2005, Nguyen *et al.* 2011). One of the most important properties of Lf is its ability to act as natural anti-bacterial, anti-viral, anti-fungal, and anti-parasitic agent. It has bacteriostatic activity due to sequestration of iron in the medium required for microbial metabolism as well as direct bactericidal effect involving N-terminal antimicrobial domain (lactoferricin) which damages bacterial cell membranes (Yamauchi *et al.* 1993). Mitra and Zhang (1994) reported bactericidal activity of truncated recombinant human Lf, produced in transgenic tobacco cells, against four phytopathogenic bacteria. It has been shown that expression of full length human Lf displayed bacteriostatic activity and thus conferred resistance to bacterial wilt in transgenic tobacco and tomato (Zhang *et al.* 1998, Lee *et al.* 2002). Significant resistance against bacterial seedling blight disease has been also observed in rice plants expressing human Lf as well as lactoferricin (Takase *et al.* 2005).

Our study showed that the alfalfa plants expressing human Lf were less susceptible to two bacterial pathogens: *Pseudomonas syringae* pv. *syringae* and *Clavibacter michiganensis*. The obtained results indicate that transgenic plants could be potentially used for controlling bacterial stem blight and bacterial wilt diseases, both of economic importance, reducing yields and altering quality of alfalfa plantations.

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