Plant Cell Reports (2019) 38:391–401
https://doi.org/10.1007/s00299-019-02377-2

ORIGINAL ARTICLE

Production of functional human interleukin 37 using plants

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Received: 10 November 2018 / Accepted: 7 January 2019 / Published online: 18 January 2019

Abstract

Key message **We demonstrate for the first time that a fully bioactive human IL-37, a newly discovered cytokine acting as a fundamental inhibitor of innate immunity, can be recombinantly produced in plant cells.**

Abstract Interleukin 37 (IL-37), a newly discovered member of the interleukin (IL)-1 family of cytokines, plays a pivotal role in limiting innate inflammation and suppressing acquired immune responses, thus holding high potential for treating a wide array of human inflammatory and autoimmune disorders. In this study, we have developed transgenic plants as a novel expression platform for production of human IL-37 (IL-37). Plant transformation vectors synthesizing various forms of the b isoform of IL-37, including an unprocessed full-length precursor form (proIL-37b), a mature form (matIL-37b) and an IL-37 fusion protein in which IL-37b was fused to soybean agglutinin (SBA-IL-37b), have been constructed and introduced into tobacco plants. The expression of all forms of IL-37b was driven by a strong constitutive 35S promoter. Transgenic tobacco plants were generated with each of these constructs. Depending on the form of IL-37b being produced, the expression level of proIL-37b reached approximately 1% of TSP, while matIL-37b expression was substantially lower (0.01% TSP). Fusion to SBA substantially increased the expression of matIL-37b, with the expression level of fusion protein accounting for 1% of TSP. Functional analysis using a cell-based in vitro assay showed that plant-made matIL-37b and proIL-37b are both biologically active, but plant-made matIL-37b exhibited significantly greater biological activity than proIL-37b. These results demonstrate that plants have great potential of being a green bioreactor for low-cost, large-scale production of biologically active IL-37.

Keywords Human IL-37 · Cytokine · Anti-inflammatory property · Plant molecular farming · Genetic transformation · Recombinant protein · Functional characterization

Introduction

Interleukin-37 (IL-37), formerly known as IL-1F7, is a newly discovered member of the IL-1 cytokine family, which functions as a natural suppressor of inflammatory and immune responses. It has been shown that overexpression of IL-37 in epithelial cells or macrophages almost completely suppressed production of pro-inflammatory cytokines such as IL-1α, IL-1β and TNFα, whereas the silencing of endogenous IL-37 increased the abundance of these cytokines in human blood cells (Nold et al. 2010; Tete et al. 2012). Mice with transgenic overexpression of IL-37 were protected from lipopolysaccharide (LPS)-induced shock (Nold et al. 2010), dextran sulfate sodium (DSS)-induced colitis (McNamee et al. 2011), ischemic heart injury (Xiao et al. 2018), ischemic cerebral injury (Patel et al. 2014), ischemic hepatic injury (Sakai et al. 2012) and obesity-induced insulin resistance and type 2 diabetes (Ballak et al. 2014). Furthermore, administration of exogenous IL-37 in mice was shown to ameliorate experimental psoriasis (Teng et al. 2014), alleviate rheumatoid arthritis (Ye et al. 2015), bleomycin induced experimental lung injury/fibrosis (Li et al. 2018) and atherosclerosis (Ji et al. 2017), decrease renal ischemia–reperfusion injury (Yang et al. 2015) and inhibit the growth of cancer cells (Deng et al. 2018). While the anti-inflammatory mechanisms of IL-37 are still not fully

Communicated by Baochun Li.

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understood yet, several studies have suggested that IL-37 binds to the receptor IL-18Rα instead of IL-18 and recruits the orphan decoy IL-1R8, which leads to suppression of innate and acquired immunity, along with augmentation of the anti-inflammatory pathway (Riva et al. 2012; Lunding et al. 2015; Nold-Petry et al. 2015; Li et al. 2015). Other studies also suggest that following activating cleavage of signal peptide by caspase-1, mature IL-37 translocates to the nucleus, where it suppresses transcription of pro- inflammatory genes (Bulau et al. 2014; Dinarello et al. 2016; Cavalli and Dinarello 2018). Taken together, these findings indicate that IL-37 is a potent anti-inflammatory cytokine with the ability to reduce and suppress immune responses and inflammation, offering a promising new drug target for treatment of a variety of inflammatory and autoimmune diseases. To make it useful therapeutically, however, IL-37 needs to be available in large and affordable quantities.

Currently, E. coli-made IL-37 is available in small quantities. While E. coli is one of the most commonly used systems for the expression of recombinant proteins, it has several inherent limitations, including low protein solubility, inclusion body formation and possible product contamination by endotoxin. Furthermore, this expression system is cell culture/fermentation dependent, making scaling-up production of recombinant proteins inconvenient and expensive. Therefore, there is an urgent need for the development of new alternative expression systems that allow high-yield and low-cost IL-37 production. Plants as expression systems for protein production offer considerable advantages. As bioreactors, plants offer virtually unlimited scalability and low-cost potential. Plant-based production would also reduce the risk of contamination with mammalian pathogens, as organisms causing human or animal diseases do not infect plant cells (Ma et al. 2005, 2015; Tremblay et al. 2010). Like animal cells, plant cells are eukaryotic cells, enabling the performance of complex post-translational modifications that are often required for biological and/or immunological functions of many therapeutic proteins. Furthermore, plant bioreactors have a short turnaround time needed to obtain gram quantities of a recombinant protein in a matter of weeks when the protein is transiently expressed in plants. This is not only economically advantageous, but also critical to meeting challenges related to quick access to life-saving biotechnology drugs and therapies. There has been a rapid increase in the number of recombinant proteins of pharmaceutical importance produced in plants, ranging from monoclonal antibodies, vaccines and hormones to enzymes. Many of the plant-made pharmaceutical proteins have been tested in preclinical animal models with promising results and, moreover, several plant-derived monoclonal antibodies and vaccines were advanced to Phase I and Phase II human clinical trials and found to be safe and well tolerated (Paul and Ma 2011; Edgue et al. 2017). Developed by Protalix Biotherapeutics and Pfizer, taliglucerase alfa (Elelyso) is the first plant cell-expressed therapeutic protein approved by the US Food and Drug Administration for use in patients with Gaucher's disease (Fox 2012).

It should be pointed out that human IL-37 possesses five different isoforms (named as IL-37 a–e) resulting from alternative gene splicing. No mouse homolog of IL-37 has been found, but human IL-37 is active on mouse cells (Boraschi et al. 2011). It has now become clearer that a variety of normal tissues and diseased tissues express IL-37 with differential expression of its five different isoforms. As with other IL-1 family members, IL-37 is synthesized as a precursor molecule that contains no classical signal peptide, and processing to remove the signal peptide to yield the mature form of IL-37 requires the proteolytic activity of caspase-1 (Cavalli and Dinarello 2018). Both the precursor and the mature form of IL-37 are biologically active (Li et al. 2015). Among the five IL-37 variants, IL-37b is the largest, most abundant, and most studied isoform. Most of the current knowledge concerning the functional characteristics and mechanisms of action of IL-37 was attained through the study of IL-37b.

Here, we report the production of recombinant IL-37b in plants and characterization of the plant-made protein. A set of plant transformation expression vectors were created for the expression of IL-37b in different forms, including a precursor form, a mature form and an IL-37 fusion protein with soybean agglutinin (SBA). Transgenic tobacco plants were generated with each of these constructs. Western blot analysis revealed the expression of IL-37b in the desired form in plants with the level of proIL-37b expression being significantly higher than that of matIL-37b (1% vs 0.01% TSP). The expression level of matIL-37b was markedly enhanced when fused to SBA. Functional cell-based assays demonstrated that both the plant-derived proIL-37b and matIL-37b were biologically active, but matIL-37b possesses considerably greater biological activity compared to proIL-37b.

Materials and methods

Plasmid construction

A cDNA clone encoding human IL-37b was obtained from OriGene (Rockville, MD, USA). To construct a plant transformation vector synthesizing the full-length, unprocessed precursor form of IL-37b (proIL-37b), the entire coding region of IL-37b including its signal peptide coding sequence was amplified by PCR using designed pair primers (forward: 5′-ATTAACATGTGTTCATACAAA GAT TTTCTTT-3′ and reverse: 5′-TATATCTAGATCAATC GGACACCTCTGAAGGAGA-3′). The forward primer contained a PciI site (underlined) that overlaps the translation start site, whereas the reverse primer contained a XbaI

site (underlined) after the stop codon. PCR reaction was performed under the following parameters: denaturing at 95 °C for 30 s, annealing at 60 °C for 1:30 min and elongation at 72 °C for 1 min, for a total of 35 cycles followed by a final elongation at 72 °C for 10 min. The PCR product was blunt-end ligated into the SmaI site of plasmid pUC19. Following DNA sequencing verification, the PCR-cloned hIL-37b gene was released from pUC19 as a PscI/XbaI fragment and then ligated to plasmid pTRL-GUS in replacement of the GUS gene (Carrington and Freed 1990). The IL-37b expression cassette, consisting of 35S promoter and 5′untranslated region from tobacco etch virus (TEV), IL-37b and 3′ untranslated region from Agrobacterium nopaline synthase gene, was released from pTRL-IL-37b as a single HindIII fragment and cloned into binary plant transformation vector pBI101.1 (Brandsma et al. 2010), generating vector pBI-proIL-37b.

A second vector construct synthesizing the mature form of IL-37b without its N-terminal 45-aa signal peptide (matIL-37b) was made by replacing the endogenous signal peptide coding sequence of IL-37b with that of barley α-amylase (Rogers and Milliman 1983) or pathogenensis-related protein 1b (PR1b) from tobacco (Matsuoka et al. 1987) combined with the addition of an endoplasmic reticulum (ER) retention signal KDEL at the C-terminus using similar PCR techniques as described above. The modified IL-37b gene was cloned into pTRL-GUS and then into pBI101.1, generating vectors pBI-sp(amy)-IL37b and PBI-sp(pr1b)-IL-37b, respectively.

A third vector construct synthesizing IL-37b as a fusion protein with SBA was created through several steps using PCR techniques. The endogenous signal peptide coding sequence of IL-37b was removed, while its C-terminus was modified by the addition of an ER retention signal KDEL followed by a stop codon. The C-terminus of the SBA coding sequence with its signal peptide coding sequence was modified by removing its stop codon. The modified IL-37b gene was then fused in-frame to the C-terminal end of SBA gene via a flexible linker (3xGGGGS), followed by a tobacco etch virus (TEV) protease cleavage site (ENLYFQS). The resulting chimeric fusion gene was cloned into pTRL-GUS and then into pBI101.1, generating vector pBI-SBA-IL-37b. To facilitate downstream purification, constructs that incorporated a 6xHis-tag at the C terminus of IL-37b were additionally produced.

Tobacco genetic transformation

Prior to plant transformation, constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating (Ma et al. 2005). Transformation of low-alkaloid *Nicotiana tabacum* cv. 81V9 was achieved by using an *Agrobacterium*-mediated leaf disc method as described by Horsch et al.(1985). Primary transgenic plants were selected on MS medium containing 100 mg/L kanamycin. As regenerated plants matured, they were transferred into a greenhouse and maintained for further analysis.

Immunoblot analysis of IL-37b expression in transgenic plants

Total leaf protein was extracted from individual transgenic tobacco lines as described previously (Ma et al. 2005). Protein concentration was determined based on the Bradford dye-binding method (Bradford 1976). Protein samples were mixed with the sample buffer and boiled for 10 min prior to loading on the SDS–PAGE gel (12.5%). Separated proteins were electrophoretically transferred from the gel to PVDF membranes and analysed by Western blotting using IL-37-specific antibodies. In brief, blots were blocked for 1 h in 5% skimmed milk–TBST (20 mm Tris, 150 mm NaCl, 0.02% Tween 20, pH 7.6), and then incubated overnight at 4 °C with a 1:2000 dilution (v/v) of rabbit anti-human IL-37 primary antibody (ab116282, Abcam), followed by goat anti-rabbit secondary antibody conjugated with peroxidase (074-1506, KLP) at 1:5000 dilution. Protein bands on the blots were detected with enhanced chemiluminescence reagent (Merck Millipore) and imaged with the FluorChem Q imaging system (ProteinSimple, San Jose, CA, USA) or blot images were obtained with X-ray film.

Quantification of plant-derived IL-37b

The concentration of plant-derived IL-37b in tobacco leaves was determined by enzyme linked immunosorbent assay (ELISA). Briefly, total protein samples were coated on a 96-well mictrotiter plate and incubated overnight at 4 °C. The plate was blocked with 3% BSA in PBS-T (phosphate-buffered saline containing 0.05% (v/v) Tween-20) for 2 h at room temperature. The plate was then washed with PBS-T and polyclonal goat anti-hIL-37 antibody (Sigma-Aldrich) diluted to 1:5000 (v/v) was added. After incubation overnight at 4 °C, the plate was washed with PBS-T and the second detecting antibody (peroxidase-conjugated, anti-goat IgG antibody; Sigma-Aldrich, 1:2000) was added. After incubation for 1 h at room temperature, bound peroxidase-conjugated antibody was visualized with tetramethylbenzidine (TMB) substrate system (R&D Systems). The reaction was stopped by the addition of 100 µL/well stop solution (R&D Systems) and the absorbance read at 450 nm. Absorbance readings were used to calculate the concentration of plant-derived IL-37b by comparison to a standard curve generated using known concentrations of IL-37b standard (R&D Systems).

The level of plant-derived SBA-IL-37b fusion protein was quantified by SBA ELISA as described previously (Tremblay et al. 2011a, b).

Purification of plant-derived IL-37b and SBA-IL-37b

His-tagged proIL-37b and matIL-37b were purified from total leaf extracts using immobilized metal ion affinity chromatography (IMAC) on a nickel–nitrilotriacetic acid (Ni–NTA) column according to the manufacturer's instructions. Eluted proIL-37b or matIL-37b fractions were dialysed extensively against PBS buffer and concentrated using a speed vacuum at 4 °C.

Purification of SBA-IL-37b fusion protein was achieved by using an N-acetly-D-galactosamine-agarose column as described previously (Tremblay et al. 2011a, b). Washes were monitored with absorbance at 280 nm using a spectrophotometer and once the readings reached zero, the fusion protein was eluted with 0.5 M galactose/0.1 M NaCl. Eluted SBA-IL-37b fractions were dialysed extensively against 0.5× PBS buffer to remove salt and galactose, and then concentrated as described above.

In vitro processing of SBA-IL-37b fusion protein

Partially purified SBA-IL-37b fusion protein (20 µg) was digested with 10 units of AcTEV protease (Cat No. 12,575,015, Invitrogen) in a total reaction volume of 150 µL of digestion buffer as recommended by the manufacturer. A small aliquot was removed from the reaction at time 0 to serve as an undigested control. The reaction was incubated at 30 °C. Aliquots were removed after 1, 2, 4 and 6 h of incubation and mixed with an equal volume of 2× SDS sample buffer. Samples were boiled at 100 °C for 5 min prior to analysis by SDS–PAGE followed by immunoblotting.

Determination of biological activity of plant-derived recombinant protein

Primary mouse renal cells were used as an in vitro cell-based bioassay to determine the biological activity of plant-made IL-37b. In brief, primary renal tubule epithelial cells were isolated from the kidney cortex of B6 mice as described previously (Jevnikar et al. 1991). Cells were grown in K1 medium (50:50 DMEM and Ham's F12; Invitrogen) supplemented with 10% (v/v) FBS, hormone mix (5 µg/mL insulin, 34 pg/mL triiodothyronine, 5 µg/mL transferrin, 1.73 ng/mL sodium selenite, 8 ng hydrocortisone and 25 ng/mL epidermal growth factor), 100 U/mL penicillin and 0.1 mg/mL streptomycin, and maintained at 37 °C in 5% CO_2. To perform the assay, cells were seeded in triplicate in 96-well tissue culture plates in complete K1 medium at a density of $1–5 \times 10^4$ cells/well and grown overnight at 37 °C to allow the cells to attach to the plate. Cells were then treated with different concentrations of proIL-37b, matIL-37b or commercial recombinant IL-37b standard. The medium was removed after 24 h and the cells were treated for 24 h with 1 µg/mL lipopolysaccharide (LPS), an endotoxin from Gram-negative bacteria that acts as a potent inducer of pro-inflammatory cytokines. Cells treated with LPS only were used as a control. The culture supernatants were then collected and analysed for TNF-α concentrations using a commercial TNF-α-specific ELISA kit according to the manufacturer's instructions (R&D Systems).

Statistical analysis

The data are expressed as mean ± SE (standard error). Statistical significance of differences was analysed by unpaired, two-tailed Student's *t* test. Differences were considered to be statistically significant when $P < 0.05$.

Results

Construction of expression vector and production of transgenic plants

The plasmid vectors constructed for overexpression of IL-37b in plants are illustrated in Fig. 1. Expression of IL-37b was under the control of an enhanced cauliflower mosaic virus (CaMV) 35S promoter with a 5′-untranslated leader sequence from the tobacco etch virus. Vector pBI-proIL-37b contains the entire coding region for IL-37b including a 45-aa N-terminal signal peptide. Since processing of the signal peptide of IL-37b precursor requires the proteolytic activity of caspase-1 and no functional homologs of known animal caspase-1 are present in plant cells (Bonneau et al. 2008), protein expression from pBI-proIL-37b would therefore be expected to produce an unprocessed, full-length precursor IL-37b (proIL-37b). For production of a mature form of IL-37, vectors pBI-sp(amy)-IL37b and PBI-sp(pr1b)-IL-37b were constructed in such a way that the IL-37 gene was modified by replacing its native signal peptide with the signal peptide from the natural secretory protein barley α-amylase or tobacco pathogenesis-related PR1b protein to ensure proper processing of the N-terminus to yield a mature IL-37 protein product, combined with addition of a C-terminal ER retention signal KDEL to increase their expression levels through selective targeting to the ER. Vector pBI-SBA-IL-37b was designed to express IL-37b as a fusion protein consisting of mature IL-37b lacking its native signal fused to the C-terminus of the full-length SBA with its native signal peptide. We have previously shown that SBA as a fusion partner can not only increase the expression levels of heterologous proteins, but also simplify the downstream

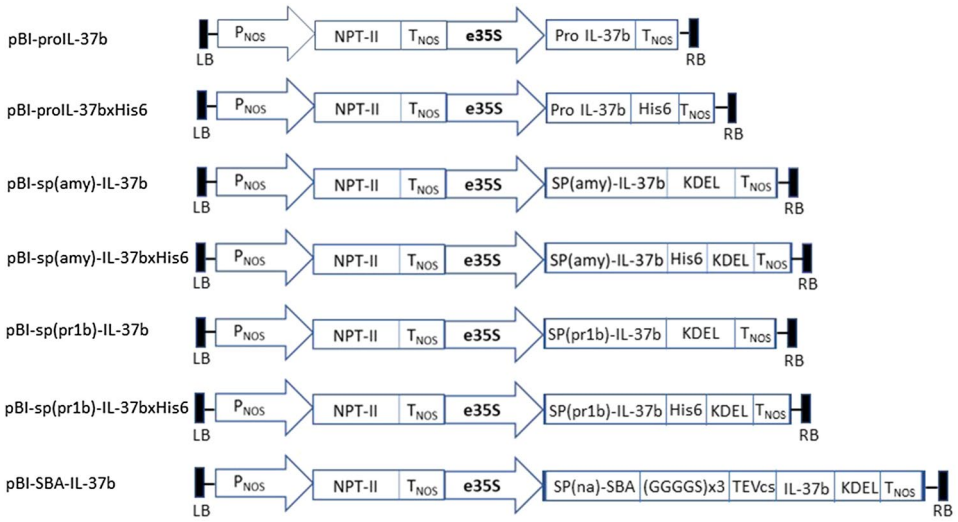

Fig. 1 Schematic diagrams of T-DNA regions of plant transformation vectors. The right and left borders of the T-DNA are indicated with RB and LB, respectively. P_{NOS} nopaline synthase promoter, *NPT II* neomycin phosphotransferase gene, T_{NOS} nopaline synthase terminator, *e35S* enhanced CaMV 35S promoter including a tobacco etch virus 5′nontranslated leader sequence, *SP(amy)* barley α-amylase signal peptide, *SP(pr1b)* signal peptide of tobacco PR-1b protein, *SP(na)* the native signal peptide of SBA, *GGGGSx3* a 15-aa linker, TEV_{CS} tobacco etch virus protease cleavage site, *KDEL* ER retention signal

protein purification process (Tremblay et al. 2011a, b). To maximize the expression yield, an ER retention motif KDEL was similarly added.

Low-alkaloid *Nicotiana tabacum* cultivar 81V9 was transformed with *Agrobacterium* strains harbouring a specific vector construct. Approximately, 25 independent transgenic plants were generated for each construct. There were no morphological differences between transgenic and untransformed control plants.

Accumulation of IL-37b protein in transgenic plants

Accumulation of proIL-37b protein in transgenic tobacco plants was analysed by immunoblot analysis of total crude leaf extracts. Western blot data, obtained with film-based detection system, showed that two major bands were reacted with anti-hIL-37 antibody (ab116282, Abcam) in extracts from tobacco plants harbouring pBI-proIL-37b: the larger and more dominant band was around 50 kDa, which corresponds to the dimeric form of proIL-37b, whereas the smaller band had an apparent molecular mass of 25 kDa corresponding to the monomeric form of proIL-37b (Fig. 2). The mature form of commercial recombinant human IL-37b (aa 46–218) with a molecular weight of 19 kDa was used as a standard. As expected, under the same experimental conditions, no protein bands of similar sizes were detected in extracts prepared from untransformed wild-type plants. The expression levels of proIL-37b in primary transgenic plants (T0) reached up to 1% of TSP. Similar results were obtained when total extracts from tobacco plants transformed with a

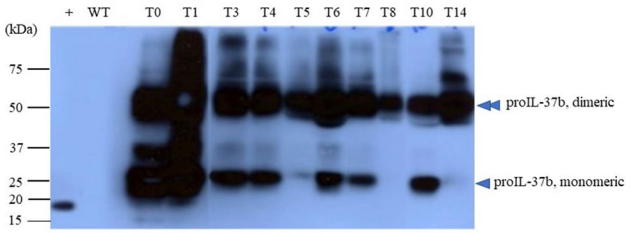

Fig. 2 Immunoblot analysis of expression of the IL-37b precursor (proIL-37b) in transgenic tobacco plants. Protein samples (40 µg/per well) were separated by 12.5% SDS–PAGE and reacted with anti-hIL-37 antibodies. The single arrow indicates the monomeric form of plant-made IL-37b precursor and the double arrow indicates its dimeric form. *WT* wild-type tobacco, + recombinant human IL-37b mature protein (aa 46–218; MW, 19 kDa; R&D Systems) standard (20 ng loaded). T0–T14, representative transgenic tobacco lines. Numbers on the left indicate the positions of protein size markers. Blot images were obtained with X-ray film

His-tagged version of pBI-proIL-37b, pBI-proIL-37bxHis6, were analysed by immunoblotting using the same anti-IL-37 antibody (data not shown).

A faint band at around 20 kDa, corresponding in size to the mature IL-37b monomeric form, was detected by immunoblotting performed on crude leaf extracts from tobacco plants harbouring pBI-sp(α-amy)-IL37bxHis6 following prolonged exposure time, visualized using FluorChem Q imaging system (matIL-37b; Fig. 3a). A slight difference in mobility on SDS–PAGE between plant-made matIL-37b and commercial recombinant mature IL-37b standard is likely due to the addition of a few extra amino acids (ER retention

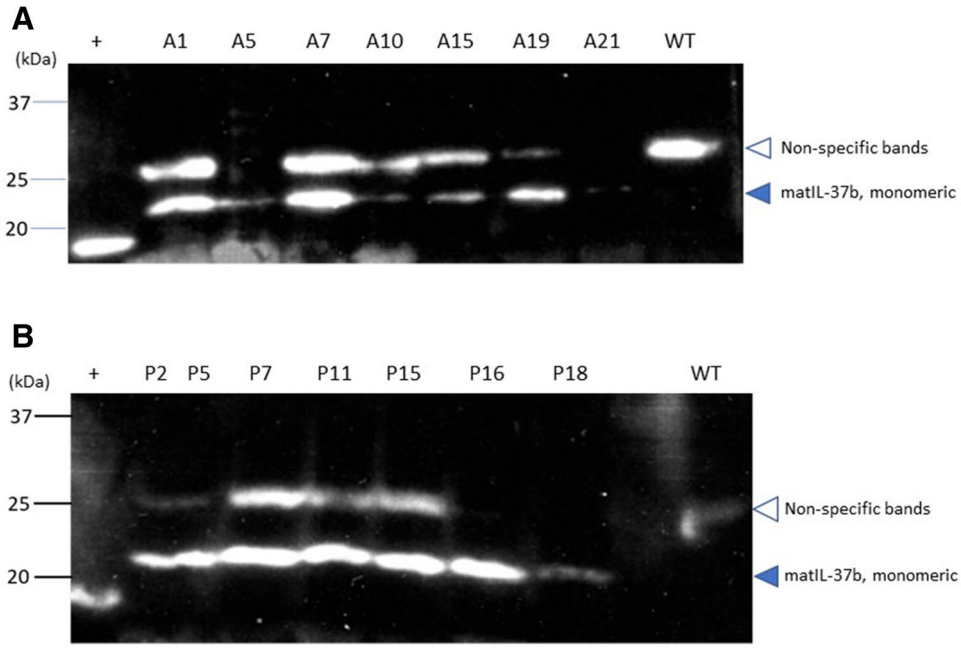

Fig. 3 Immunoblot analysis of expression of the mature form of IL-37b (matIL-37b) in transgenic tobacco plants. Protein samples (50 µg/per well) from pBI-sp(amy)-IL37bxHis6 (**a**) and PBI-sp(pr1b)-IL-37bxHis6 (**b**) plants were separated by 12.5% SDS–PAGE and reacted with anti-hIL-37 antibodies. Blot signals were detected using a FluorChem Q system after a prolonged exposure time (4–5 min). The position of matIL-37b is indicated by a solid triangle. The nonspecific bands at 25 kDa are indicated by an empty triangle. *A1–A21* representative transgenic lines harbouring pBI-sp(amy)-IL37bxHis6; P2 to P18, representative transgenic lines harbouring PBI-sp(pr1b)-IL-37bxHis6. *WT* wild-type tobacco, + recombinant human IL-37b mature protein (aa 46–218)

signal KDEL and 6xHis) to the C-terminus of plant-made matIL-37b. Upon prolonged blot exposure, an additional band of 25 kDa (indicated by an empty triangle in Fig. 3a, b) was detected, which is likely to be non-specific because the 25-kDa band was also detected in extracts from untransformed wild-type tobacco plants. No dimeric form of the plant-made matIL-37b was observed under these conditions. Similar results were obtained in immunoblotting analysis of extracts from tobacco plants harbouring PBI-sp(pr1b)-IL-37bxHis6 under the same experimental conditions (matIL-37b; Fig. 3b). Compared to proIL-37b expression, the levels of matIL-37b accumulation in tobacco plants harbouring either pBI-sp(α-amy)-IL37bxHis6 or PBI-sp(pr1b)-IL-37bxHis6 were much lower, accounting for approximately 0.01% of TSP.

Immunoblotting analysis, using a film-based system for detection, of leaf extracts from tobacco plants containing pBI-SBA-IL-37b revealed the presence of multiple bands when probed with an antibody directed against the C-terminal of IL-37 (C-terminal ab153889, Abcam). As shown in Fig. 4, the band at 50 kDa is likely to represent the monomeric form of the fusion protein, as the calculated molecular weight of SBA-IL-37b monomer is around 50 kDa. Other bands with molecular weights higher than 75 kDa may represent the dimeric, trimeric and tetrameric forms of SBA-IL-37b, respectively. None of these bands were detected in control plant extracts. Similar results were obtained with the use of anti-SBA antibodies (data not shown). The expression level of SBA-hIL-37b reached about 1% of TSP.

Digestion of SBA-IL-37b fusion protein in vitro

As SBA-IL-37b fusion protein is separated by an engineered TEV cleavage sequence (ENLYFQS), this gives flexibility to remove the tag from the target protein after expression if a tag-free native target protein is desirable. To demonstrate that the fusion protein could be cleaved so that a tag-free IL-37 is produced, SBA-IL-37b was isolated from total extracts using an *N*-acetly-D-galactosamine-agarose column and partially purified SBA-IL-37b was then subjected to TEV protease digestion in vitro. As shown in Fig. 5, it appears that after 1-h incubation, the SBA-IL-37 fusion protein was completely cleaved by TEV protease at the designed recognition sites. Continued incubation resulted in no cleavage of the protein at non-canonical sites, suggesting the efficiency and stringent sequence specificity of the TEV protease.

Plant-derived IL-37b is biologically active

The biological activity of plant-made hIL-37 was determined using an in vitro cell model system based on mouse renal

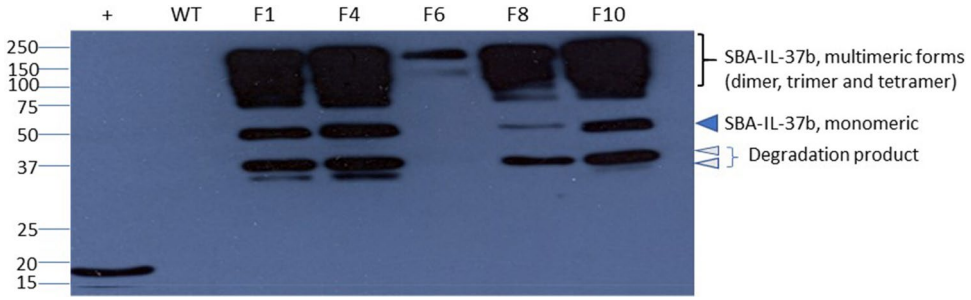

Fig. 4 Immunoblot analysis of expression of the SBA-IL-37b fusion protein in transgenic tobacco plants. Protein samples (40 µg/per well) were separated by 12.5% SDS–PAGE and reacted with antibody directed against a C-terminal sequence of IL-37 (C-terminal ab153889, Abcam). Solid triangle indicates the bands corresponding to the SBA-IL-37b monomer; empty triangle indicates the likely product of degradation. Bands with molecular weights higher than 75 kDa likely represent the SBA-hIL-37b dimer, trimer and tetramers, respectively, indicated by a bracket. *F1–F10* representative PBI-SBA-hIL-37b transgenic tobacco lines, *WT* wild-type tobacco, + recombinant human IL-37b mature protein (aa 46–218). Blot images were obtained with X-ray film

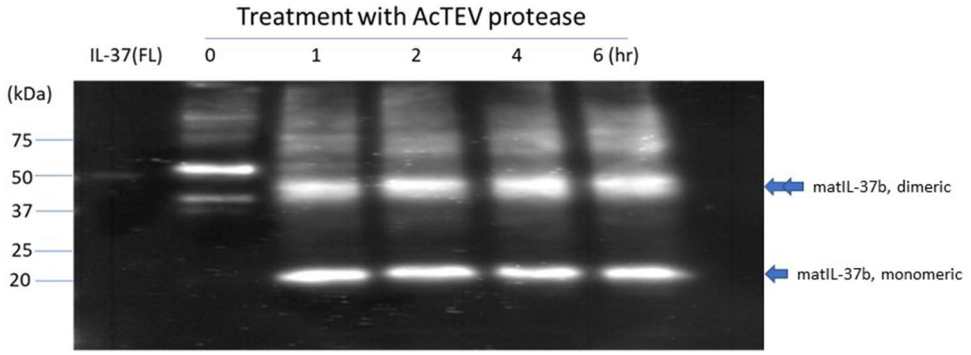

Fig. 5 Digestion of SBA-hIL-37b fusion protein in vitro by TEV protease. The assay was performed as described in "Materials and methods". Sample aliquots were removed at 0, 1, 2, 4 and 6 h of incubation. Samples were analysed by SDS–PAGE (12.5%), followed by Western blotting using anti-hIL-37 antibodies. IL-37(FL), recombinant human IL-37 full-length protein standard (aa 1-218; MW, 25 kDa; Abcam), migrated at a position of around 50 kDa under our experimental conditions due to dimerization. The single-headed arrow indicates the monomeric form of IL-37b and the double-headed arrow indicates the dimeric IL-37b protein

tubule epithelial cells, as Yang et al. (2015) demonstrated that IL-37 inhibited LPS-induced production of pro-inflammatory cytokines such as TNF-α in mouse renal tubular epithelial cells. In this end, His-tagged proIL-37b and matIL-37b were partially purified from total leaf extracts by IMAC. Prior to its use in the assay, the integrity of the partially purified protein was analysed by Western blotting. A clear single band at the expected size for proIL-37b or matIL-37b was observed after Western blot using anti-IL-37 antibody, with no degradation products with lower molecular weight detected (data not shown). To carry out the assay, mouse primary renal tubular epithelial cells were treated with different concentrations of partially purified prohIL-37b, mathIL-37b or commercial hIL-37b standard and then exposed to LPS. Cells treated with LPS only were used as a control. The concentrations of TNF-α in the culture supernatants were measured by ELISA. As shown in Fig. 6, pre-treatment with proIL-37b, matIL-37b or IL-37b mature protein standard (aa 46-218) at all test concentrations significantly decreased LPS-induced TNF-α secretion in a dose-dependent manner in mouse renal tubular epithelial cells compared with the LPS-stimulated control cell ($p < 0.05$), except for those cells that received the lowest concentration of proIL-37b (0.625 nM). The inhibitory effect of plant-made matIL-37b on the secretion of TNF-α in the LPS-induced mouse renal tubular epithelial cells was similar to the effects of the IL-37 standard (aa 46-218). Compared with proIL-37b, however, the inhibitory effect displayed by both plant-made matIL-37b and IL-37 standard (aa 46-218) was significantly greater ($p < 0.05$).

Discussion

Human IL-37, a non-glycoprotein, is a new member of IL-1 family cytokine with potent anti-inflammatory and immunosuppressive properties, which could provide a powerful

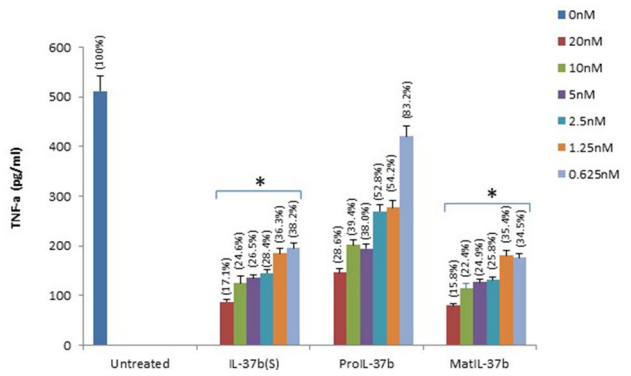

Fig. 6 Analysis of TNF-α production in mouse primary renal tubular epithelial cells treated with plant-made IL-37b. Cells were incubated with various doses of proIL-37b, matIL-37b or IL-37b standard for 24 h and then treated with 1 μg/ml of LPS for 24 h. Control cells were treated with LPS only. TNF-α levels in the supernatants were assayed by ELISA. The assay was performed in triplicate and repeated twice. Representative data are shown with standard error bars. The cells treated with LPS alone were assigned a value of 100% as the baseline and the actual values of others were calculated relative to the baseline value. *Statistically significant difference ($p < 0.05$) in TNF- α levels released from LPS-induced cells treated with either plant-made matIL-37b or recombinant human IL-37 mature protein standard (aa 46–218) vs plant-made proIL-37b. *Untreated* cells treated with LPS only, *IL-37b(S)* recombinant human IL-37 mature protein standard

new therapeutic approach for treating inflammatory and autoimmune diseases. Currently, *E. coli*-made recombinant IL-37 is commercially available only in limited quantity that is insufficient to meet the need for clinical applications. In the present study, plants were evaluated as an alternative host for the expression of recombinant IL-37. To this aim, transgenic tobacco plants synthesizing various forms of IL-37b were generated. Plasmid pBI-proIL-37b was designed to express a precursor form of IL-37b containing its N-terminal signal peptide. As shown in Fig. 2, plant-made proIL-37 majorly exists as a dimer and its expression level in plant cells reaches as high as 1% of TSP. Both the unprocessed precursor and the processed mature form of native human IL-37 form homodimers in solution that are principally stabilized by noncovalent bonds. The dimerization status of human IL-37 is not affected by the presence of a reducing agent such as dithiothreitol (DTT) (Kumar et al. 2002). Since plant extracts used for immunoblotting analysis were mixed with the sample buffer containing DTT and boiled for 10 min prior to protein separation by SDS–PAGE gel electrophoresis, the detection of dimer as a major form on the immunoblot under reducing conditions suggests that plant-made proIL-37b has a structure similar to its native counterpart. For matIL-37b expression, our immunoblotting data indicate that both pBI-sp(amy)-IL37b- and PBI-sp(pr1b)-IL-37b-transformed tobacco plants synthesized a protein at the expected size of monomeric mature IL-37b (Fig. 3a, b), suggesting that, as expected, the engineered plant signal peptide is capable of directing efficient and accurate processing of IL-37b, resulting in a mature form of IL-37 product. Compared to proIL-37b, however, the accumulation level of matIL-37b was significantly lower. The highly significant differences in expression levels between matIL-37b and proIL-37b may be due to several factors. First, the ER may not serve as a suitable subcellular compartment for accumulation of matIL-37b. In plant-based protein expression systems, targeting of proteins to intracellular organelles (ER, chloroplast, and vacuole) has been shown to influence both their yield and stability (Schnell et al. 2010). While our group as well as others have previously shown that targeting of proteins to the lumen of the ER is an effective approach to enhance the production of heterologous proteins including human IL-4 (Ma et al. 2005), human IL-13 (Wang et al. 2008) and orange fluorescent protein (Man et al. 2012), the effectiveness of this strategy may be associated with the protein being expressed. Secondly, unlike matIL-37b, plant-made proIL-37b is most likely retained in the cytoplasm due to the inability of plant cell's machinery to recognize and process IL-37's native signal peptide, which may provide an environment that favours its high-level accumulation. Thirdly, a much higher accumulation of proIL-37 in plant cells may also be due to the presence of its native signal peptide of 1–45 amino acids that may play a pivotal role in the stabilization of IL-37 precursor. While the role of the N-terminal signal peptide of IL-37 precursor in stabilizing the protein is presently not yet fully understood, one possibility is that its presence may induce some major structural changes of proIL-37b that helps render the protein more stable. IL-37 is generally regarded as an unstable protein and, like some of the IL-1 family members, contains functional mRNA instability elements in its coding region that act as a negative feedback mechanism to regulate its expression at both mRNA and protein levels (Bufler et al. 2004). Given that IL-37, as an inhibitor of inflammation, plays an important regulatory role in both innate immune response and adaptive immune response (Cavalli and Dinarello 2018), it is conceivable that if IL-37 production is dysregulated (overproduction or underproduction), consequently it can cause the body's immune system to become imbalanced, which in turn can cause chronic inflammation, autoimmune conditions and allergic responses. Deletion of these instability sequences has been shown to result in increased expression of IL-37 protein (Bufler et al. 2004). In contrast to plant-made proIL-37, plant-made matIL-37 exists as a monomer, with none of its dimeric form being detected. Although more research is needed to gain a better understanding of the mechanism that favours accumulation of matIL-37b monomer, it is speculated that dimerization of IL-37 may be concentration dependent. Since plant-expressed matIL-37 accumulated at a level much lower than proIL-37, this level of accumulation may not reach the threshold concentration

required for dimer formation. This appears to be the case, indeed. When His-tagged matIL-37b was purified, concentrated and then re-examined by immunoblotting analysis under the same reducing conditions, the dimeric form was observed as a major form of the protein (data not shown).

Results obtained from plant expression of SBA-hIL-37b indicate that the yield of matIL-37b production can be significantly improved when expressed as a fusion with SBA, with the amount of SBA-hIL-37b fusion protein reaching approximately 1% of TSP. These results suggest that using SBA as a fusion tag is an effective strategy to improve the production of a target protein, especially those that are unstable in nature. While a number of fusion tags have been developed to aid in the expression and purification of recombinant proteins (Banerjee et al. 2009), SBA as a new fusion tag offers unique advantages: (1) being a tetrameric glycoprotein and present abundantly in soybean seeds, SBA has a very high degree of stability and its use as a fusion partner can often lead to high-level expression of target proteins; (2) SBA is a sugar-binding lectin that specifically binds to *N*-acetyl-D-galactosamine and, as such, SBA-tagged proteins can be conveniently purified by affinity chromatography using an *N*-acetyl-D-galactosamine-linked agarose column as we have demonstrated previously (Tremblay et al. 2011a, b); (3) in case of the need to separate the target protein from the SBA tag, a protease cleavage site can be engineered between SBA and the target protein to allow removal of the SBA moiety from the target recombinant protein after purification. Following protease cleavage in solution, the digested sample is reapplied to the column and the target protein will flow through the column (i.e., in the unbound fraction), whereas the SBA and any uncleaved SBA fusion protein will bind to the column. Since SBA-hIL-37b fusion protein contains an engineered TEV protease recognition site between the two moieties, we show here that the SBA tag can be removed by endoproteolytic cleavage at a designed site using TEV protease.

The biological activity of plant-derived IL-37b was determined by an in vitro bioassay based on its ability to inhibit proinflammatory cytokine TNF-α in LPS-stimulated primary mouse renal epithelial cells. Our results indicate that both plant-derived matIL-37b and proIL-37b are biologically active and act in a dose-dependent manner, similar to the commercial IL-37b standard. Compared to proIL-37b, however, both matIL-37b and IL-37b standard (aa 46-218) displayed significantly greater biological activity when the same test concentrations were used. These results are consistent with previous studies that show that the biological activity of the mature form of IL-37 is several-fold greater that that of the full-sequence precursor molecule (Li et al. 2015). It has been recently suggested that the mature IL-37b molecule (aa 46-218) in the absence of its N-terminal 45-aa signal peptide sequence undergoes a major conformational change that leads to a structure that helps promote receptor binding, resulting in increased IL-37 activity (Cavalli and Dinarello 2018).

In conclusion, we have produced transgenic plants synthesizing various forms of IL-37b, with the expression level of both proIL-IL-37b and SBA-IL-37b fusion protein reaching as high as 1% of TSP. Like the native protein, plant-made proIL-37 exists predominantly as a homodimer. Using an in vitro cell-based bioassay, we demonstrated that plant-made mature and precursor form of IL-37b are both biologically active. To the best of our knowledge, there has been no previous report on the use of plants for production of human IL-37. The availability of large amounts of IL-37 at affordable low cost will allow for the development of new and more effective treatment alternatives for many inflammatory and autoimmune diseases.

Author contribution statement NA participated in the design of the experiment and performed the majority of the experimental work. HD performed some experimental work including immunoblotting, ELISA and some statistical analysis of experimental data. AMJ participated in project planning. SM designed and guided intellectually all processes of the work. AN and SM wrote the manuscript. All authors have read and approved the manuscript.

Acknowledgements AN was supported by a Graduate Student Scholarship from Saudi Arabia government. This work was supported in part by the London Health Sciences Centre, London, Ontario, Canada.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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