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Transplastomic Biofactory for the Production of Functional Human α-Lactalbumin for Nutritional and Therapeutic Applications

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Abstract

The sustainable production of functional human proteins in plants offers a transformative path for developing animal-free nutritional and therapeutic compounds. In this study, we report one of the first expression of human α-lactalbumin (hLA), a key milk protein with nutritional and anticancer properties, via chloroplast genome engineering in *Nicotiana tabacum*. A codon-optimized hLA gene was introduced into the plastid genome using a synthetic expression cassette under the control of strong plastid regulatory elements. Homoplasmic transplastomic lines were obtained and confirmed via PCR and Southern blot analysis. Immunoblotting and ELISA quantification revealed that hLA accumulated to 23.4% of total soluble protein (TSP), one of the highest levels reported for plastid-expressed human proteins. Far-UV circular dichroism (CD) spectroscopy confirmed that the chloroplast-derived hLA adopted a native-like α-helical structure. Functionally, the recombinant protein successfully activated galactosyltransferase (GalT) *in vitro*, enabling lactose synthesis at 93% of the rate observed with native hLA. Furthermore, chloroplast-derived hLA was converted into a bioactive HAMLET complex by combining with oleic acid under mild thermal conditions. This complex induced potent apoptosis in human colorectal (WiDr) and breast cancer (MCF-7) cells, reducing viability to less than 8%, as confirmed by MTT and Annexin V/PI assays. These findings establish chloroplasts as an effective platform for high-yield, correctly folded, and functional production of human milk proteins. The ability to generate both enzymatically active and therapeutically functional products from a single plant-based system underscores its potential in food engineering and synthetic biology. This work offers a

scalable, sustainable, and dual-purpose strategy for the development of recombinant milk proteins applicable in infant nutrition, functional foods, and plant-made biotherapeutics.

Key words: Bioengineering, Chloroplast transformation, Functional food proteins, Human α-lactalbumin, Nutrition, Synthetic biology

Introduction

Milk proteins are fundamental constituents of dairy products, and are crucial for nutrition. Currently, they constitute approximately 10% of the protein consumed globally (Smith et al., 2022). Dairy proteins exhibit versatility in numerous applications because of their highly desirable solubility, gelling, and emulsification properties. These characteristics render milk proteins essential not only for nutritional purposes, but also for diverse industrial applications (Behm et al., 2022).

Breast milk, the optimal source of nutrition for infants, is a critical protein source for humans. It comprises numerous complex proteins, lipids, and carbohydrates, the concentrations of which fluctuate significantly during a single feeding session and throughout the lactation period, reflecting the infant's nutritional requirements (Andreas et al., 2015). A key component of milk proteins is α-lactalbumin (α-LA), which accounts for approximately 22% of human breast milk proteins and 3.5% of bovine milk proteins. This protein plays a vital role as a coenzyme in lactose biosynthesis and provides an essential energy source (Heine et al., 1991). Structurally, α-lactalbumin is a small, calcium-binding protein (~14 kDa) that adopts a compact α-helical conformation (Permyakov, 2020). Importantly, it does not require post-translational modifications such as glycosylation for its folding or biological activity, which makes it particularly suitable for recombinant expression in heterologous systems (Giuffrida et al., 1997). Furthermore, α-LA offers valuable bioactive peptides and indispensable amino acids, including tryptophan, lysine, branched-chain amino acids, and sulfur-containing amino acids, all of which are fundamental to infant nutrition (Kunz & Lönnerdal, 1992). α-LA exhibits a role in reducing the incidence of certain malignancies, including breast and colon cancer, through the inhibition of cell division in intestinal cell lines. This whey protein contains approximately 6% tryptophan, a serotonin precursor that is crucial for maintaining the necessary serotonin levels in the brain and is associated with mitigating chronic stress-induced cognitive decline (Layman et al., 2018). Owing to its superior protein quality, characterized by high digestibility, rich amino acid content, neutral flavor

profile, and excellent solubility across a broad pH range (2.0−9.0), α-LA preparations have been widely proposed as ingredients in functional foods and beverages, including infant formulas and nutritional supplements (Enomoto et al., 2009; Lien, 2003; Lönnerdal, 2014; Mossberg et al., 2010; Permyakov & Berliner, 2000). Beyond its nutritional and functional food roles, α-LA has also drawn attention as a molecular building block in synthetic biology applications aimed at creating multifunctional bioactive complexes such as HAMLET.

The traditional dairy sector faces numerous challenges in meeting the market demand. Livestock-related greenhouse gas (GHG) emissions constitute 18% of global emissions (Hettinga & Bijl, 2022). Furthermore, the requirement for extensive acreage or confinement of farm animals raises concerns regarding milk contamination at dairy farms owing to hormone treatments, disease outbreaks, and antibiotic misuse (Awasthi et al., 2012; Behm et al., 2022). These factors, in conjunction with environmental fluctuations and natural climate, impact productivity and contribute to market uncertainty (Geistlinger et al., 2022; Vestergaard et al., 2016). As a result, there is increasing interest in biotechnological approaches for sustainable production of milk proteins, enabling the development of plant-based systems that can act as biofactories for high-value nutritional proteins while reducing environmental footprints (Geistlinger et al., 2022). In particular, plant synthetic biology enables the rational design and engineering of standardized, scalable, and animal-free protein production systems, directly addressing both environmental and ethical concerns.

The production of various biopharmaceutical and enzymatic proteins in the food processing industry is a common application of recombinant protein technology, which, with further development, can overcome the drawbacks of conventional dairy methods by providing independence from animals and seasonality, reduced environmental impact, enhanced supply chain flexibility, and increased cost efficiency (Hettinga & Bijl, 2022; Vestergaard et al., 2016). This aligns closely with the goals of biological engineering, where programmable expression systems are developed to achieve predictable and controllable biomolecule production in plants.

Human α-lactalbumin has been expressed in a wide range of recombinant platforms with variable efficiency. While *E. coli* offers high expression levels, the protein often requires complex refolding procedures (Chaudhuri et al., 1999; Overton, 2014). Yeast systems (e.g., *Pichia pastoris* and *Komagataella phaffii*) can achieve high secretory expression (~3–4 g/L), but non-human glycosylation can alter protein properties (Deng et al., 2022; Saito et al., 2002). Mammalian cell cultures, particularly CHO cells, produce correctly folded and bioactive hLA at ~20–50 mg/L, but their high-cost limits large-scale applications (Demain & Vaishnav, 2009). Mammalian cell cultures are

high-cost and yield less (~20–50 mg/L) (Demain & Vaishnav, 2009), and while transgenic animals (e.g., goats) can secrete high concentrations (~2–3 g/L), ethical and biosafety regulations pose significant barriers (Long, 2014). Among these, plant-based systems, especially when integrated with synthetic biology tools, have demonstrated the ability to produce food-grade proteins in a scalable, safe, and environmentally sustainable manner, overcoming limitations of traditional and microbial hosts.

Nuclear transgenic plants have been explored, but reported expression levels are typically low ($\sim 0.01-0.5\%$ of total soluble protein) (Bicar et al., 2008; Salmon et al., 1998). In contrast, the expression of animal proteins in plants, particularly milk proteins, represents a cutting-edge approach that diversifies the protein supply and improves food security. Foreign gene expression in chloroplasts offers distinct advantages among the various genetic engineering techniques for recombinant protein expression in plant cells, including elevated transgene expression levels due to the high copy number of the plastidial genome (up to 70% of total soluble protein), multi-gene expression, absence of epigenetic effects, and transgene containment through maternal inheritance (Bock, 2007; Chebolu & Daniell, 2009; Koop et al., 2007; Maliga, 2002; Oey, Lohse, Kreikemeyer, et al., 2009; Oey, Lohse, Scharff, et al., 2009). Chloroplast expression systems also provide bioencapsulation for protecting proteins. In the context of bioengineering, plastid genome transformation offers a robust chassis for stable and high-level expression of synthetic genes, meeting criteria for safety, scalability, and functionality for the production of high-value nutritional proteins and biopharmaceuticals (Daniell et al., 2001; Fischer et al., 2004; Ma et al., 2005).

However, despite the increasing use of nuclear plant systems or microbial hosts for recombinant milk protein production, no study has yet reported the successful expression of functionally active human α-lactalbumin in chloroplasts. Although progress has been made in expressing recombinant milk proteins in various systems, chloroplast-based platforms for such proteins remain underexplored, which leaves significant opportunities for developing plant-derived dairy analogues and bioactive formulations.

This study aims to fill that gap by engineering the plastid genome of *Nicotiana tabacum* to express functional human α-lactalbumin, using codon optimization, modular vector design, and chloroplast transformation strategies. By harnessing the inherent benefits of plastid genetic engineering, this work seeks to develop a novel plant-based platform for producing human milk proteins with superior yield and functionality. To the best of our knowledge, this is one of the first report of plastid-mediated expression of human α-lactalbumin, integrating synthetic biology tools and functional protein assays to bridge food biotechnology and biopharmaceutical innovation. The approach aligns

with synthetic biology's goal of redesigning biological systems for improved food production and therapeutic potential, and addresses critical environmental, economic, and health challenges associated with current milk protein production methods.

Materials and Methods

Construction of chloroplast transformation vector

The amino acid sequence of human α-lactalbumin was retrieved from the UniProt database (accession ID: P00709), and the 19-residue N-terminal signal peptide was excluded from the construct design. To facilitate the detection and purification of the expressed protein, a hexahistidine (6×His) tag was appended to the N-terminus of the coding sequence, followed by a TEV protease cleavage site positioned between the His-tag and α-lactalbumin coding region. This amino acid sequence was reverse-translated into a nucleotide sequence and subsequently codon-optimized for efficient expression in the chloroplast genome of *N. tabacum*. To simplify downstream cloning procedures, *Nco*I and *Xho*I restriction sites were introduced at the 5′ and 3′ ends of the synthetic gene, respectively. A complete codon-optimized gene construct measuring 425 base pairs was commercially synthesized (GenScript, USA).

To develop the plastid expression cassette, the synthesized hLA gene replaced the DARPin G3 coding region in the pPRV-DARPin vector (Ehsasatvatan et al., 2022b), which was engineered based on the backbone of the pPRV111A chloroplast transformation vector (Zoubenko et al., 1994). The pPRV111A system facilitates the site-specific integration of foreign sequences into the rps12/7–rrn16 intergenic region of the tobacco plastome and includes the *aadA* gene, which encodes aminoglycoside 3′-adenyltransferase, as a selectable marker for spectinomycin resistance. To ensure robust transgene expression, the hLA coding region was placed under the transcriptional control of the plastid ribosomal RNA operon promoter (Prrn), coupled with the 5′ untranslated region (5′ UTR) of bacteriophage T7 gene 10 (T7g10), which is known to enhance translation in plastids. The construct also contained the *E. coli* rrnB 3′ UTR as a transcriptional terminator. To enhance translation, the 5′ UTR harbored the epsilon motif 'TTAACTTTA', promoting ribosome binding to 16S rRNA. The final expression construct, designated pPRV-hLA, was used to mediate chloroplast transformation in tobacco plants.

Plant material, growth conditions and stable plastid transformation

N. tabacum cv. Perega plants were propagated under sterile conditions using Murashige and Skoog (MS) basal medium (Duchefa Biochemie, Netherlands) supplemented with B5 vitamins (Duchefa Biochemie, Netherlands), 3% (w/v) sucrose (Merck, Germany), and 0.7% (w/v) agar (Merck, Germany). *N. tabacum* cv. Perega plants were propagated aseptically in vitro from laboratory-maintained stocks. This is a standard cultivated research cultivar routinely used for chloroplast transformation studies. No field collection of plant material was performed, no collection permits were required, and deposition of a voucher specimen is not applicable. The medium pH was adjusted to 5.8 prior to autoclaving. Cultures were maintained at 24 ± 2 °C under a 16-hour photoperiod with a light intensity of approximately 40 μmol photons m^{-2} s^{-1}. Fully expanded mature leaves from these *in vitro*-grown plants were used as the target tissue for transformation.

Chloroplast transformation was performed using the biolistic method. Tungsten particles (0.7 μm, Bio-Rad, USA) were coated with 1 μg of the pPRV-hLA plasmid DNA and delivered into leaf explants using a Bio-Rad PDS-1000/He particle delivery system, following established protocols (Ehsasatvatan et al., 2022a). Bombarded explants were transferred to RMOP medium containing 500 mg/L spectinomycin dihydrochloride for selection. Spectinomycin-resistant shoots were isolated and subjected to additional rounds of regeneration on the same selective medium to promote homoplasmy. A secondary selection step was performed using RMOP medium supplemented with spectinomycin and streptomycin (500 mg/L each), and plantlets that remained resistant were regenerated again on medium containing only spectinomycin. Rooting was induced on MS medium supplemented with B5 vitamins, 3% sucrose, 0.7% agar, and 500 mg/L spectinomycin. Rooted plantlets were maintained under aseptic conditions prior to subsequent molecular and protein analyses.

Molecular analysis of transplastomic plants

DNA was extracted from 100–150 mg of leaf tissue collected from *in vitro*-grown transplastomic and wild-type *N. tabacum* plants using the cetyltrimethylammonium bromide (CTAB) method (Murray & Thompson, 1980). The presence of the human α-lactalbumin (hLA) transgene in the chloroplast genome was initially confirmed by polymerase chain reaction (PCR) using a primer pair specific to the hLA coding sequence: hLA-F (5′-AGGTACTTGAGAAGATTTACACCA-3′) and hLA-R (5′-TGGAGGAATTGCTTTACCTGAA-3′), which amplified a 148 bp product. To verify the site-specific integration of the transgene into the plastid genome, a PCR analysis was performed using primers annealing to the aadA selectable marker and the flanking plastid sequences.

Specifically, the aadA-R primer (5′-AAGAATTTGTCCACTACGTGA-3′) and the 16S-F primer (5′-AACTAAACACGAGGGTTGC-3′) were used, resulting in the amplification of an expected fragment of approximately 1.8 kb. This fragment is only produced when the transgene is correctly integrated into the targeted region of the plastome, confirming site-specific insertion. To further assess the integrity of the inserted construct, a second PCR was performed using primers located within the flanking plastid sequences, namely 16S-F and rps7/12-R primer (5′-AGTATTAGTTAGTGATCCCGAC-3′). In this assay, amplification of a 4 kb fragment was observed exclusively in transplastomic plants harboring an intact transgene, thereby confirming both the correct insertion site and the structural integrity of the integrated construct. All primers were synthesized by GenScript, USA.

Southern blot analysis was conducted for further validation of homoplasmy in the transplastomic lines. DNA (2 μg) extracted from leaf tissue was digested with the restriction enzyme *Bgl*II (New England Biolabs, USA), separated by agarose gel electrophoresis, and transferred to a nylon membrane for hybridization. A 232 bp DIG-labeled DNA probe corresponding to the rrn16 flanking region of the tobacco plastid genome was used for hybridization. Probe labeling and detection were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany) according to the manufacturer's instructions. The presence of a single hybridizing fragment of 6.5 kb in transplastomic samples and the absence of the 4.5 kb wild-type band confirmed both correct integration and homoplasmic status of the transgenic lines.

Protein extraction and immunoblot analysis

Total soluble proteins (TSP) were extracted from the leaves of transplastomic tobacco plants expressing human α-lactalbumin (hLA) using a protein extraction buffer composed of 200 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.05% (v/v) Tween-20, 0.1% SDS, 400 mM sucrose, 2 mM PMSF, and a commercial protease inhibitor cocktail (Sigma-Aldrich, Cat. #P9599). Following homogenization, the lysates were clarified by centrifugation, and the supernatants were collected for further analyses. Protein concentration was determined using the Bradford assay. About 500 ng of total soluble protein of each sample were separated on 12.5% SDS-PAGE gels and transferred onto PVDF membranes (Millipore, USA). For immunodetection, the membranes were probed with a rabbit anti-His in 1:1,000 dilution as primary antibody (Abcam, UK, Cat. #ab9108), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Abcam; Cat. #ab6721) secondary antibody (1:10,000 dilution). Protein bands were visualized using 3,3′-diaminobenzidine (DAB) substrate (Sigma-Aldrich). Protein extracts from wild-type

tobacco plants were used as negative controls to confirm the specificity of hLA expression in transplastomic lines.

Quantitation of human alpha-lactalbumin

The expression levels of human α-lactalbumin in transplastomic tobacco plants were quantified using an indirect enzyme-linked immunosorbent assay (ELISA). Ninety-six-well ELISA plates (Nunc MaxiSorp, Thermo Fisher Scientific, USA) were coated with 50 ng/well of total soluble proteins extracted from both transplastomic and wild-type leaves. Samples were applied in triplicate and incubated overnight at 4 °C. After washing, the wells were blocked with a blocking buffer containing 1% bovine serum albumin (BSA, Sigma-Aldrich) in 1× phosphate-buffered saline (PBS) supplemented with 0.1% Tween-20, and incubated at 37 °C for 1 h. Following the three washing steps, the wells were incubated with rabbit anti-His-tag primary antibody (1:1000 dilution in blocking buffer) for 2 h at 37 °C. After another washing cycle, goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) was added at a dilution of 1:10,000 and incubated at 37 °C for 1 h. The plates were washed again before adding the tetramethylbenzidine (TMB) substrate (Bio-Rad), and the reaction was allowed to proceed for 10 min at room temperature. The enzymatic reaction was stopped by adding 2 M sulfuric acid (H_2SO_4), and the absorbance was measured at 450 nm using an ELISA plate reader (BioTek ELx800, USA). For quantification, a standard curve was generated using known concentrations (5–25 ng) of recombinant human α-lactalbumin (Abcam, cat. # ab276670). All ELISA measurements were performed using three independent biological replicates, each analyzed in triplicate wells.

Affinity purification

Human α-lactalbumin (hLA) protein was purified from the total soluble protein extract of transplastomic tobacco leaves using immobilized metal affinity chromatography (IMAC) with the QIAexpress Ni–NTA Protein Purification System (QIAGEN). The crude extract was incubated in batch mode with 2 mL of Ni-NTA Agarose resin (Qiagen), pre-equilibrated with 4× binding buffer composed of 2 M NaCl and 2× PBS, at a ratio of 4:1 (extract:resin) at 4 °C for 2 h with gentle agitation. Then the protein–resin complex was packed into a column for the washing and elution steps. The protein–resin complex was then packed into a gravity-flow column, during which unbound proteins were allowed to pass through the column by flow-through. The column was washed with a buffer containing 0.5 M NaCl, 0.5× PBS, and 25 mM imidazole to remove non-specifically bound proteins. Bound proteins were eluted using

10 mL of elution buffer composed of 0.5 M NaCl and 0.5× PBS containing 200 mM imidazole. Elution was performed manually under gravity flow with a constant flow rate of 0.5 mL/min maintained by adjusting the column outlet. The protein concentration in the collected fractions was assessed using the Bradford assay, and purity was evaluated by SDS–PAGE.

To eliminate Rubisco, which is highly abundant in plant tissues, the purified protein fractions were treated with 10 mM sodium phytate (phytic acid sodium salt hydrate; Sigma) and 10 mM $CaCl_2$ at 37 °C for 10 min. The samples were centrifuged at 14,000 rpm for 10 min at room temperature. Supernatants were recovered, dialyzed against PBS, and stored at −20 °C until further use.

To remove the N-terminal His-tag, purified hLA was dialyzed against 20 mM Tris-HCl (pH 7.5), followed by digestion with TEV protease (New England Biolabs, #P8112) at a concentration of 0.5 units per µg of protein. The cleavage reaction was carried out at 30 °C for 1 h in 50 mM Tris-HCl (pH 7.5). The reaction mixture was passed through a Ni-NTA column to remove the cleaved His-tag and TEV protease from the mixture. The flow-through containing tag-free hLA was collected and analyzed using 12.5% SDS–PAGE followed by Coomassie Brilliant Blue staining. All functional assays, including the assessment of Lactose Synthase activity and the preparation of the HAMLET complex, were conducted using the re-purified, tag-free human α-lactalbumin.

Secondary structure characterization by circular dichroism spectroscopy

The secondary structure of recombinant human α-lactalbumin (hLA) expressed in tobacco chloroplasts was analyzed using far-ultraviolet (UV) circular dichroism (CD) spectroscopy. The concentration of purified protein was determined using the Bradford method and adjusted to 0.2 mg/mL for CD measurements. Samples were prepared in a low-ionic-strength phosphate buffer (10 mM sodium phosphate, pH 7.0) to minimize baseline interference in the far-UV region. CD spectra were recorded at 25 °C using a Jasco J-815 spectropolarimeter equipped with a quartz cuvette with a 0.1 mm path length. Measurements were taken over the wavelength range of 180–260 nm, using a bandwidth of 1 nm, a response time of 1 s, and a scanning speed of 50 nm/min. Each spectrum was the average of three independent scans. The baseline spectrum of the buffer was subtracted from each protein spectrum to correct for the background absorbance. To estimate the secondary structure composition, the processed spectra were submitted to the DichroWeb server (http://dichroweb.cryst.bbk.ac.uk) and analyzed using the SELCON3 algorithm. The percentages of α-helix, β-sheet, and random coil elements were calculated and compared with the values

reported for native human α-lactalbumin. For direct comparison, commercially available native human α-lactalbumin (Abcam, cat. # ab276670) was included as a standard control and analyzed under identical conditions. Each spectrum represents the average of three independent scans, and CD measurements were repeated using three independently purified protein samples.

Enzymatic assay for lactose synthesis

To confirm the functional activity of chloroplast-derived human α-lactalbumin (hLA), an *in vitro* lactose synthesis assay was performed. The assay was designed to evaluate the ability of recombinant hLA to activate galactosyltransferase (GalT), catalyzing the formation of lactose from UDP-galactose and D-glucose. Purified chloroplast-derived hLA was incubated with commercially available GalT (Sigma-Aldrich) in a reaction mixture containing 10 mM UDP-galactose (Sigma-Aldrich), 20 mM D-glucose, 50 mM Tris-HCl (pH 7.5), 10 mM MnCl2, and 1 mg/mL bovine serum albumin (BSA) in a total volume of 200 µL. The reaction was initiated by adding GalT (final concentration 0.1 U/mL) and incubated at 37°C for 30 min. As controls, reactions were set up with (i) GalT and native human α-lactalbumin (Abcam, cat. # ab276670), and (ii) GalT without any α-lactalbumin. A negative control consisting of total soluble protein purified from non-transformed wild-type tobacco plants was included in parallel to exclude interference by endogenous plant components. Authentic standards of lactose, D-glucose, and UDP-galactose (all purchased from Sigma-Aldrich) were analyzed under identical HPLC conditions to determine retention times and confirm peak identity in reaction mixtures. Chromatograms were recorded in a 0–10 min retention window using a refractive index detector. All experiments were conducted in three biological replicates, each with three technical repeats unless otherwise stated.

Detection and quantification of lactose

At the end of incubation, reactions were terminated by boiling for 2 min. Samples were analyzed by High-Performance Liquid Chromatography (HPLC, Agilent 1200 Series, Agilent Technologies, USA) using a carbohydrate analysis column (Waters XBridge Amide column, 4.6 x 250 mm) with an isocratic mobile phase of acetonitrile:water (75:25 v/v) and refractive index detection. Lactose was quantified by comparison with standard curves generated using known concentrations of lactose. All experiments were performed using three independent biological replicates, each analyzed in technical triplicates.

Formation of the HAMLET complex

The HAMLET complex was generated by combining purified human α-lactalbumin (hLA), expressed in tobacco chloroplasts, with oleic acid using the heat-induced unfolding method described previously (Kamijima et al., 2008). Purified hLA was diluted in phosphate-buffered saline (PBS) to a final concentration of 210 μM. The protein solution was incubated at 50 °C for 15 min with gentle agitation to induce partial unfolding. Following the initial incubation, oleic acid was added to the protein solution at a molar ratio of 120:1 (oleic acid:hLA). The mixture was then incubated for an additional 10-minute incubation at 50 °C with continuous agitation to facilitate complex formation. After cooling the solution to room temperature, the unbound oleic acid was removed by centrifugation. The resulting HAMLET complex was aliquoted and stored at −80 °C until further use in cytotoxicity and apoptosis assays. For direct comparison, an additional HAMLET complex was prepared using commercially available native human α-lactalbumin (Abcam, cat. # ab276670) under the same conditions (protein concentration, oleic acid ratio, and heat-induced unfolding protocol) to serve as a positive control.

Cell viability assay

The MTT assay was used to evaluate the inhibitory effect of the chloroplast-derived HAMLET complex on the proliferation of human colorectal adenocarcinoma (WiDr) and human breast adenocarcinoma (MCF-7) cells. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin (10,000 units/mL), 1% streptomycin (10 mg/mL), and 10% fetal bovine serum (FBS) under humidified conditions at 37°C with 5% CO_2. Both cell lines were routinely tested for mycoplasma contamination using a commercial PCR-based detection kit (Sigma-Aldrich, Cat. #MP0025) and authenticated via short tandem repeat (STR) profiling. Cells were seeded in 96-well plates at a density of 2×10^4 cells/well and incubated for 24 h to allow attachment. The HAMLET complex was then added to the culture medium at a final concentration of 21 μM, as determined in prior studies (Žilinskas et al., 2023), and incubated for 6 h. After treatment, the medium was replaced with fresh growth medium, and the cells were incubated for an additional 18 h. After incubation, 20 μL of MTT reagent (5 mg/mL in PBS) was added to each well, and the plates were incubated for 3-4 hours at 37°C to allow formazan crystals to form. The medium was then carefully removed, and the formazan crystals were dissolved in 100 μL dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm with a reference wavelength of 620 nm using a microplate reader.

Cell viability was expressed as a percentage relative to that of the untreated control cells. The controls included total soluble protein from wild-type plants, purified chloroplast-derived human α-lactalbumin, and oleic acid. In addition, a HAMLET complex prepared from native human α-lactalbumin was included as a reference control to evaluate the functional equivalence of chloroplast-derived HAMLET. All experiments were conducted in three biological replicates, each with three technical repeats unless otherwise stated. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test to determine differences between each treatment and the untreated control. Statistical significance was defined as ***$p < 0.001$.

Annexin V-FITC/PI Apoptosis Detection Assay

To further investigate the mechanism of HAMLET-induced cytotoxicity, Annexin V-FITC/PI (Propidium Iodide) dual staining was performed following treatment of WiDr and MCF-7 cells with HAMLET. After seeding in 6-well plates at a density of 1×10^5 cells per well and allowing overnight adhesion, cells were exposed to 21 μM of the chloroplast-derived HAMLET complex for 6 h. Untreated cells were used as negative controls. Apoptotic and necrotic cell populations were assessed using flow cytometry, as described previously (Miller, 2004).

Following treatment, the cells were harvested, washed twice with cold phosphate-buffered saline (PBS), and resuspended in 100 μL of binding buffer from the Annexin V-FITC/PI apoptosis detection kit (BD Biosciences). Annexin V-FITC and PI were added according to the manufacturer's instructions, and the samples were incubated at room temperature for 15 min in the dark. Cells were immediately analyzed using a MACSQuant 10 Flow Cytometer (Miltenyi Biotec, Germany), and 10,000 events were collected per sample. Cells were first gated on FSC-A vs SSC-A to select the population of interest, followed by FSC-H vs FSC-A to exclude doublets and select singlets. The data were analyzed to determine the proportions of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic cells (Annexin V^-/PI^+). All experiments were performed on three independent biological replicates, and for each replicate, 10,000 events were collected per sample. No statistical testing was performed for the apoptosis detection assay; results are presented as descriptive analyses based on three independent biological replicates.

Statistical analysis

All quantitative experiments, including ELISA, enzymatic assays, and cell viability studies, were performed in at

least three independent biological replicates, each with three technical replicates, unless otherwise stated. Data are presented as mean ± standard deviation (SD). Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test, with $p < 0.05$ considered significant. All statistical analyses were performed using SPSS software (version 26.0; IBM Corp., Armonk, NY, USA).

Results

Chloroplast transformation vectors

A synthetic chloroplast expression cassette was engineered to drive high-level expression of human α-lactalbumin (hLA) in *N. tabacum*, using modular regulatory elements and plastid-targeted integration. The hLA coding sequence was placed under the transcriptional control of the tobacco plastid *Prrn* promoter and the *T7g10* 5′ untranslated region, with transcription terminated by the *E. coli rrnB* 3′ UTR. The expression cassette was cloned into the pPRV111A chloroplast transformation vector, which includes homologous flanking regions (*rrn16* and *rps7/12*) to facilitate targeted integration into the plastid genome. Selection of transplastomic lines was achieved via the *aadA* gene encoding aminoglycoside 3′-adenyltransferase. The finalized construct, referred to as pPRV-hLA, was sequence-verified (Supplementary Figure S1) and subsequently used for plastid transformation. A schematic overview of the construct, including primer and probe positions used for molecular confirmation, is shown in Figure 1A.

Creation and evaluation of hLA producing homoplasmic transplastomic tobacco lines

The hLA expression cassette was introduced into the chloroplast genome of *N. tabacum* via biolistic transformation. Schematic representation of the wild-type plastome, including the homologous recombination regions used for integration and the restriction enzyme (BglII) cutting sites employed for Southern blot analysis is shown in Figure 1B. Following bombardment, leaf explants were cultured on spectinomycin-containing medium (500 mg/L) to enable selective regeneration. Resistant shoots were subjected to three consecutive rounds of selection under identical conditions to promote the emergence of homoplasmic lines (Figure 2A). The recovered lines were successfully rooted in selective media (Figure 2B), acclimated within a biodome, and subsequently transferred to greenhouse settings, where they developed to full maturity and produced seeds (Figure 2C). Comparative phenotypic evaluation revealed that hLA-transplastomic plants exhibited no discernible differences in morphology

or development relative to wild-type controls (Supplementary Figure S2). Plant height, leaf size and shape, flowering time, and seed production were comparable between transplastomic and WT lines under both *in vitro* and greenhouse conditions. The ability of transplastomic plants to reach maturity and produce viable progeny further confirmed that plastid-based hLA expression did not exert adverse physiological effects on plant growth or fertility.

PCR amplification confirmed integration of the hLA gene into the plastid genome, with a 148 bp product observed in transplastomic lines using gene-specific primers (Figure 3A). Further confirmation of site-specific insertion into the plastome was obtained using primers annealing to the *aadA* marker gene and *16S* flanking region, producing a 1.8 kb amplicon exclusively in transplastomic samples (Figure 3B). To assess construct integrity, PCR using primers located in the plastid flanking regions (16S-F and rps7-R) yielded a 4 kb fragment in transplastomic plants, whereas a ~2 kb product was observed in wild-type controls (Figure 3C). These results collectively confirm both the correct site-specific integration and the structural integrity of the transgene cassette in the plastid genome.

Southern blot hybridization validated the homoplasmic status of the transformed plants. Upon digestion of total DNA with *BglII* and hybridization with a probe specific to the *rrn16* flanking region, only a single band of 6.5 kb was detected in transplastomic lines, whereas wild-type plants showed a 4.5 kb fragment (Figure 3D). Homoplasmy was further confirmed by over-exposed Southern blot (Supplementary Figure S3), in which no residual wild-type fragment could be detected even after prolonged exposure. The absence of the wild-type signal confirmed that the plastid genomes of all selected lines were fully replaced by the transformed version, indicating successful establishment of homoplasmy.

Expression and quantification of chloroplast-made hLA protein in transplastomic lines

Total soluble proteins (TSP) were extracted from homoplasmic transplastomic tobacco plants expressing hLA and analyzed by immunoblotting using an anti-His-tag antibody. The presence of a 16 kDa protein, corresponding to human α-lactalbumin, was detected exclusively in the transplastomic plant samples, with no signal detected in the wild-type plants (Figure 4A, Figure S4). Based on quantification via enzyme-linked immunosorbent assay (ELISA), it was estimated that hLA comprised 23.4 ± 1.99% of the total soluble protein content in transplastomic tobacco plants, as confirmed by serial dilutions and standard curve analysis (Supplementary Figure S5), corresponding to 3.9 ± 0.4 mg/g fresh leaf weight (3.9 g/kg fresh leaf biomass). This accumulation level is among the highest reported for plastid-expressed therapeutic proteins, reinforcing the potential of chloroplasts as high-yield biofactories.

Purification of chloroplast-made hLA protein

Recombinant human α-lactalbumin was purified using affinity chromatography with a Ni-NTA column. The elution fractions were analyzed by SDS-PAGE followed by Coomassie blue staining, and a 16 kDa band corresponding to hLA was clearly visible in the purified fractions. The dominant 55 kDa band corresponding to the Rubisco large subunit, a major plant protein (Jensen, 2000), was also observed in crude extracts but was significantly reduced in the final purified fractions. To eliminate Rubisco, sodium phytate precipitation was applied (Krishnan & Natarajan, 2009), which successfully removed most of the Rubisco from the samples. Complete removal of the His-tag was achieved by TEV protease cleavage followed by a second Ni-NTA passage (Figure 4B). The resulting tag-free hLA migrated at the expected 14.1 kDa. No residual His-tagged protein or TEV protease was detectable. The multi-step purification protocol, combining affinity capture and selective Rubisco removal, enabled efficient downstream processing suitable for food-grade protein production pipelines.

Secondary structure analysis of chloroplast-expressed human α-lactalbumin by circular dichroism spectroscopy

Far-UV circular dichroism (CD) spectroscopy was conducted on both plastid-derived recombinant hLA and commercial native hLA. The spectra of the two proteins were nearly superimposable, each showing the characteristic α-helix-rich profile with negative minima at ~208 nm and ~222 nm and a positive maximum near 195 nm (Figure 5). Deconvolution analysis indicated that both proteins contained approximately 40% α-helix, 14–15% β-sheet, and ~45% unordered structures, values consistent with those previously reported for native hLA (Mossberg et al., 2010; Ramboarina & Redfield, 2003).

The observed structural similarity strongly suggests that chloroplast-derived hLA maintains correct folding, which is essential for its biological functionality. This native-like secondary structure is particularly critical for downstream applications such as the formation of the HAMLET complex and its associated bioactivities. The data affirm the structural integrity of the recombinant protein, validating the chloroplast system as a reliable platform for producing functionally competent human milk proteins.

Functional activity of chloroplast-derived human α-lactalbumin

Chloroplast-derived hLA successfully activated GalT, resulting in the production of lactose *in vitro*. The lactose yield in the presence of chloroplast-derived hLA was comparable to that obtained with commercial native human α-lactalbumin (Figure 6A). In the GalT-only control, no lactose peak was observed, as expected. However, substrate peaks corresponding to UDP-galactose (~1.2 min) and D-glucose (~3.3 min) were clearly detectable, confirming the presence of unreacted substrates in the absence of hLA. The retention times of lactose (~6.3 min), glucose, and UDP-galactose in reaction samples matched those of authentic standards run under the same chromatographic conditions. To rule out potential interference by co-purified plant proteins, an identical amount of total soluble protein purified from wild-type tobacco leaves was tested in the lactose synthesis assay. No lactose formation was observed, confirming that only the recombinant hLA is responsible for the observed galactosyltransferase-activating activity.

Quantitative analysis revealed that chloroplast-derived hLA achieved a lactose production rate of 0.95 ± 0.08 μmol/min/mg protein, which was 93% of the activity observed with native hLA (Figure 6B). These findings demonstrate that chloroplast-derived hLA retains its functional integrity and biological activity, validating its potential use as a functional component in dairy alternatives and food systems.

HAMLET induces apoptosis and reduces viability in cancer cells

The functionality of chloroplast-derived human α-lactalbumin was further assessed through its ability to form HAMLET complexes with oleic acid. Following thermal processing and complex formation, the resulting HAMLET preparations markedly reduced the viability of WiDr and MCF-7 cancer cells. MTT assays demonstrated a ~95% reduction in WiDr and ~90% reduction in MCF-7 cell survival compared with untreated controls ($p < 0.001$) (Figure 7A). HAMLET derived from commercial hLA showed a comparable cytotoxic profile, while controls including purified hLA alone, oleic acid alone, and wild-type plant extracts had no statistically significant effect ($p > 0.05$).

To investigate the mechanism of reduced cell viability, apoptosis was quantified using Annexin V/PI flow cytometry. In WiDr cells, treatment with chloroplast-derived HAMLET reduced the viable population to 5%, with 53% of cells in early apoptosis, 38% in late apoptosis, and 4% necrotic. In MCF-7 cells, 10% of cells remained viable, whereas 47% were early apoptotic, 35% late apoptotic, and 8% necrotic (Figure 7B). Representative flow cytometry dot plots illustrating the complete gating strategy are provided in Supplementary Figure S6. Together, these results show that chloroplast-derived HAMLET exerts potent cytotoxic effects against both cell lines, primarily through the induction

of apoptosis.

Discussion

Recombinant milk protein production has a significant potential to address the limitations and challenges associated with traditional dairy manufacturing. The increasing demand for sustainable and ethically sourced alternatives to animal-derived products, driven by concerns about animal welfare and environmental implications, has accelerated the development of this technology. Through the use of genetically modified organisms, it is feasible to synthesize milk proteins that closely resemble their natural counterparts (Piazenski et al., 2024). The landscape of recombinant milk protein technology has undergone substantial transformation, particularly in the selection of host organisms. Initial research focused on utilizing *E. coli*, a widely recognized model organism in the field of molecular biology. However, concerns regarding safety and potential contamination have impeded the progress of recombinant *E. coli* applications, particularly in the food sector (Batt et al., 1990; Chaudhuri et al., 1999; Goda et al., 2000; Kim et al., 1997). In recent years, innovative approaches have facilitated the adoption of alternative host organisms, including yeast (Deng et al., 2022; Kalidas et al., 2001; Kim et al., 1999; Saito et al., 2002; Totsuka et al., 1990), fungi (Sun et al., 1999), other bacterial species, and plants (Bicar et al., 2008; Chong et al., 1997; Salmon et al., 1998). These alternatives have garnered significant attention from industry leaders owing to their inherent advantages.

Plants, and particularly chloroplasts, represent a powerful chassis for the bioengineering of food-grade proteins, due to their scalability, safety, and capacity for post-translational modification. Our study reinforces this potential by demonstrating that rationally designed plastid expression cassettes can drive high-level production of complex human proteins such as α-lactalbumin. Notably, the methodology for expressing recombinant milk proteins in plants has been patented extensively. Several corporations, including Nobell Foods, Ventria BioScience, Alpine Roads, and Mozza Foods, have secured intellectual property rights to this approach and utilize a diverse range of plant species, such as soy, rice, tobacco, and thale cress, to produce caseins, whey proteins, and other milk-associated proteins (Huang et al., 2014; Lanquar & Magi, 2022; Tobin, 2022). However, these systems mostly rely on nuclear expression, which suffers from position effect variation and gene silencing. In contrast, plastid genome engineering ensures homoplasmy, high gene copy number, and stable inheritance, which are essential parameters in the rational design of reliable protein production platforms.

Recent advances in genetic engineering have enhanced the capacity of plant chloroplasts to express exogenous

proteins at high levels, offering advantages such as cost-effectiveness, scalability, and safety for recombinant protein production (Ehsasatvatan & Kohnehrouz, 2023; Scotti et al., 2013). Our study is the first report on the expression of functionally active human α-lactalbumin (hLA) in the chloroplast genome of *N. tabacum*. The successful development of homoplasmic transplastomic lines, coupled with robust protein accumulation, validates the efficiency of plastid-based systems. Recombinant hLA accumulated up to 3.9 ± 0.4 mg per gram fresh weight (FW) of leaf tissue (3.9 g/kg fresh leaf biomass), corresponding to ~23.4% of TSP. Following Ni–NTA affinity purification, the overall recovery was approximately 60%, yielding ~2.34 mg hLA per gram FW of processed leaf tissue. These values indicate that the downstream process is efficient in maintaining a substantial fraction of the recombinant protein while reducing host background proteins. This yield significantly exceeds prior reports using nuclear or microbial expression systems, demonstrating the impact of plastid bioengineering in overcoming key production bottlenecks. In bacterial hosts such as *E. coli*, soluble recombinant hLA production after refolding typically remains below 100 mg/L (Chaudhuri et al., 1999). Yeast expression systems like *K. phaffii* have achieved considerably higher yields, with recent reports describing up to 3.5 g/L of hLA in optimized fermentation setups (Deng et al., 2022). Mammalian CHO cell lines produce correctly folded protein at ~20–50 mg/L, suitable for therapeutic use but cost-prohibitive at scale (Demain & Vaishnav, 2009). Transgenic goats secrete 2–3 g/L of hLA in milk (Long, 2014),F yet animal containment and biosafety challenges restrict their industrial applicability. Plant nuclear expression systems report much lower values, generally in the range of 0.01–0.5% TSP (Salmon et al., 1998).

Importantly, the plant-derived hLA maintained its native-like structure and biofunctionality. Circular dichroism analysis revealed secondary structure content highly similar to native hLA, suggesting proper folding — a crucial prerequisite for downstream applications in nutrition and therapeutics. The presence of correctly folded protein is essential not only for structural integrity but also for functionality in lactose biosynthesis and complex formation with oleic acid.

A key novelty of this study is the demonstration that chloroplast-derived hLA retains its biological activity by activating galactosyltransferase (GalT) *in vitro*, catalyzing lactose formation. Quantitative analysis revealed a lactose synthesis rate equivalent to 93% of that achieved by commercial native hLA. This finding confirms the correct conformation and cofactor activity of the plant-made protein, which is a critical step in demonstrating functional bioequivalence**.**

From a biological engineering perspective**,** the successful lactose biosynthesis represents a synthetic metabolic function that was reconstituted *in vitro* using a plant-expressed human protein. This highlights the broader potential of plant chassis in producing synthetic metabolic components for use in reconstituted food systems or diagnostic platforms. Moreover, the ability to program plastids for functional protein output strengthens their role in modular, predictable biofactory design — a cornerstone of synthetic biology.

The high catalytic efficiency of the chloroplast-derived hLA at 0.95±0.08 μmol/min/mg protein, which is 93% of the native protein's activity, carries significant physiological and industrial implications. From a physiological perspective, this near-native activity is critical as hLA is a key regulatory subunit of the lactose synthase enzyme in the mammary gland. This high functional fidelity confirms that the α-lactalbumin produced in the chloroplast system is correctly folded and capable of forming a fully active, stable complex with beta-1,4-galactosyltransferase 1 (GalT). This validates the use of Ch-hLA as a functional replacement for native human α-lactalbumin in applications like fortification of humanized infant formulas, where the nutritional quality and biological role of the protein are paramount.

From an industrial standpoint, the high specific activity suggests that Ch-hLA is suitable for large-scale enzymatic applications. This includes the potential use of the protein as a reusable biocatalyst in the production of valuable prebiotics, such as specific galacto-oligosaccharides (GOS), which are synthesized by GalT in the presence of hLA. This functional confirmation supports the economic viability of the transplastomic system as an efficient biofactory for industrial-grade functional proteins.

In addition, the recombinant hLA was successfully purified using a two-step strategy: Ni-NTA affinity chromatography and Rubisco depletion. This approach yielded highly enriched hLA suitable for both structural and functional assays. While Ca^{2+}/phytate treatment of crude extracts has been reported to remove up to 85% of Rubisco (Krishnan & Natarajan, 2009), the method is not fully selective and may cause loss of recombinant proteins. For this reason, we applied phytate precipitation after Ni–NTA enrichment. Similar strategies have been reported in other chloroplast-based systems, where post-purification phytate treatment was successfully used to improve purity (Boyhan & Daniell, 2011). Nonetheless, employing this step earlier in the process could further reduce host protein background and potentially improve downstream recovery, and this option warrants investigation in future optimization studies. The downstream purification pipeline employed here is scalable and food-grade compatible, providing a basis for future bioprocessing development. Considering that downstream processing constitutes a major

cost component in protein biomanufacturing, the simplicity and effectiveness of this strategy reinforce the economic feasibility of chloroplast-based systems.

Beyond nutritional applications, the formation of HAMLET — a cytotoxic protein-lipid complex composed of α-lactalbumin and oleic acid — demonstrates that chloroplast-derived hLA is amenable to functionalization for therapeutic purposes**.** Upon thermal processing, the plant-made hLA was capable of forming a bioactive HAMLET complex that induced robust apoptosis in both colorectal (WiDr) and breast (MCF-7) cancer cell lines. In line with these results, purified hLA alone did not reduce cell viability significantly ($p > 0.05$), confirming that the cytotoxic effect is strictly dependent on HAMLET complex formation. Flow cytometry analysis confirmed apoptosis as the predominant mode of cell death, with over 90% of cells undergoing programmed cell death after 6-hour exposure. These results are in line with reports on native HAMLET (HÅKANssoN et al., 1995; Ho Cs et al., 2012; Svensson et al., 1999) confirming that plant-made hLA can match the therapeutic efficacy of its mammalian-derived counterpart.

An important consideration in evaluating plastid-based hLA production is the choice of host species. In this study, *Nicotiana tabacum* was selected as a model system due to its well-established plastid transformation protocols, high plastome copy number, and suitability for proof-of-concept demonstration. However, tobacco is a non-food crop and therefore not a direct candidate for producing bioengineered nutritional proteins intended for infant consumption. This raises legitimate biosafety and food security concerns, particularly in the context of scaling production for human nutrition. Several strategies may mitigate these limitations. First, complete downstream purification of recombinant hLA, as demonstrated here, eliminates co-purified plant metabolites such as alkaloids, thereby ensuring food-grade purity. Second, edible plant species with established plastid transformation systems, including lettuce (*Lactuca sativa*), potato (*Solanum tuberosum*), and maize (*Zea mays*) offer promising alternatives for future translation of this platform into safe, food-compatible biofactories (Bicar et al., 2008; Boyhan & Daniell, 2011; Daniell et al., 2001). Third, plastid genome engineering inherently restricts gene flow through maternal inheritance, addressing environmental containment issues (Bock, 2007; Maliga, 2002). Collectively, these approaches provide a roadmap for transferring proof-of-concept achievements from tobacco into edible plant systems, ensuring biosafety and compliance with food security standards while retaining the high yield and functional advantages of plastid biofactories.

This dual functionality — nutritional and anti-cancer — makes α-lactalbumin a prime candidate for synthetic food

design and plant-based therapeutic production. Its high tryptophan content, digestibility, and structural integrity position it as a central component for infant formula design, brain health supplements, and functional dairy alternatives (Kunz & Lönnerdal, 1992; Layman et al., 2018). By enabling its scalable production in plants, this work paves the way for next-generation food engineering strategies that are animal-free, sustainable, and multifunctional.

In summary, this study demonstrates how rational plastid genome engineering can be used to develop programmable plant platforms for producing human therapeutic and nutritional proteins. The integration of synthetic biology principles — including codon optimization, standardized expression cassettes, and functional validation — showcases the feasibility of this system for biological engineering applications. Future efforts could expand this platform to other milk proteins (e.g., lactoferrin, caseins) or co-express multiple components in polycistronic constructs to mimic complete milk protein blends. Overall, our findings support the emerging paradigm that chloroplasts are not only viable expression hosts, but strategic bioengineering platforms for next-generation synthetic foods and plant-made pharmaceuticals.

Conclusion

This study reports the first successful expression of functional human α-lactalbumin (hLA) in tobacco chloroplasts, achieving high-level accumulation (~23% TSP) via rational plastid genome engineering. The plant-derived hLA retained native-like folding and biological activity, evidenced by its capacity to activate galactosyltransferase for lactose synthesis and form cytotoxic HAMLET complexes. Future studies will focus on assessing the *in vivo* bioavailability and safety of this plant-derived protein in animal models, as well as transferring this genetic system into edible crops such as lettuce or spinach to eliminate downstream purification costs for oral delivery applications. This work establishes a foundation for sustainable, animal-free production of dual-purpose nutritional and therapeutic milk proteins.

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Author contribution statement

M.E., designed the study and experiments, performed the experiments, analyzed the data, and wrote the manuscript.

B. B. K., conceived, designed, organized, and supervised the study, provided specialized scientific and technical support, and revised the paper. All authors read and approved the final manuscript.

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Data availability

The codon-optimized nucleotide sequence of the synthetic human α-lactalbumin (hLA) gene generated in this study is available in GenBank at NCBI under the accession number PX828338. All other datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare no competing interests.

Ethical approval and consent to participate

Not applicable. This study does not report the results of a clinical trial.

Clinical trial number

Not applicable.

Consent for publication

Not applicable.

References

Andreas, N. J., Kampmann, B., & Le-Doare, K. M. (2015). Human breast milk: A review on its composition and bioactivity. *Early Human Development*, *91*(11), 629-635.

Awasthi, V., Bahman, S., Thakur, L. K., Singh, S. K., Dua, A., & Ganguly, S. (2012). Contaminants in milk and impact of heating: an assessment study. *Indian Journal of Public Health*, *56*(1), 95-99.

Batt, C. A., Rabson, L. D., Wong, D. W., & Kinsella, J. E. (1990). Expression of recombinant bovine β-lactoglobulin in Escherichia coli. *Agricultural and Biological Chemistry*, *54*(4), 949-955.

Behm, K., Nappa, M., Aro, N., Welman, A., Ledgard, S., Suomalainen, M., & Hill, J. (2022). Comparison of carbon footprint and water scarcity footprint of milk protein produced by cellular agriculture and the dairy industry. *The International Journal of Life Cycle Assessment*, *27*(8), 1017-1034.

Bicar, E. H., Woodman-Clikeman, W., Sangtong, V., Peterson, J. M., Yang, S. S., Lee, M., & Scott, M. P. (2008).

Transgenic maize endosperm containing a milk protein has improved amino acid balance. *Transgenic Research*, *17*, 59-71.

Bock, R. (2007). Structure, function, and inheritance of plastid genomes. In *Cell and Molecular Biology of Plastids* (pp. 29-63). Springer.

Boyhan, D., & Daniell, H. (2011). Low-cost production of proinsulin in tobacco and lettuce chloroplasts for injectable or oral delivery of functional insulin and C-peptide. *Plant Biotechnology Journal*, *9*(5), 585-598.

Chaudhuri, T. K., Horii, K., Yoda, T., Arai, M., Nagata, S., Terada, T. P., Uchiyama, H., Ikura, T., Tsumoto, K., & Kataoka, H. (1999). Effect of the extra N-terminal methionine residue on the stability and folding of recombinant α-lactalbumin expressed in Escherichia coli. *Journal of Molecular Biology*, *285*(3), 1179-1194.

Chebolu, S., & Daniell, H. (2009). Chloroplast-derived vaccine antigens and biopharmaceuticals: expression, folding, assembly and functionality. *Plant-produced Microbial Vaccines*, 33-54.

Chong, D., Roberts, W., Arakawa, T., Illes, K., Bagi, G., Slattery, C., & Langridge, W. (1997). Expression of the human milk protein β-casein in transgenic potato plants. *Transgenic Research*, *6*, 289-296.

Daniell, H., Streatfield, S. J., & Wycoff, K. (2001). Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends in Plant Science*, *6*(5), 219-226.

Demain, A. L., & Vaishnav, P. (2009). Production of recombinant proteins by microbes and higher organisms. *Biotechnology Advances*, *27*(3), 297-306.

Deng, M., Lv, X., Liu, L., Li, J., Du, G., Chen, J., & Liu, Y. (2022). Efficient Bioproduction of Human Milk Alpha-Lactalbumin in Komagataella phaffii. *Journal of Agricultural and Food Chemistry*, *70*(8), 2664-2672.

Ehsasatvatan, M., & Kohnehrouz, B. B. (2023). The lyophilized chloroplasts store synthetic DARPin G3 as bioactive encapsulated organelles. *Journal of Biological Engineering*, *17*(1), 63.

Ehsasatvatan, M., Kohnehrouz, B. B., Gholizadeh, A., Ofoghi, H., & Shanehbandi, D. (2022a). Physical and Biologically Effective Parameters in Developing Transplastomic Tobacco Plants by Particle Bombardment Method using PDS-1000/He. *Optimization*, *10*(2).

Ehsasatvatan, M., Kohnehrouz, B. B., Gholizadeh, A., Ofoghi, H., & Shanehbandi, D. (2022b). The production of the first functional antibody mimetic in higher plants: the chloroplast makes the DARPin G3 for HER2 imaging in oncology. *Biological Research*, *55*(1), 1-18.

Enomoto, H., Hayashi, Y., Li, C., Ohki, S., Ohtomo, H., Shiokawa, M., & Aoki, T. (2009). Glycation and phosphorylation of α-lactalbumin by dry heating: Effect on protein structure and physiological functions. *Journal of Dairy Science*, *92*(7), 3057-3068.

Fischer, R., Stoger, E., Schillberg, S., Christou, P., & Twyman, R. M. (2004). Plant-based production of biopharmaceuticals. *Current Opinion in Plant Biology*, *7*(2), 152-158.

Geistlinger, T., Jensen, H., Jhala, R., Meerman, H., Ramesh, B., Wagoner, T., Johnson, T. S., Wu, V. W.-X., & Manea, F. (2022). Recombinant components and compositions for use in food products. In: Google Patents.

Giuffrida, M. G., Cavaletto, M., Giunta, C., Neuteboom, B., Cantisani, A., Napolitano, L., Calderone, V., Godovac-Zimmermann, J., & Conti, A. (1997). The unusual amino acid triplet Asn-Ile-Cys is a glycosylation consensus site in human alpha-lactalbumin. *Journal of Protein Chemistry*, *16*(8), 747-753. https://doi.org/10.1023/a:1026359715821

Goda, S., Sharman, A., Yates, M., Mann, N., Carr, N., Minton, N., & Brehm, J. (2000). Recombinant expression analysis of natural and synthetic bovine alpha-casein in Escherichia coli. *Applied Microbiology and Biotechnology*, *54*, 671-676.

Hakansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H., & Svanborg, C. (1995). Apoptosis induced by a human milk protein. *Proceedings of the National Academy of Sciences*, *92*(17), 8064-8068.

Heine, W. E., Klein, P. D., & Reeds, P. J. (1991). The importance of α-lactalbumin in infant nutrition. *The Journal of Nutrition*, *121*(3), 277-283.

Hettinga, K., & Bijl, E. (2022). Can recombinant milk proteins replace those produced by animals? *Current Opinion in Biotechnology*, *75*, 102690.

Ho Cs, J., Rydstrom, A., Manimekalai, M. S. S., Svanborg, C., & Grüber, G. (2012). Low resolution solution structure of HAMLET and the importance of its alpha-domains in tumoricidal activity. *PloS One*, *7*(12), e53051.

Huang, N., Rodriguez, R. L., & Hagie, F. E. (2014). Expression of human milk proteins in transgenic plants. In: Google Patents.

Jensen, R. G. (2000). Activation of Rubisco regulates photosynthesis at high temperature and CO2. *Proceedings of the National Academy of Sciences*, *97*(24), 12937-12938.

Kalidas, C., Joshi, L., & Batt, C. (2001). Characterization of glycosylated variants of β-lactoglobulin expressed in

Pichia pastoris. *Protein Engineering*, *14*(3), 201-207.
Kamijima, T., Ohmura, A., Sato, T., Akimoto, K., Itabashi, M., Mizuguchi, M., Kamiya, M., Kikukawa, T., Aizawa, T., & Takahashi, M. (2008). Heat-treatment method for producing fatty acid-bound alpha-lactalbumin that induces tumor cell death. *Biochemical and Biophysical Research Communications*, *376*(1), 211-214.
Kim, Y.-K., Yu, D.-Y., Kang, H.-A., Yoon, S., & Chung, B.-H. (1999). Secretory Expression of Human $\alpha_{s1} $-Casein in Saccharomyces cerevisiae. *Journal of Microbiology and Biotechnology*, *9*(2), 196-200.
Kim, Y., Chung, B. H., Yoon, S., Lee, K.-K., Lönnerdal, B., & Yu, D.-Y. (1997). High-level expression of human α s1-casein in Escherichia coli. *Biotechnology Techniques*, *11*, 675-678.
Koop, H.-U., Herz, S., Golds, T. J., & Nickelsen, J. (2007). The genetic transformation of plastids. *Cell and Molecular Biology of Plastids*, 457-510.
Krishnan, H. B., & Natarajan, S. S. (2009). A rapid method for depletion of Rubisco from soybean (Glycine max) leaf for proteomic analysis of lower abundance proteins. *Phytochemistry*, *70*(17-18), 1958-1964.
Kunz, C., & Lönnerdal, B. (1992). Re-evaluation of the whey protein/casein ratio of human milk. *Acta Paediatrica*, *81*(2), 107-112.
Lanquar, V., & Magi, E.-R. (2022). Recombinant milk proteins and food compositions comprising the same. In: Google Patents.
Layman, D. K., Lönnerdal, B., & Fernstrom, J. D. (2018). Applications for α-lactalbumin in human nutrition. *Nutrition Reviews*, *76*(6), 444-460.
Lien, E. L. (2003). Infant formulas with increased concentrations of α-lactalbumin. *The American Journal of Clinical Nutrition*, *77*(6), 1555S-1558S.
Long, C. (2014). Transgenic livestock for agriculture and biomedical applications. In *BMC Proceedings* (Vol. 8, No. Suppl 4, p. O29). London: BioMed Central. doi: 10.1186/1753-6561-8-S4-O29.
Lönnerdal, B. (2014). Infant formula and infant nutrition: bioactive proteins of human milk and implications for composition of infant formulas. *The American Journal of Clinical Nutrition*, *99*(3), 712S-717S.
Ma, J. K. C., Barros, E., Bock, R., Christou, P., Dale, P. J., Dix, P. J., Fischer, R., Irwin, J., Mahoney, R., & Pezzotti, M. (2005). Molecular farming for new drugs and vaccines: current perspectives on the production of pharmaceuticals in transgenic plants. *EMBO Reports*, *6*(7), 593-599.
Maliga, P. (2002). Engineering the plastid genome of higher plants. *Current Opinion in Plant Biology*, *5*(2), 164-172.
Miller, E. (2004). Apoptosis measurement by annexin v staining. *Cancer Cell Culture: Methods and Protocols*, 191-202.
Mossberg, A. K., Hun Mok, K., Morozova-Roche, L. A., & Svanborg, C. (2010). Structure and function of human α-lactalbumin made lethal to tumor cells (HAMLET)-type complexes. *The FEBS journal*, *277*(22), 4614-4625.
Murray, M., & Thompson, W. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, *8*(19), 4321-4326.
Oey, M., Lohse, M., Kreikemeyer, B., & Bock, R. (2009). Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. *The Plant Journal*, *57*(3), 436-445.
Oey, M., Lohse, M., Scharff, L. B., Kreikemeyer, B., & Bock, R. (2009). Plastid production of protein antibiotics against pneumonia via a new strategy for high-level expression of antimicrobial proteins. *Proceedings of the National Academy of Sciences*, *106*(16), 6579-6584.
Overton, T. W. (2014). Recombinant protein production in bacterial hosts. *Drug Discovery Today*, *19*(5), 590-601.
Permyakov, E. A. (2020). α-Lactalbumin, amazing calcium-binding protein. *Biomolecules*, *10*(9), 1210.
Permyakov, E. A., & Berliner, L. J. (2000). α-Lactalbumin: structure and function. *FEBS Letters*, *473*(3), 269-274.
Piazenski, I. N., Candelário, J. P. M., Soccol, V. T., de Souza Vandenberghe, L. P., de Melo Pereira, G. V., & Soccol, C. R. (2024). From Lab to Table: the path of recombinant milk proteins in transforming dairy production. *Trends in Food Science & Technology*, 104562.
Ramboarina, S., & Redfield, C. (2003). Structural characterisation of the human alpha-lactalbumin molten globule at high temperature. *Journal of Molecular Biology*, *330*(5), 1177-1188. https://doi.org/10.1016/s0022-2836(03)00639-9
Saito, A., Usui, M., Song, Y., Azakami, H., & Kato, A. (2002). Secretion of glycosylated α-lactalbumin in yeast Pichia pastoris. *The Journal of Biochemistry*, *132*(1), 77-82.
Salmon, V., Legrand, D., Slomianny, M.-C., El Yazidi, I., Spik, G., Gruber, V., Bournat, P., Olagnier, B., Mison, D., & Theisen, M. (1998). Production of human lactoferrin in transgenic tobacco plants. *Protein Expression and Purification*, *13*(1), 127-135.
Scotti, N., Bellucci, M., & Cardi, T. (2013). The chloroplasts as platform for recombinant proteins production. In

Translation in Mitochondria and Other Organelles (pp. 225-262). Springer.
Smith, N. W., Fletcher, A. J., Hill, J. P., & McNabb, W. C. (2022). Modeling the contribution of milk to global nutrition. *Frontiers in Nutrition*, *8*, 716100.
Sun, X.-L., Baker, H. M., Shewry, S. C., Jameson, G. B., & Baker, E. N. (1999). Structure of recombinant human lactoferrin expressed in Aspergillus awamori. *Acta Crystallographica Section D: Biological Crystallography*, *55*(2), 403-407.
Svensson, M., Sabharwal, H., Håkansson, A., Mossberg, A.-K., Lipniunas, P., Leffler, H., Svanborg, C., & Linse, S. (1999). Molecular characterization of α–lactalbumin folding variants that induce apoptosis in tumor cells. *Journal of Biological Chemistry*, *274*(10), 6388-6396.
Tobin, C. J. (2022). Recombinant micelle and method of in vivo assembly. In: Google Patents
Totsuka, M., Katakura, Y., Shimizu, M., Kumagai, I., Miura, K.-i., & Kaminogawa, S. (1990). Expression and secretion of bovine β-lactoglobulin in Saccharomyces cerevisiae. *Agricultural and Biological Chemistry*, *54*(12), 3111-3116.
Vestergaard, M., Chan, S. H. J., & Jensen, P. R. (2016). Can microbes compete with cows for sustainable protein production-A feasibility study on high quality protein. *Scientific Reports*, *6*(1), 36421.
Žilinskas, J., Stukas, D., Jasukaitienė, A., Šapauskienė, J., Banienė, R., Trumbeckaitė, S., Švagždys, S., Cicciu, M., Dambrauskas, Ž., & Gulbinas, A. (2023). HAMLET effect on cell death and mitochondrial respiration in colorectal cancer cell lines with KRAS/BRAF mutations. *Journal of Cancer Research and Clinical Oncology*, *149*(11), 8619-8630.
Zoubenko, O. V., Allison, L. A., Svab, Z., & Maliga, P. (1994). Efficient targeting of foreign genes into the tobacco plastid genome. *Nucleic Acids Research*, *22*(19), 3819-3824.

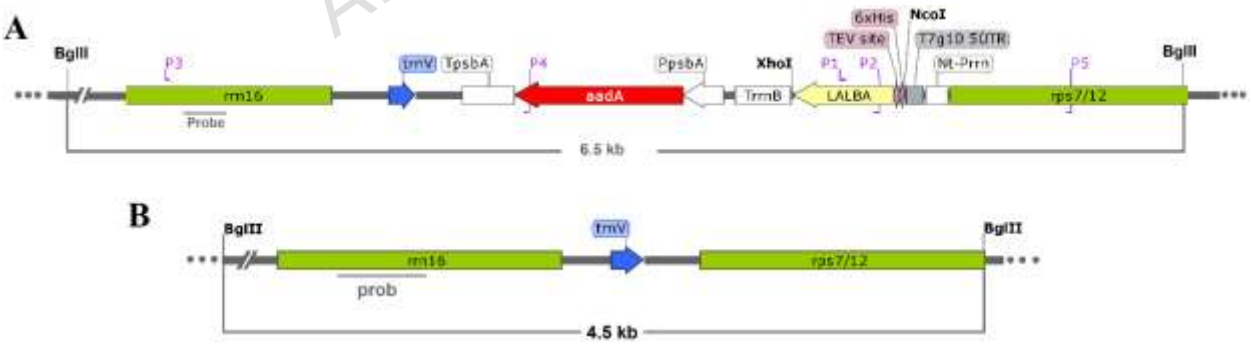

Figure 1. A) Physical map of plastid transformation vectors of the pPRV111A harboring hLA expression cassettes (PRV-hLA), and B) homologous recombination regions of the wild-type plastome. Nt-Prrn: ribosomal RNA operon promoter from tobacco; T7g10 5′ UTR: 5′ untranslated region of bacteriophage T7 gene 10; hLA: coding sequence of human alpha-lactalbumin, TrrnB: rrnB 3′ untranslated region from *E. coli;* PpsbA: promoter and 5′ UTR of *psbA* gene; *aadA*: aminoglycoside 3′- adenylytransferase gene; TpsbA: terminator of *psbA* gene. The transgenes are targeted to the intergenic region between the rrn16 and rps7/12 plastid genes. The expected sizes of the DNA

fragments in Southern blot analyses with the restriction enzyme *Bgl*II are indicated. The location of the Southern blotting probe is shown as a black bar.

Figure 2. Regeneration of hLA transplastomic tobacco plants A) appearance and growth of the single green and resistant seedling on RMOP medium containing 500 mg/l of spectinomycin 35 days after shooting; B) rooted homoplasmic transplastomic plant in MS medium containing 500 mg/l spectinomycin 3 months after shooting; C) adapted transplastomic plants to natural conditions.

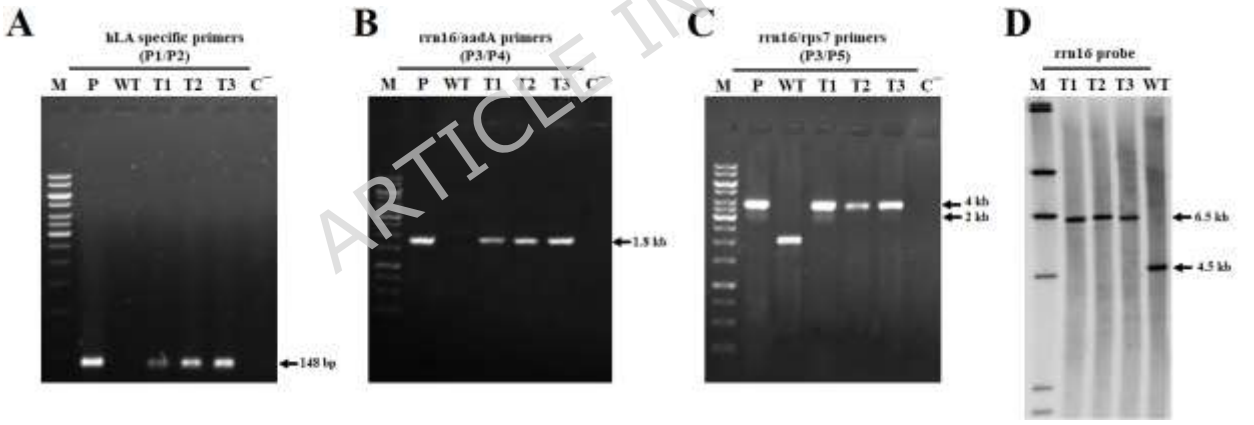

Figure 3. A) PCR analysis of hLA transplastomic plants with primers P1/P2 which land on the hLA coding sequence, generating a 148 bp fragment. B) PCR analysis of hLA transplastomic plants with primers P3/P4 which land on the rrn16 flanking sequence and *aadA* gene, generating 1.8 kb fragments in transplastomic. C) PCR analysis of hLA transplastomic plants with primers P3/P5 which land on the rrn16 and rps7/12 flanking sequences, generating 4 kb fragment in transplastomic and 2 kb fragment in wild-type plant. D) Southern blot analysis of hLA transplastomic plants. A single band of 6.5 kb after digestion of gDNA (2 μg) with *Bgl*II confirms hLA gene integration and homoplasmy in hLA transplastomic.

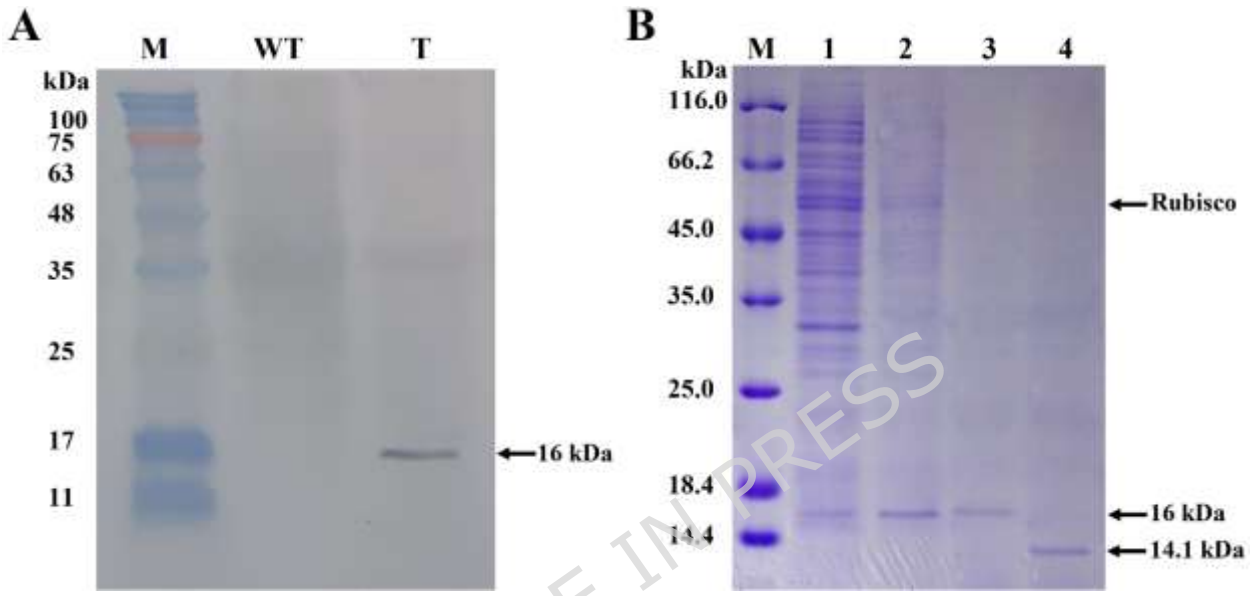

Figure 4. A) Western blot analysis of total soluble protein of hLA transplastomic plants probed with anti-his-tag antibody. M: prestained molecular mass markers (CinnaGen, Iran, Cat. #SL7011); WT: 500 ng of total soluble protein from untransformed plant; T: 500 ng total soluble protein from transplastomic plant leaves. Sizes are shown on the left-hand side in kilodaltons (kDa). B) Coomassie blue–stained SDS-PAGE of purified hLA protein from the total soluble protein using affinity chromatography. M: unstained protein MW marker (Thermo Fisher Scientific, USA, Cat. #26610); Lane 1: total soluble protein from transplastomic plants (300 ng); Lane 2: purified fraction from transplastomic plants (~150 ng); Lane 3: Purified hLA after phytate precipitation to remove Rubisco (~100 ng). Lane 4: purified fraction after his-tag removal (~100 ng).

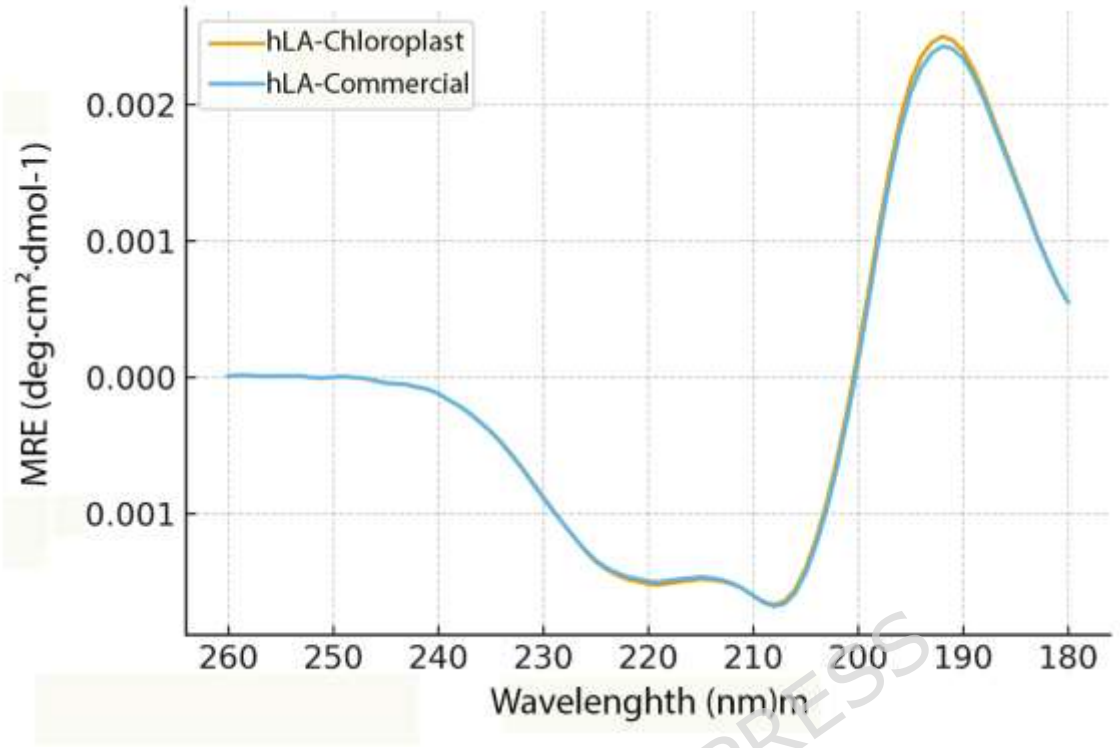

Figure 5. Comparative Far-UV circular dichroism (CD) spectra of recombinant hLA expressed in tobacco chloroplasts (Orange) and commercial native hLA (blue). Data are expressed as mean residue ellipticity (deg·cm²·dmol⁻¹). Both spectra overlap and display typical α-helical features, with minima at 208 nm and 222 nm. Secondary structure analysis indicated similar compositions (~40% α-helix, ~15% β-sheet, ~45% coil), confirming structural equivalence of plastid-derived and native hLA. Data represent mean ± SD of three independent biological replicates, each measured in technical triplicates.

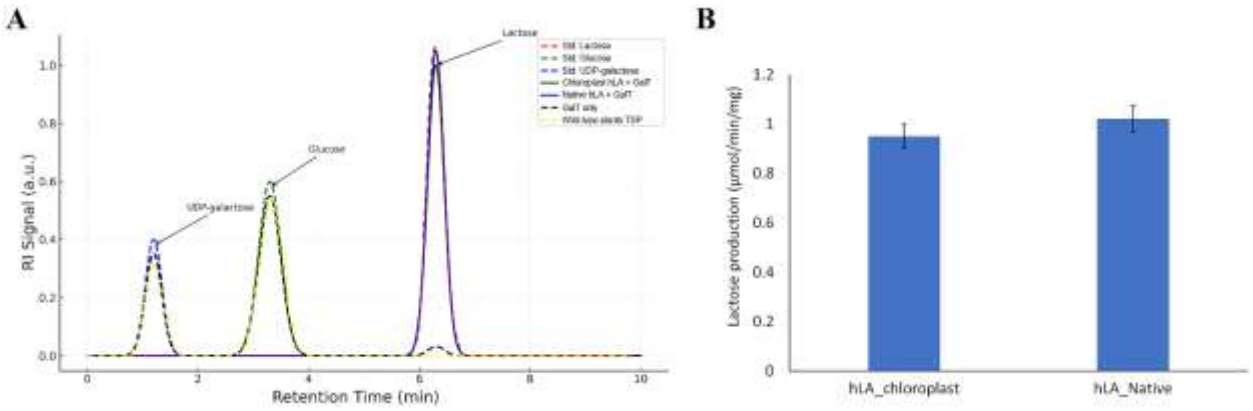

Figure 6. Functional validation of chloroplast-derived human α-lactalbumin (hLA) through *in vitro* lactose synthesis. (A) HPLC chromatogram demonstrating lactose formation. Authentic standards of lactose (red dashed line), D-glucose (green dashed line), and UDP-galactose (blue dashed line) were analyzed alongside reaction mixtures. Reaction mixtures contained galactosyltransferase (GalT) with chloroplast-derived hLA (dark green), native human hLA (blue), GalT only (black dashed line), or TSP rom wild-type tobacco plants (yellow dashed line). The lactose peak (~6.3 min) is clearly visible in reactions containing hLA but absent in the GalT-only control, in which only the substrate peaks for glucose (~3.3 min) and UDP-galactose (~1.2 min) appear. No lactose peak was detected in the wild-type TSP control, confirming that plant-derived components do not interfere with the assay (B) Quantitative analysis of lactose production by galactosyltransferase (GalT) in the presence of either chloroplast-derived hLA or native human hLA. Recombinant hLA purified from tobacco chloroplasts supported lactose synthesis at a rate of 0.95 ± 0.08 µmol/min/mg protein, equivalent to 93% of the activity observed with native hLA (1.02 ± 0.05 µmol/min/mg), while the GalT-only control showed negligible activity. Data represent mean ± SD of three independent biological replicates, each measured in technical triplicates.

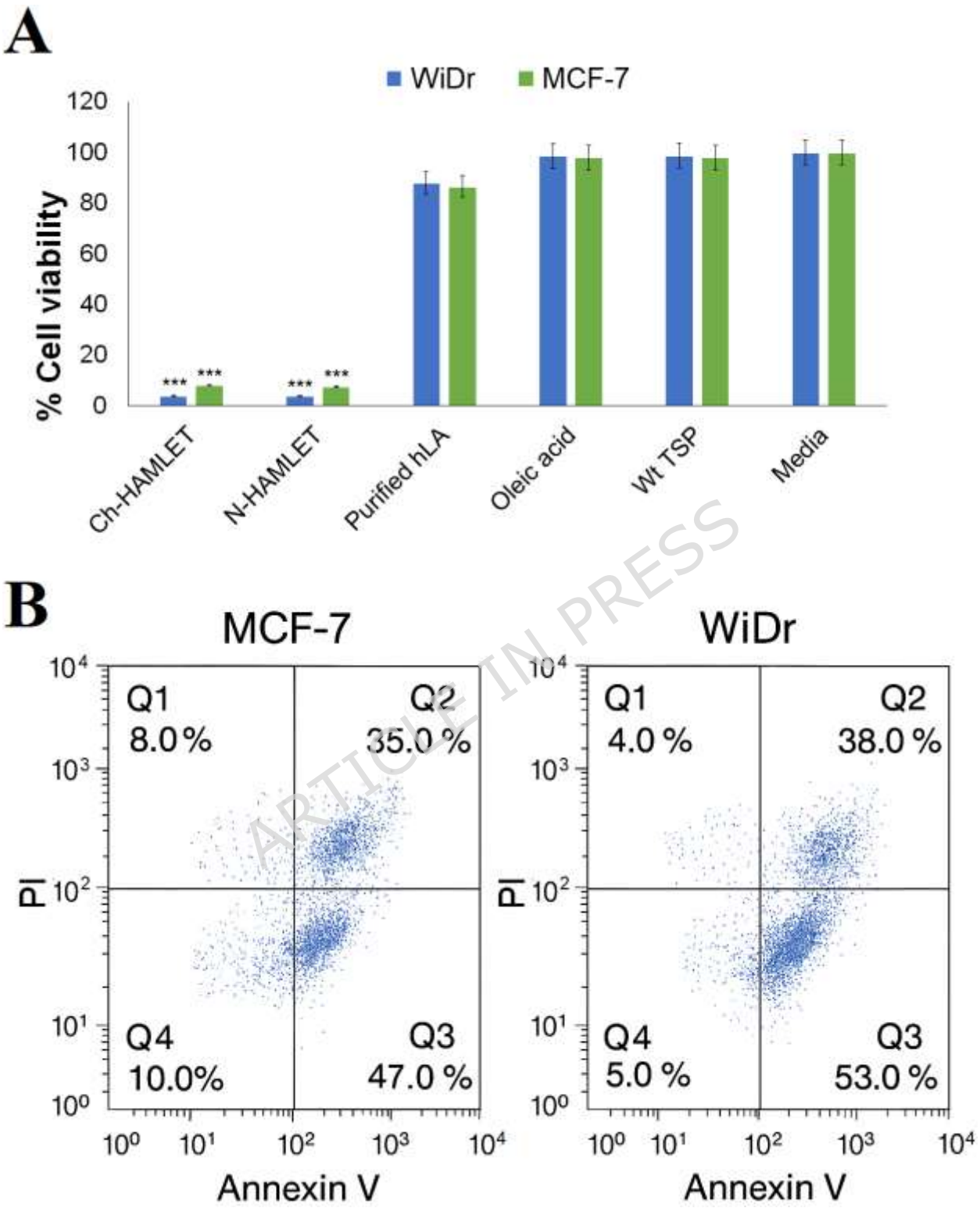

Figure 7. Tumoricidal activity and apoptosis induction by chloroplast-derived HAMLET complex in human cancer cells. (A) Cell viability of WiDr (colorectal adenocarcinoma) and MCF-7 (breast adenocarcinoma) cell lines after treatment with 21 μM HAMLET complexes prepared from plastid-derived hLA (Ch-HAMLET), 21 μM HAMLET

complexes prepared from commercial hLA (N-HAMLET), purified hLA, oleic acid, and total soluble proteins (TSP) from wild-type (WT) plants, measured by MTT assay. HAMLET treatment significantly reduced cell viability in both cell lines (~96% reduction in WiDr and ~92% in MCF-7) compared to controls. Data are presented as mean ± SD (n = 3). Statistical significance was determined by one-way ANOVA followed by a post-hoc test. Asterisks denote significant differences compared to the control group: ***$p < 0.001$. No statistically significant cytotoxicity ($p > 0.05$ vs. Control) was observed in cells treated with purified α-lactalbumin, oleic acid, or WT extracts. (B) Flow cytometric analysis of apoptosis using Annexin V-FITC/PI (Propidium Iodide) staining after 6-hour treatment with HAMLET (21 μM). In WiDr cells, 53% were early apoptotic, 38% late apoptotic, and 4% necrotic, with only 5% viable. In MCF-7 cells, 47% were early apoptotic, 35% late apoptotic, 8% necrotic, and 10% viable. The data indicate that HAMLET induces potent apoptosis-mediated cytotoxicity in both cancer cell lines.