https://doi.org/10.1038/s44383-025-00005-5

Sustainable production of functional human serum albumin in *Nicotiana benthamiana*: a green biomanufacturing approach

Ittichai Sujarittham[1,2], Utapin Ngaokrajang[3], Pipob Suwanchaikasem[3], Kaewta Rattanapisit[3], Kitti Jirananon[3], Pornpoom Angsuwattana[4,5], Pornchai Rojsitthisak[4,6], Christine Joy I. Bulaon[3] ✉ & Waranyoo Phoolcharoen[1,2] ✉

Human serum albumin (HSA) is widely used in medicine and biotechnology, yet its demand exceeds plasma-derived supply. Recombinant production in microbial and mammalian systems presents cost and sustainability limitations. This study optimized transient expression of recombinant HSA (rHSA) in *Nicotiana benthamiana* and evaluated its ecological impact. The highest yield of 30 µg/g fresh weight was achieved at 4 days post-infiltration using Agrobacterium OD600 of 0.2 and 21-day-old seedlings. Purified rHSA exhibited 95% purity and significantly enhanced NIH3T3 fibroblast proliferation in a dose-dependent manner. Sustainability assessment using ComplexMoGAPI indicated moderate solvent efficiency (0.083 L/mg) and energy consumption (6.73 kWh/mg) but identified waste generation (9350 g/mg) and process mass intensity (10,166.67 g/mg) as major environmental constraints. While *N. benthamiana* offers a scalable rHSA production platform, improvements in waste valorization and solvent recovery are needed to enhance environmental performance. This study advances plant-based biomanufacturing as a sustainable strategy for biopharmaceutical production.

Human serum albumin (HSA) is an essential plasma protein with widespread applications in medicine and biotechnology. It functions as a stabilizer in vaccine formulations, a therapeutic agent in conditions such as severe burns and hemorrhagic shock, and a vital supplement in serum-free cell culture media[1]. The global demand for HSA exceeds 500 metric tons annually, largely fulfilled through plasma fractionation. However, this method is constrained by limited donor availability, high production costs, and potential biosafety risks associated with blood-borne pathogens[2]. To address these limitations, alternative recombinant expression platforms including microbial, yeast, and mammalian cell cultures have been explored. Yet, each presents inherent challenges: *Escherichia coli* lacks post-translational modifications (PTMs) machinery critical for protein activity[3], while yeast and mammalian systems require complex culture conditions, bioreactor infrastructure, and long production cycles, driving up costs and complexity[4–6]. These challenges underscore the pressing need for more scalable, cost-effective, and sustainable production systems for biopharmaceuticals.

Plant molecular farming has emerged as a versatile and increasingly validated platform for recombinant protein production. It offers several advantages, including lower production costs, rapid scalability, intrinsic biosafety, and the capacity for PTMs[6,7]. Among transient expression systems, *Nicotiana benthamiana* has become a workhorse due to its susceptibility to *Agrobacterium tumefaciens*-mediated transformation and its amenability to scalable agroinfiltration techniques[8–10]. Transient expression enables high-level protein production within days, without requiring stable transformation or tissue culture. Previous efforts have demonstrated the feasibility of expressing recombinant HSA (rHSA) in plants[4,11,12], but few studies have systematically optimized expression parameters or assessed protein functionality alongside environmental sustainability, leaving critical gaps in process development for plant-based rHSA production.

[1]Center of Excellence in Plant-Produced Pharmaceuticals, Chulalongkorn University, Bangkok, Thailand. [2]Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. [3]Baiya Phytopharm Co. Ltd., Bangkok, Thailand. [4]Center of Excellence in Natural Products for Ageing and Chronic Diseases, Chulalongkorn University, Bangkok, Thailand. [5]Department of Analytical Development, Pharma Nueva Co. Ltd., Bangkok, Thailand. [6]Department of Food and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. ✉e-mail: christine.b@baiyaphytopharm.com; waranyoo.p@chula.ac.th

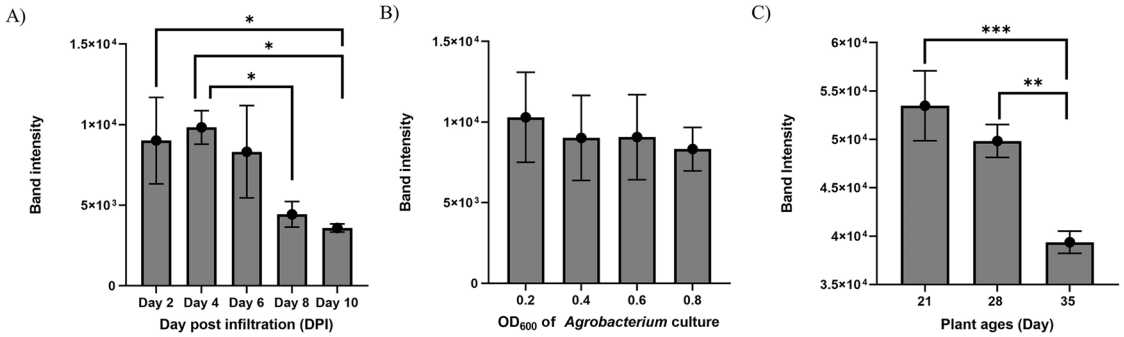

Fig. 1 | Optimization of rHSA expression in *N. benthamiana*. **A** Western blot analysis of rHSA accumulation at 2, 4-, 6-, 8-, and 10-dpi. **B** Western blot analysis of rHSA expression at different *Agrobacterium* OD_{600} values (0.2, 0.4, 0.6, and 0.8). **C** Western blot analysis of rHSA expression in plants of different ages (21-, 28-, and 35-dpg). Data were analyzed using ImageJ for densitometric quantification. Data are presented as mean ± SD ($n = 3$). Statistical significance was determined using one-way ANOVA ($p < 0.05$).

In addition to production efficiency, sustainability and regulatory compliance are key considerations in biomanufacturing. Plasma-derived and mammalian cell-based HSA productions involve significant water, energy, and chemical inputs, contributing to high environmental footprints[13,14]. Even microbial systems require energy-intensive fermentation and generate considerable downstream waste[15]. While plant-based systems are often considered greener alternatives, quantitative assessments of their environmental performance remain scarce. Sustainability metrics such as solvent efficiency, energy consumption, waste-to-product ratio (WPR), process mass intensity (PMI), the Green Analytical Procedure Index (GAPI), and its extension, the Complex Modified GAPI (Complex-MoGAPI), provide structured frameworks for evaluating solvent usage, reagent toxicity, energy demands, and waste generation throughout both production and analytical workflows[16,17]. Applying these tools to plant-based recombinant protein production can yield critical insights to guide both process improvement and environmental benchmarking.

This study aims to optimize the transient expression of rHSA in *N. benthamiana* by systematically evaluating agroinfiltration parameters, including days after infiltration, bacterial optical densities, and plant age, to maximize protein yield. The biological functionality of purified protein is validated using NIH3T3 fibroblast proliferation assays, and a comprehensive sustainability assessment evaluation is conducted using the Complex-MoGAPI flamework. Matrices including solvent use, energy consumption, and waste-to-product ratio are quantified to identify key environmental challenges and opportunities. By integrating biotechnological and sustainability perspectives, this study contributes to the growing body of work advancing plant molecular farming as a commercially viable and environmentally responsible solution for producing essential recombinant proteins such as HSA. Importantly, the findings also support the future regulatory potential of plant-derived biotherapeutics by demonstrating product integrity, functional equivalence, and a lower ecological impact—key criteria in emerging biosafety and sustainability guidelines[18,19].

Results

Optimization of rHSA expression efficiency in *N. benthamiana*

In the present study, specific infiltration parameters were systematically assessed to identify optimal conditions that enhance rHSA protein yield in *N. benthamiana*. The pBYR2e geminiviral vector and *Agrobacterium tumefaciens* strain GV3101 were used for transient expression in planta.

Effect of days post-infiltration (dpi) on rHSA accumulation

To determine the optimal harvest time for rHSA production, leaves were collected at 2, 4, 6, 8, and 10 dpi, and protein expression levels were quantified using Western blot densitometry (Fig. 1A and Supplementary Fig. S1). The results revealed a time-dependent increase in rHSA accumulation, reaching a peak at 4 dpi. Expression levels remained relatively high at 6 dpi but declined significantly after 8 dpi ($p < 0.05$) (Supplementary Table S1).

The observed reduction in expression beyond 4 dpi is likely due to protein degradation, transient gene silencing, or plant immune responses, which are common challenges in agroinfiltration-based expression systems. Previous studies have reported that silencing mechanisms such as post-transcriptional gene silencing can lead to decreased transgene expression at later time points[9]. These findings suggest that harvesting leaves at 4–6 dpi is optimal for maximizing rHSA yield before significant degradation occurs.

Effect of Agrobacterium optical density (OD_{600}) on expression levels

To optimize bacterial concentration during infiltration, *Agrobacterium tumefaciens* harboring the pBYR2e-rHSA-His vector was resuspended in infiltration buffer to OD_{600} values of 0.2, 0.4, 0.6, and 0.8, and leaves were harvested at 4 dpi for analysis. Western blot quantification (Fig. 1B and Supplementary Fig. S2) indicated no significant difference in rHSA expression across OD_{600} values ranging from 0.2 to 0.8 ($p > 0.05$), as also summarized in Supplementary Table S2. However, leaves infiltrated with $OD_{600} = 0.2$ showed slightly higher expression levels, while higher OD_{600} values (0.6 and 0.8) resulted in localized necrosis on leaf surfaces.

This lack of a clear dose-dependent increase suggests that higher *Agrobacterium* concentrations do not necessarily enhance transient expression efficiency and may instead increase stress responses and leaf damage, which can negatively affect protein production. Similar findings have been reported in previous transient expression studies, where excessive bacterial concentrations triggered reactive oxygen species production, leading to localized cell death[20]. Based on these results, an OD_{600} of 0.2 was selected as the optimal concentration for subsequent experiments to balance expression efficiency and plant health.

Influence of plant age on transient expression efficiency

To assess the impact of plant developmental stage on rHSA expression, *N. benthamiana* plants were agroinfiltrated at 21, 28, and 35 days post-germination (dpg), and protein accumulation was measured at 4 dpi. The results showed that rHSA expression level was highest in 21-day-old plants (Fig. 1C and Supplementary Fig. S3). Meanwhile expression in 28-day-old plants was moderately lower, and 35-day-old plants showed a significant decline in expression ($p < 0.05$), as summarized in Supplementary Table S3.

The reduction in expression in older plants may be attributed to changes in leaf physiology, cuticle thickening, and a decrease in metabolic activity, which could reduce the efficiency of *Agrobacterium*-mediated gene delivery. Younger leaves generally have higher transcriptional activity and greater susceptibility to bacterial infiltration, facilitating higher transgene expression[21]. These results are consistent with previous findings, where younger leaves supported higher protein yields due to enhanced permeability and reduced lignification[22].

Based on these findings, 21-day-old plants at 4 dpi and an OD_{600} of 0.2 were determined to be the optimal conditions for transient rHSA expression

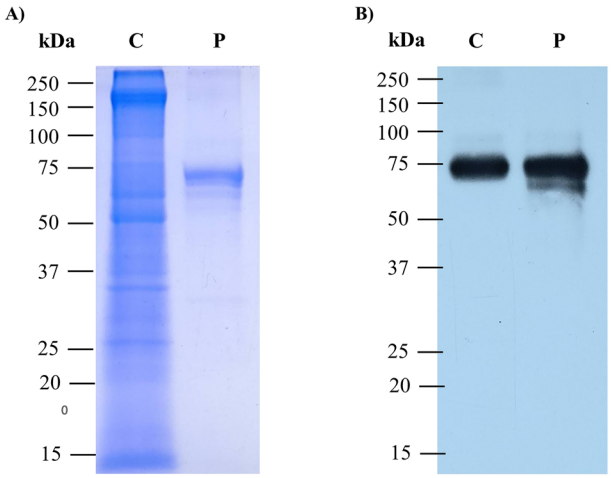

Fig. 2 | Purification of HSA from *Nicotiana benthamiana using* Ni-NTA affinity chromatography. A SDS-PAGE analysis of crude extract and purified HSA, showing a single band for purified HSA at approximately 70 kDa. **B** Western blot analysis confirmed a band at approximately 70 kDa in both the crude extract and purified HSA, consistent with the SDS-PAGE results. The blot was probed with a 1:5,000 dilution of anti-His antibody. C: crude extract; P: purified HSA.

in *N. benthamiana*. Quantification using Bradford protein assay yielded 30 mg of rHSA per kg of fresh leaf weight. These optimized parameters maximize protein yield while maintaining plant health, supporting the feasibility of using transient expression systems for sustainable recombinant protein production.

Purification and characterization of plant-derived rHSA. To ensure the production of high-quality rHSA, the purity, protein integrity, and structural confirmation were assessed using Ni-NTA affinity chromatography, SDS-PAGE, Western blot, and LC-MS analysis.

Purification using Ni-NTA affinity chromatography

Purification of plant-expressed rHSA was carried out using Ni-NTA affinity chromatography, which selectively binds the C-terminal His-tag incorporated into the recombinant protein. The purity of HSA was compared to the total protein crude extract and quantified using Image J program. Relative band intensity indicated an overall purity of 95.6%, as summarized in Supplementary Table S4. These results demonstrate that Ni-NTA chromatography is an effective purification method for plant-derived rHSA, facilitating high recovery with minimal impurities.

SDS-PAGE and Western blot analysis of rHSA purity and integrity

The purified rHSA was analyzed using SDS-PAGE under reducing conditions, followed by Western blotting to assess protein integrity. Figure 2A and Supplementary Fig. S4 show a distinct band at approximately 70 kDa against protein standard, corresponding to the expected molecular weight of monomeric HSA. Densitometric analysis confirmed an rHSA purity of 95.6%, with minimal contamination from plant-derived host proteins.

Western blotting using an anti-His antibody confirmed the identity of the recombinant protein (Fig. 2B and Supplementary Fig. S5). Minor degradation bands were observed at lower molecular weights, likely representing partial proteolysis products or truncated forms of rHSA. These degradation bands may be due to residual plant protease activity, which has been reported in transient expression systems[23]. The inclusion of protease inhibitors or optimized harvesting conditions may help mitigate protein degradation and further improve yield consistency.

Intact mass and peptide mapping analysis by LC-MS

The purified rHSA was analyzed using reverse-phase liquid chromatography (RP-LC) coupled with high-resolution mass spectrometry (HR-MS).

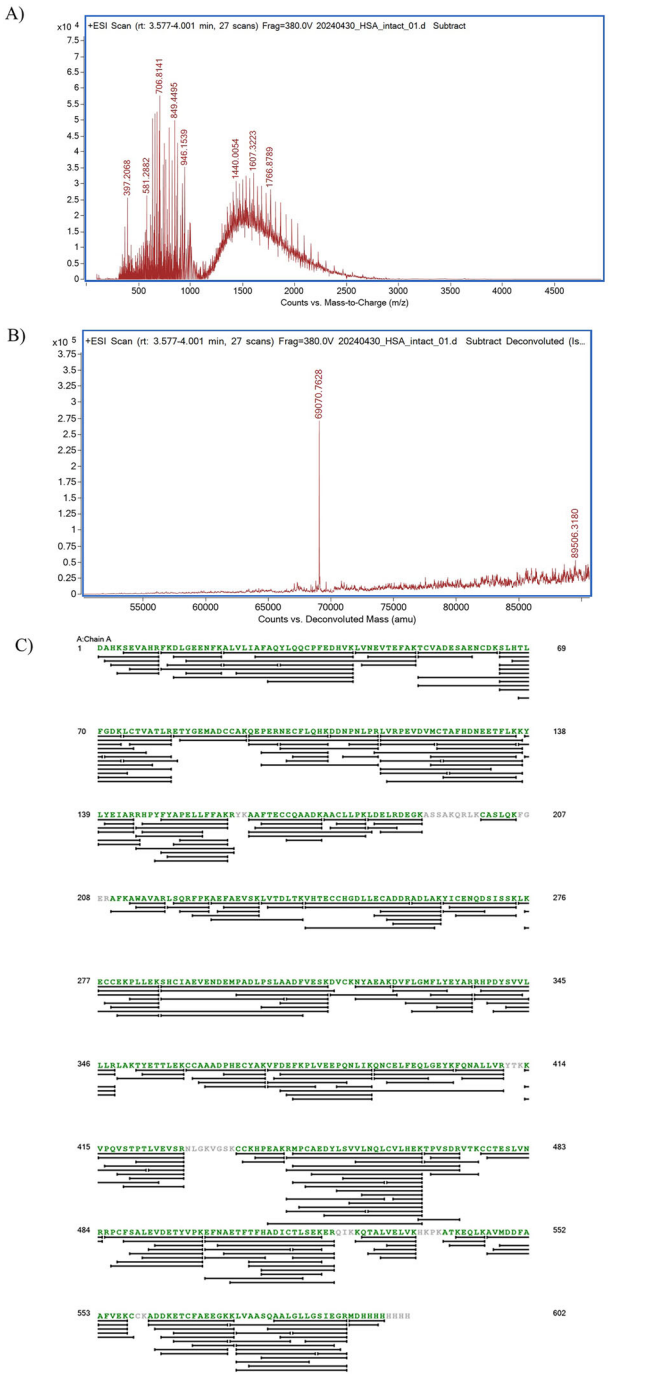

Fig. 3 | Characterization of plant-produced HSA using LC/MS. A Reverse-phase liquid chromatography chromatogram during intact mass analysis. **B** Intact mass analysis chromatogram of purified HSA showing a single peak indicating the exact mass of the purified plant-produced HSA. **C** Peptide mapping analysis of purified plant-produced HSA, displaying 93.25% sequence coverage compared to the UniProt HSA sequence (P02768; ALBU_HUMAN).

The LC separation was performed on an Agilent PLRP-S column with gradient elution from 75% aqueous (0.1% formic acid) to 90% organic (0.1% formic acid in acetonitrile) over six minutes. The total ion chromatogram showed a single major peak, corresponding to intact rHSA (Fig. 3A). The deconvoluted mass spectrum confirmed a molecular weight of ~69.07 kDa, which aligns with the calculated mass of full-length HSA (Fig. 3B). No significant truncations or modifications were detected, indicating that plant-derived rHSA maintains its expected structure without substantial degradation or unexpected PTMs.

Peptide mapping analysis was conducted to further confirm the primary sequence of plant-derived rHSA. The purified protein was enzymatically digested using trypsin, followed by LC-MS analysis of the resulting peptides. Sequence coverage analysis revealed that 93.25% of the expected peptide fragments were detected (Fig. 3C), confirming that the recombinant protein was correctly synthesized and processed. A comparison of the detected peptide fragments with the theoretical HSA sequence showed no significant differences, confirming that the plant-expressed rHSA maintains structural fidelity to native HSA.

Cell proliferation activity. The biological activity of plant-derived HSA was evaluated using the NIH3T3 mouse fibroblast cell proliferation assay (Fig. 4). At 24 h, there was no significant difference in cell proliferation between HSA-treated and untreated cells across all concentrations. However, by 48 h, a significant increase in cell proliferation was observed in cells treated with 10 µg/mL rHSA compared to non-treated controls ($p < 0.001$) (Supplementary Table S5). This trend continued at 72 h, where cells treated with 0.1 and 1 µg/mL rHSA showed a statistically significant increase in proliferation compared to untreated cells ($p < 0.001$).

The results demonstrate a dose- and time-dependent effect of plant-derived HSA on fibroblast proliferation. Notably, while higher concentrations (10 µg/mL) induced earlier cell growth at 48 h, lower concentrations (0.1 and 1 µg/mL) showed delayed but significant effects by 72 h, suggesting a potential cumulative or delayed response to rHSA treatment. These findings confirm that plant-derived rHSA retains its biological functionality and supports fibroblast proliferation, comparable to its native human counterpart.

Sustainability assessment of plant-based rHSA production. To evaluate the environmental impact of plant-derived rHSA production, a sustainability assessment was performed by analyzing solvent efficiency, energy consumption, waste-to-product ratio (WPR), process mass intensity (PMI), and the GAPI using the ComplexMoGAPI framework.

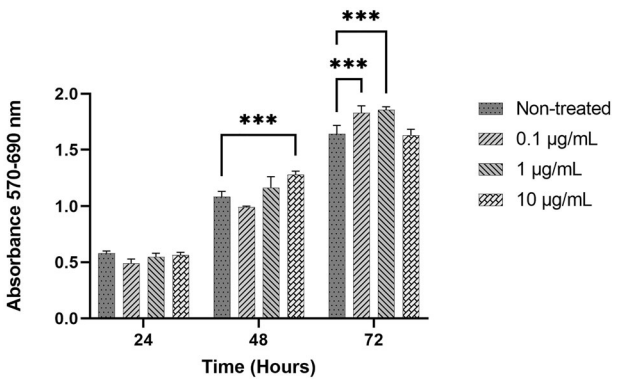

Fig. 4 | NIH3T3 fibroblast cell proliferation in response to purified plant-derived rHSA treatment at 0.1, 1, and 10 µg/mL. Cell viability was assessed at 24, 48, and 72 h using the MTT assay. Data are presented as mean ± SD ($n = 3$). Statistical significance was determined using one-way ANOVA ($p < 0.001$) by comparing treatment groups to non-treated cells.

These parameters provided quantitative insights into the resource efficiency and environmental footprint of the process (Table 1).

Solvent efficiency and energy consumption analysis

The solvent efficiency of the plant-based rHSA production process was evaluated based on the total solvent usage in protein extraction, purification, and buffer exchange steps. *N. benthamiana* extraction was performed using an extraction buffer ratio of 1:2 (w/v), requiring 20 L of solvent per 10 kg of fresh leaves, with an additional 5 L of solvent utilized during the purification process. Applying Eq. (1), the solvent efficiency was calculated to be 0.083 L/mg. This value reflects the total solvent required per mg of purified rHSA. Compared to conventional biopharmaceutical production platforms, solvent demand in the plant-based system was lower due to the absence of cell culture media and serum supplements, reducing the overall environmental footprint.

The energy consumption for rHSA production was assessed by measuring the total electricity usage across the entire workflow, from plant cultivation to final purification. A total of 2020 kWh was required to cultivate plants yielding 10 kg of fresh biomass over a three-week period, including all associated laboratory activities. Using Eq. (2), the specific energy consumption was determined to be 6.73 kWh/mg, highlighting the energy-intensive nature of the process.

Waste-to-Product and Process Mass Intensity

The WPR quantifies the total waste generated per unit of purified rHSA, encompassing biomass residue, liquid waste, and solid waste. To obtain 10 kg of fresh *Nicotiana benthamiana* leaves, the total waste output was 2805 kg, comprising 5 kg of spent biomass, 2700 kg of liquid waste, and 100 kg of solid waste. Using Eq. (3), the WPR was calculated as 9350 g/mg, reflecting the substantial material input associated with plant-based rHSA production.

Approximately 80% of the total waste was derived from spent plant biomass, which presents an opportunity for valorization strategies such as biofuel production, composting, or extraction of secondary metabolites. Additionally, process optimizations aimed at reducing chromatography buffer consumption and implementing liquid waste recycling could further enhance the sustainability of the production system.

The PMI provides a measure of the total material input relative to the purified rHSA yield. The cumulative material input for rHSA production was 3050 kg, consisting of 3000 kg of water, 25 kg of solvents, and 15 kg of chemicals. Applying Eq. (4), PMI was calculated as 10,166.67 g/mg, indicating the overall resource demand per unit of purified protein.

While the PMI of plant-based rHSA production is higher than that of microbial fermentation systems, the platform offers key advantages, including a lower reliance on costly culture media and bioreactors. Further improvements, such as water recirculation systems, solvent recovery, and process streamlining, could enhance the material efficiency and cost-effectiveness of *N. benthamiana*-derived recombinant protein production.

Green analytical procedure assessment. The ComplexMoGAPI evaluation provided a detailed sustainability assessment of the analytical procedures used in rHSA characterization, focusing on pre-analysis and analytical stages. The pre-analysis stage, which included extraction and purification, required the processing of 10 kg of infiltrated *N. benthamiana* leaves in imidazole-based extraction buffer, classified as

Table 1 | Summary of sustainable metrics evaluated for *N. benthamiana*-derived rHSA production

Sustainable Metric	Value	Unit	Description
Solvent efficiency	0.083	L/mg	Volume of solvent used per mg of purified rHSA
Energy consumption	6.73	kWh/mg	Energy required per mg of purified rHSA
Waste-to-Product Ratio	9,350	g/mg	Mass of waste generated per mg of purified rHSA
Process Mass Intensity	10,166.67	g/mg	Total mass of materials used per mg of purified rHSA

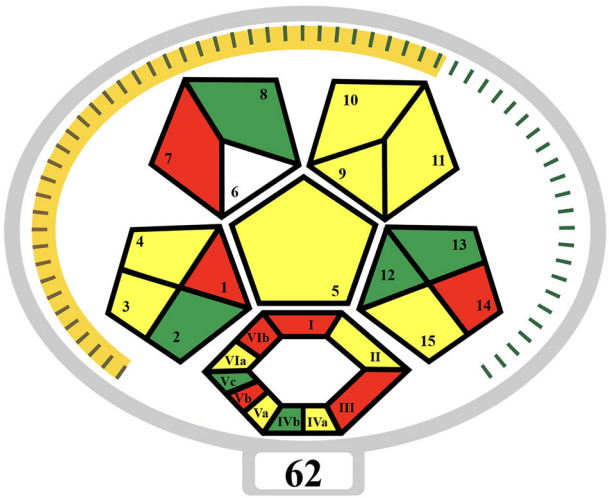

Fig. 5 | ComplexMoGAPI assessment of the analytical procedure used for plant-derived rHSA production. The pictogram provides a visual summary of sustainability performance across different stages of the analytical process. Color-coded symbols indicate the environmental impact of each step: green (low impact), yellow (moderate impact), and red (high impact). The five pentagons represent sustainability parameters related to sample preparation and instrumental analysis, while the hexagon corresponds to pre-analytical factors.

moderately hazardous with an NFPA health rating of 2 (imidazole). The purification process, involving centrifugation, multiple filtration steps, and Ni-NTA affinity chromatography, was categorized as a semi-advanced process in green chemistry evaluations. The estimated energy consumption per sample was above 1.5 kWh, with refrigerated centrifugation being the most energy-intensive step. The final rHSA yield was 30 µg/g fresh leaf weight, translating to an overall yield of 0.003%, with a purity of 95%.

In the analytical stage, the LC-MS-based intact mass and peptide mapping analyses were identified as the most resource-intensive procedures. Sample collection was performed off-line, and no specific preservation measures were applied before processing. The total solvent usage for sample preparation and LC-MS analysis exceeded 100 mL, with formic acid and acetonitrile identified as key hazardous reagents. Formic acid had an NFPA health hazard rating of 3[24], while acetonitrile, classified as the most flammable solvent used, had an NFPA flammability rating of 3[25]. Despite these solvent hazards, the estimated energy consumption per sample for LC-MS analysis remained low, at less than 0.1 kWh. However, waste generation from mobile phase elution reached approximately 5 mL per injection, necessitating proper hazardous waste management through outsourced treatment services.

The ComplexMoGAPI assessment of the environmental sustainability of the analytical procedure is presented in Fig. 5. This analysis identifies areas where the procedure meets green chemistry principles and highlights steps where improvements can enhance overall sustainability. The ComplexMoGAPI evaluation identified waste generation and hazardous solvent use as the major environmental concerns in the analytical workflow. These findings suggest that further improvements, such as reducing solvent dependency, implementing solvent recovery strategies, and utilizing energy-efficient purification methods, could significantly enhance the sustainability of plant-based rHSA production.

Discussion

The increasing global demand for HSA in medical and pharmaceutical applications necessitates the development of alternative, sustainable production platforms beyond plasma-derived sources[2,26]. In this study, the transient expression of rHSA in *N. benthamiana* was successfully optimized. The highest expression level was achieved under the conditions of 4 dpi, an *Agrobacterium* OD_{600} of 0.2, and 21-day-old plants. Western blot analysis confirmed that the plant-produced rHSA appeared as a single band (~ 70 kDa) with 95.6% purity, comparable to bacterially expressed HSA. These findings suggest that *N. benthamiana* serves as an efficient, scalable expression host capable of producing structurally intact rHSA with minimal purification steps[27,28]. Functional validation using NIH3T3 fibroblast cells further demonstrated that plant-derived rHSA retains biological activity. The observed dose-dependent stimulation of cell proliferation supports its biocompatibility and functional equivalence to plasma-derived HSA[4,29]. Nevertheless, it should be noted that previous studies have reported that HSA does not consistently promote cell growth across all cell types[30]. Here, NIH3T3 cells were used as a model for preliminary functional validation. Future work should thus include additional cell lines with higher sensitivity to growth factors, as well as broader dose ranges and complementary assays, to ensure a more comprehensive assessment of the biological activity of plant-derived rHSA.

The *Agrobacterium tumefaciens* strain GV3101 was selected based on prior reports showing superior expression efficiency compared to other strains such as EHA105 and LBA4404[31,32]. Optimization of agroinfiltration parameters revealed that younger plants (21 days old) supported the highest rHSA accumulation, consistent with previous findings that immature leaf tissues enhance gene transfer efficiency and protein synthesis[21]. An *Agrobacterium* OD_{600} of 0.2 was found to provide the optimal balance between protein yield and phytotoxicity, as increasing the OD_{600} beyond this threshold did not lead to further improvements. Expression levels declined at both lower and higher OD values, corroborating results reported by our group and others[9,33,34]. On the other hand, delaying harvest beyond 4 dpi resulted in reduced expression, prospectively due to protein degradation and senescence[35]. These findings emphasize the importance of carefully defining protein-specific expression kinetics to maximize yield in transient systems[9]. Despite optimization, the rHSA yield achieved (30 µg/g leaf fresh weight) remains lower than the yields reported from stable platforms such as transgenic rice seeds (2.75 g/kg) and microbial systems like *E. coli* (18.92 mg/L)[4,36]. However, our yield is comparable to previous transient expression studies in *N. benthamiana*, such as Sijmons et al. (11–36 µg/g)[11] and Fernandez-San Millan et al. (20 µg/g via chloroplast transformation)[37]. Our approach offers advantages such as shorter production timeline (four days) and simplified downstream processing, although overall yields remain within a similar range. These results suggest that fundamental biological limitations of leaf-based transient expression may restrict further yield improvements without adopting alternative strategies such as vector redesign, subcellular targeting, or metabolic engineering. Compared to seed-based systems, leaf-based expression platforms are inherently limited by lower protein storage capacity, higher levels of protease activity, and a shorter window for protein accumulation[38,39]. In this study, we attempted to minimize proteolytic degradation by maintaining harvested leaves at temperatures below 4 °C, a condition known to reduce protease activity[39]. To further improve rHSA yield and stability, future work could explore strategies targeting the protein to storage organelles, co-expressing protease inhibitors, or using gene silencing to suppress endogenous proteases. Additionally, we recognize the possibility for His-tag cleavage by plant proteases, which may have contributed to reduced purification efficiency. Although not confirmed in the present study, we plan to analyze the flow-through fractions using rHSA-specific antibodies or mass spectrometry to assess the presence of tag-free rHSA and better understand potential yield losses due to proteolytic tag removal.

Critically, this study not only evaluates the environmental sustainability of plant-based rHSA production but also includes the analytical procedures, an often overlooked yet essential component of biomanufacturing. By accounting for the material, energy, and waste contributions of these workflows, we offer a more realistic evaluation of the true environmental footprint across the full production lifecycle. Quantified sustainability metrics, including solvent efficiency (0.083 L/mg), energy consumption (6.73 kWh/mg), waste-to-product ratio (9350 g/mg), and process mass intensity (10,166.67 g/mg), reveal major environmental

hotspots, particularly related to solvent use, energy input, and waste generation. While plant molecular farming offers benefits such as lower infrastructure costs and biosafety compared to mammalian and microbial platforms, challenges related to solvent consumption, energy input, and waste management remain[7]. Process innovations such as solvent recovery, enzyme-assisted extraction, and valorization of residual biomass could help address the environmental and economic performance of the process[40].

To contextualize our findings, we estimated the sustainability metrics for bacterial- and yeast-based rHSA production using data from literature. For bacterial expression, it was assumed that 1 kg of biomass requires 10.96 kWh of electricity and 2.41 kg of raw material input, generating approximately 0.11 kg of waste[41]. Based on prior yields, 1 kg of *E. coli* biomass was estimated to produce 600 mg of HSA with a downstream recovery of 60%[10,41]. Under these assumptions, the energy consumption, PMI, and WPR for bacterial-derived rHSA were calculated to be 3.04×10^{-5} kWh/mg HSA, 6.69×10^{-3} g/mg HSA, and 3.06×10^{-4} g/mg HSA, respectively. By contrast, yeast-based rHSA production (*Pichia pastoris*) indicated a product yield of 90 ng/mg total protein (0.01%)[42,43]. The total mass input was 34,742.8 kg per 125 kg of protein, primarily comprising 34,678 kg of water and 64.8 kg of chemicals. This primarily consists of 34,678 kg of water and 64.8 kg of chemicals used for medium preparation. Energy consumption was estimated at 7.176×10^{-2} kWh/mg, with downstream waste generation of 8000 kg. These figures translated to a PMI of 2.8×10^{3} g/mg and WPR of 6.4×10^{2} g/mg for yeast-derived rHSA. While useful for benchmarking, these estimates rely on assumptions and indirect literature values. While these estimates offer useful benchmarks, they rely on indirect assumptions, highlighting the need for future validation using standardized large-scale datasets.

Despite the relatively high PMI and WPR values observed for plant-based rHSA, the platform retains notable sustainability advantages for large-scale deployment. In contrast to microbial and mammalian platforms, which require costly stainless-steel bioreactors and validated clean-in-place systems, plant systems function as single-use bioreactors, reducing capital expenditures and simplifying operations[44]. Additionally, plant-based expression eliminates the risk of cross-contamination, a common know challenge in multi-use fermenters[7]. Moreover, the environmental footprint of plant biomass disposal is significantly lower. Unlike bacterial platforms that may generate hazardous waste such as endotoxins, plant biomass is biodegradable and generally biosafe. Residual biomass can be managed as agricultural waste or repurposed for biofuel production, composting, or metabolite extraction, adding circular value to the process[40,45]. Given that recombinant proteins often account for less than 1% of total plant biomass, valorizing the remaining material is essential for improving the overall process sustainability and aligning with circular bioeconomy goals[46–49].

Transient expression systems in *N. benthamiana*, while highly flexible and rapid, can introduce additional environmental burdens due to the need of *Agrobacterium* cultivation and the agroinfiltration process itself[8]. In contrast, stable plant-based platforms may offer greater sustainability potential by eliminating these repetitive bacterial cultivation steps; however, they are often constrained by more complex regulatory requirements, longer development times, and the potential of transgene silencing over time[50,51]. Future research should systematically compare the environmental impacts of transient and stable systems to better guide platform selection and process optimization strategies for plant-based manufacturing.

From a regulatory perspective, ensuring the safety and consistency of plant-produced rHSA is paramount. While peptide mapping confirmed 93.25% sequence coverage, PTMs such as glycosylation was not fully characterized in this study. Since plant-specific glycans can differ immunogenically from human glycans, mass spectrometry-based glycan profiling is recommended to support regulatory approval[14,52,53]. Similarly, although Ni-NTA affinity purification achieved high purity (95%), additional polishing steps, such as size-exclusion or ion-exchange chromatography, may be required to meet clinical-grade standards[54,55].

Moving forward, several molecular and bioprocess engineering strategies could further enhance plant-based rHSA production. These include optimizing expression vectors with stronger promoters or replicons, applying metabolic modifications to reduce protease activity, co-infiltrating protease inhibitors, and deploying automated agroinfiltration platforms for large-scale production[56,57]. In parallel, cost modeling studies, in-depth functional assays against plasma-derived HSA, and early engagement with regulatory frameworks will be critical to advancing plant-produced rHSA toward clinical translation and industrial adoption.

In conlusion, this study demonstrates the potential of *N. benthamiana* as a scalable and sustainable platform for the transient expression of rHSA. Under optimized agroinfiltration conditions, a maximum yield of 30 µg/g FW was achieved, and functional assays confirmed that the plant-derived rHSA retains biological activity, supporting its use as an alternative to plasma- or microbe-derived HSA. In addition to process optimization, this work presents one of the first comprehensive environmental sustainability assessments of plant-based rHSA production using the ComplexMoGAPI framework. Key metrices including solvent efficiency, energy consumption, and waste generation highlight both the strengths and the challenges of molecular farming in meeting green biomanufacturing goals. While plant-based systems offer advantages such as reduced infrastructure demands and safer waste profiles, further innovations in biomass valorization, energy-efficient processing, and downstream purification will be essential to improve overall sustainability. This study reinforces the promise of plant molecular farming as a cost-effective, environmentally responsible strategy for recombinant protein production. By integrating biotechnological advancement with structured sustainability evaluation, plant expression systems can contribute meaningfully to the future of greener, scalable biopharmaceutical manufacturing.

Methods

Vector construction and transformation

The nucleotide sequence of the rHSA coding region (GenBank accession number: AAA98797.1) was codon-optimized for expression in *N. benthamiana*. A polyhistidine (His) tag was fused at the C-terminus using an IEGRMD linker to facilitate purification. The gene was synthesized commercially (Genewiz, China) and cloned into the pBYR2e geminiviral vector between the *Xba*I and *Sac*I restriction sites[58]. This vector contains regulatory elements, including the Cauliflower Mosaic Virus (CaMV) 35S promoter,

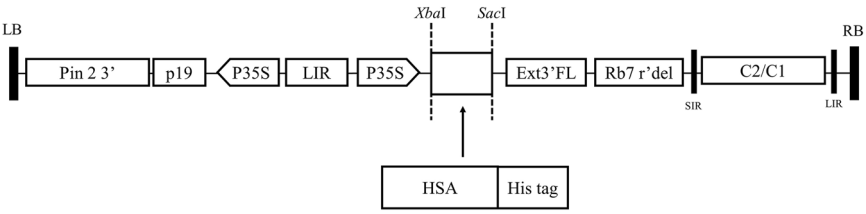

Fig. 6 | Schematic within left and right border of T-DNA located in Germiniviral plant expression vector pBYR2e fused with HSA gene. The Pin II 3'; sequence derived from potato proteinase inhibitor II, P19; RNA silencing suppressor, the Cauliflower Mosaic Virus (CaMV) 35 s promoter, P35s; the CaMV enhancer, P35s×2; the tobacco extension gene region, Ext3' FL, 3'; the tobacco RB7 promoter, Rb7 5' del; the Bean Yellow Dwarf Virus (BeYDV) short intergenic region, SIR; the BeYDV long intergenic region, LIR; and the BeYDV replication initiation proteins, Rep and RepA, along with C2/C1.

BeYDV replication elements, and the RB7 matrix attachment region, designed to enhance transient gene expression. Additionally, the RNA silencing suppressor gene P19 was also incorporated to reduce post-transcriptional gene silencing and improve recombinant protein yield[59] (Fig. 6).

For bacterial transformation, the pBYR2e-rHSA-His construct was introduced into *E. coli* DH10B via heat shock. Colonies were screened by PCR, and positive clones were cultured in LB medium containing 50 mg/L kanamycin at 37 °C with shaking (250 rpm). Plasmids were extracted using the DNA-spin Plasmid Purification Kit (iNtRON Biotechnology, Korea), verified by restriction digestion and sequencing, and subsequently transformed into *Agrobacterium tumefaciens* strain GV3101 using electroporation (MicroPulser, Bio-Rad, USA).

Positive *Agrobacterium* clones were selected on LB agar plates supplemented with kanamycin (50 mg/L), gentamicin (25 mg/L), and rifampicin (50 mg/L), and verified via colony PCR (Supplementary Fig. S6). Confirmed clones were grown in LB broth with the same antibiotics at 28 °C, 250 rpm overnight. Cultures were pelleted (14,000 rpm, 5 min, 4 °C), washed, and resuspended in infiltration buffer (10 mM MES, 10 mM $MgSO_4$, pH 5.5) to a final optical density at 600 nm (OD_{600}) of 0.2 for agroinfiltration.

Plant-based transformation and optimization

N. benthamiana seeds were obtained from Dr. Supaart Sirikantaramas (Faculty of Science, Chulalongkorn University, Thailand). Plants were cultivated in growth chambers at 22–25 °C under a 16 h light/8 h dark photoperiod and were used at 21, 25, and 35 dpg to assess age-related expression variability. *Agrobacterium* cultures harboring pBYR2e-rHSA-His were grown overnight, pelleted (4000 × g, 10 min, 4 °C), washed, and resuspended in infiltration buffer to final OD_{600} values of 0.2, 0.4, 0.6, and 0.8. Agroinfiltration was performed using syringe infiltration (into the abaxial leaf surface) and vacuum infiltration (submerging whole plants under −50 kPa for 2 min, followed by 5 min recovery). Leaves were harvested at 2, 4, 6, 8, and 10 dpi. Expression levels were quantified by Western blot densitometry, and optimum conditions were selected for further experiments.

Protein extraction and purification

Infiltrated leaves were homogenized in extraction buffer (10 mM imidazole, 20 mM Tris-HCl pH 7.4, 50 mM NaCl) at a 1:2 (w/v) ratio (1 g fresh tissue per 2 mL buffer). Homogenates were centrifuged (14000 × g, 50 min, 4 °C) and filtered through filter cloth followed by 0.45 µm paper filters (Merck, USA). Clarified extracts were loaded onto Ni-NTA resin (Cat No.17531802, Cytiva, USA). pre-equilibrated with 10 mM imidazole buffer. Columns were washed with 10–30 mM imidazole and rHSA was eluted with 250 mM imidazole in 20 mM Tris-HCl, 50 mM NaCl, pH 7.4. Eluates were desalted into PBS by dialysis. Protein purity was assessed via SDS-PAGE and Western blot, using rabbit anti-His antibody (1:5000 dilution; Southern Biotech, USA) and HRP-conjugated secondary antibody. Densitometry was performed using ImageJ software Version 1.54p[60]. To evaluate the yield of plant-produced rHSA, the fresh weight of harvested leaves was recorded prior to protein extraction and purification. The purified HSA was quantified using the Bradford protein assay, with bovine serum albumin (BSA) as a standard. Briefly, purified HSA sample was mixed with Bradford reagent, and absorbance was measured at 595 nm. The rHSA concentration was determined from the BSA standard curve. The total amount of purified HSA (µg) was calculated by multiplying the measured concentration by the total elution volume. Finally, the expression yield was expressed as micrograms of purified HSA per gram of initial fresh leaf weight, as shown in Eq. (1).

$$Yield\ of\ rHSA = \frac{Final\ concentration\ of\ purified\ HSA(\mu g/mL) \times Total\ volume\ of\ purified\ HSA(mL)}{Total\ leafs\ fresh\ weight(gram)} \quad (1)$$

Intact mass and peptide mapping analyses

To further characterize plant-derived rHSA, the purified protein was analyzed using liquid chromatography-mass spectrometry (LC-MS) on an Agilent 1290 Infinity II LC system coupled with an Agilent 6545XT Q-TOF mass spectrometer. The protein was desalted using Bio-Gel P6 Micro Bio-Spin columns (Bio-Rad, USA) and exchanged into ammonium bicarbonate buffer before injection. For intact mass analysis, 3 µL of desalted rHSA was loaded onto an Agilent PLRP-S column (1000 Å, 2.1 × 50 mm, 5 µm), and a gradient elution system was applied with 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B). The gradient gradually increased %B to 90% over six minutes to achieve optimal separation. Mass spectra were acquired in positive ion mode across an m/z range of 400–3200, with key ionization parameters listed in Supplementary Table S6.

For peptide mapping, the purified protein was reduced with 10 mM dithiothreitol (30 min, 65 °C), alkylated with 25 mM iodoacetamide (20 min, room temperature in the dark), and digested with trypsin (0.5 µg per sample). Digestion was stopped with 10% formic acid, and samples were centrifuged (14000 rpm, 10 min). The supernatant was analyzed via LC-MS, and sequence coverage was determined using BioConfirm Software version 12.0.

Cell proliferation assay

The NIH3T3 fibroblast cell line was provided by Assoc. Prof. Jittima Luckanagul (Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin under 5% CO_2 at 37 °C.

For proliferation assays, cells were seeded at 3000 cells/well in 96-well plates and serum-starved for 18 h before treatment. Plant-produced rHSA was added at 0.1, 1, and 10 µg/mL, and cell viability was assessed using the MTT assay at 24, 48, and 72 h. Absorbance was measured at 570 nm (formazan) and 690 nm (background). Statistical significance was analyzed using one-way ANOVA for comparisons across treatment groups and two-way ANOVA for time-dependent effects (GraphPad Prism 9.0, USA). A p-value < 0.05 was considered statistically significant (Supplementary Table S5).

Sustainability metrics

The environmental sustainability of rHSA production was assessed using ComplexMoGAPI to quantify the process's resource efficiency and environmental impact. This method integrates green chemistry principles with biopharmaceutical production metrics, evaluating solvent use, energy consumption, waste generation, and mass intensity. Sustainability assessments were conducted following established methodologies[16,17], with all measurements recorded from laboratory-scale experiments.

Solvent efficiency. Solvent efficiency was determined by calculating the total volume of solvents (aqueous and organic) used per mg of purified rHSA (L/mg rHSA) (See Eq. 2). This included solvents used in protein extraction, affinity purification, and buffer exchange steps. Lower solvent consumption per unit of purified protein indicates a more sustainable and efficient process. Solvent usage data were recorded throughout purification, and waste minimization strategies, such as solvent recycling and buffer optimization, were considered.

$$\text{Solvent Efficiency} = \frac{\text{Total Volume of Solvent Used(L)}}{\text{Yield of Purified HSA(mg)}} \quad (2)$$

Energy consumption. Total energy consumption was measured as the cumulative energy input (kWh) required for rHSA production, normalized per mg of purified protein (kWh/mg rHSA) (see Eq. 3). The calculation included energy consumption from plant cultivation (growth chamber lighting, ventilation, and climate control), bacterial culture incubation, vacuum infiltration, centrifugation, chromatography, and lyophilization. Energy consumption data were recorded using laboratory power meters and manufacturer specifications for equipment power ratings to ensure accurate quantification.

$$\text{Energy Consumption}\left(\text{kWh/mg}\right) = \frac{\text{Total Energy Used(kWh)}}{\text{Yield of Purified HSA(mg)}} \quad (3)$$

Waste-to-product ratio. The waste-to-product (g/mg rHSA) represents the total mass of biomass and chemical waste generated per mg of purified protein (see Eq. 4). Biomass waste included spent plant material and residual cell debris, while chemical waste included extraction solvents, chromatography buffers, and discarded culture media. A higher WPR indicates lower resource efficiency[45]. Data were collected at each stage of the workflow, and process modifications such as biomass valorization and solvent recovery were explored as potential strategies for waste reduction.

$$\text{WPR} = \frac{\text{Mass of Waste Generated}}{\text{Yield of Purified HSA(mg)}} \quad (4)$$

Process mass intensity. Process mass intensity was calculated as the total mass of all raw materials, including solvents, buffers, growth media, and biomass, used per milligram of purified rHSA to evaluate overall process efficiency (see Eq. 5). This assessment systematically quantifies solvent usage, energy consumption, waste generation, and process mass intensity, providing a comprehensive sustainability profile of plant-based rHSA production. The total mass input accounts for all materials utilized from plant cultivation through final protein purification. Lower PMI values indicate a more resource-efficient and sustainable process, facilitating comparisons with alternative biomanufacturing platforms[61].

$$\text{PMI} = \frac{\text{Mass of Input Materials(g)}}{\text{Yield of Purified HSA(mg)}} \quad (5)$$

Green analytical procedure assessment

Green analytical procedure index. The GAPI was employed to assess the sustainability of the analytical workflow, focusing on solvent and reagent selection, energy efficiency, waste management, and safety considerations. The assessment was structured using a GAPI pictogram, where each factor was color-coded: green indicating optimal sustainability and red signifying high environmental impact. This approach provided a quantitative and visual representation of the ecological footprint of the analytical process. Key parameters evaluated included sample preparation efficiency, chemical safety, waste minimization, and operational sustainability. The methodology was applied following established guidelines[16].

Complex modified GAPI

The ComplexMoGAPI extended the GAPI framework by incorporating additional sustainability parameters, including instrumental energy efficiency, analytical throughput, and waste-to-product ratios (WPR and PMI). This assessment accounted for energy consumption from LC-MS instrumentation, solvent usage in protein characterization, and analytical waste generation, enabling a more comprehensive evaluation of sustainability within the rHSA production workflow. The analysis was conducted following established sustainability assessment protocols (WPR and PMI)[17,45]. By integrating ComplexMoGAPI, the study provided quantitative insights into the environmental benefits and potential impact reductions associated with plant-based transient expression systems.

Data availability

All data presented in the study are included in the article or as electronic supplementary material. Other inquiries can be made available on reasonable requests to the corresponding author/s.

Received: 22 March 2025; Accepted: 5 June 2025;
Published online: 27 September 2025

References

1. Mishra, V. & Heath, R. J. Structural and Biochemical Features of Human Serum Albumin Essential for Eukaryotic Cell Culture. *Int. J. Mol. Sci.* **22**, 8411 (2021).
2. Chen, Z., He, Y., Shi, B. & Yang, D. Human serum albumin from recombinant DNA technology: Challenges and strategies. *Biochimica et. Biophysica Acta ((BBA)) - Gen. Subj.* **1830**, 5515–5525 (2013).
3. Mohammadinejad, R. et al. Plant molecular farming: production of metallic nanoparticles and therapeutic proteins using green factories. *Green. Chem.* **21**, 1845–1865 (2019).
4. He, Y. et al. Large-scale production of functional human serum albumin from transgenic rice seeds. *Proc. Natl. Acad. Sci. USA* **108**, 19078–19083 (2011).
5. Rybicki, E. P. Plant molecular farming of virus-like nanoparticles as vaccines and reagents. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol* **12**, e1587 (2020).
6. Kulshreshtha, A., Sharma, S., Padilla, C. S. & Mandadi, K. K. Plant-based expression platforms to produce high-value metabolites and proteins. *Front. Plant Sci.* **13**, https://doi.org/10.3389/fpls.2022.1043478 (2022).
7. Buyel, J. F. Plant Molecular Farming – Integration and Exploitation of Side Streams to Achieve Sustainable Biomanufacturing. *Front. Plant Sci.* **9**, https://doi.org/10.3389/fpls.2018.01893 (2019).
8. Golubova, D., Tansley, C., Su, H. & Patron, N. J. Engineering Nicotiana benthamiana as a platform for natural product biosynthesis. *Curr. Opin. Plant Biol.* **81**, 102611 (2024).
9. Norkunas, K., Harding, R., Dale, J. & Dugdale, B. Improving agroinfiltration-based transient gene expression in Nicotiana benthamiana. *Plant Methods* **14**, 71 (2018).
10. Ridgley, L. A. et al. Killer to cure: Expression and production costs calculation of tobacco plant-made cancer-immune checkpoint inhibitors. *Plant Biotechnol. J.* **21**, 1254–1269 (2023).
11. Sijmons, P. C. et al. Production of Correctly Processed Human Serum Albumin in Transgenic Plants. *Bio/Technol.* **8**, 217–221 (1990).
12. Sun, Q.-Y. et al. Improved expression and purification of recombinant human serum albumin from transgenic tobacco suspension culture. *J. Biotechnol.* **155**, 164–172 (2011).
13. Shirahata, H., Diab, S., Sugiyama, H. & Gerogiorgis, D. I. Dynamic modelling, simulation and economic evaluation of two CHO cell-based production modes towards developing biopharmaceutical manufacturing processes. *Chem. Eng. Res. Des.* **150**, 218–233 (2019).
14. Walsh, G. & Jefferis, R. Post-translational modifications in the context of therapeutic proteins. *Nat. Biotechnol.* **24**, 1241–1252 (2006).
15. Burnett, M. J. B. & Burnett, A. C. Therapeutic recombinant protein production in plants: Challenges and opportunities. *Plants, people, planet* **2**, 121–132 (2020).
16. Gałuszka, A., Migaszewski, Z. M., Konieczka, P. & Namieśnik, J. Analytical Eco-Scale for assessing the greenness of analytical procedures. *TrAC Trends Anal. Chem.* **37**, 61–72 (2012).
17. Płotka-Wasylka, J. & Wojnowski, W. Complementary green analytical procedure index (ComplexGAPI) and software. *Green. Chem.* **23**, 8657–8665 (2021).
18. Lomonossoff, G. P. & D'Aoust, M. A. Plant-produced biopharmaceuticals: A case of technical developments driving clinical deployment. *Sci. (N. Y., N. Y.)* **353**, 1237–1240 (2016).

19. Peyret, H. & Lomonossoff, G. P. When plant virology met Agrobacterium: the rise of the deconstructed clones. *Plant Biotechnol. J.* **13**, 1121–1135 (2015).
20. Leuzinger, K. et al. Efficient agroinfiltration of plants for high-level transient expression of recombinant proteins. *Journal of visualized experiments: JoVE* https://doi.org/10.3791/50521 (2013).
21. Zhang, Y. et al. Effects of different light conditions on transient expression and biomass in Nicotiana benthamiana leaves. **18**, https://doi.org/10.1515/biol-2022-0732 (2023).
22. Sainsbury, F. & Lomonossoff, G. P. Transient expressions of synthetic biology in plants. *Curr. Opin. Plant Biol.* **19**, 1–7 (2014).
23. Goulet, C., Khalf, M., Sainsbury, F., D'Aoust, M. A. & Michaud, D. A protease activity-depleted environment for heterologous proteins migrating towards the leaf cell apoplast. *Plant Biotechnol. J.* **10**, 83–94 (2012).
24. Formic acid; CAS RN: 64-18-6; rev. 7; Thermo Fisher Scientific, N., 2022. https://www.fishersci.com/store/msds?partNumber=AC147930250&productDescription=FORMIC+ACID+98%25+25ML&vendorId=VN00033901&countryCode=US&language=en (accessed 2025-02-16).
25. Acetonitrile; CAS RN: 75-05-8; rev. 8; Thermo Fisher Scientific, O., 2023. https://www.fishersci.com/store/msds?partNumber=A9964&productDescription=ACETONITRILE+OPTIMA+GRADE+4L&vendorId=VN00033897&countryCode=US&language=en (accessed 2025-02-16).
26. Marcucci, P. Future trends in the plasma products market. *Production of Plasma Proteins for Therapeutic Use*, 461–470 (2013).
27. Latta, M. et al. Synthesis and Purification of Mature Human Serum Albumin from E. Coli. *Bio/Technol.* **5**, 1309–1314 (1987).
28. Sharma, A. & Chaudhuri, T. K. Revisiting Escherichia coli as microbial factory for enhanced production of human serum albumin. *Microb. Cell Factories* **16**, 173 (2017).
29. Tan, H. et al. Large-scale and cost-effective production of recombinant human serum albumin (rHSA) in transgenic Bombyx mori cocoons. *Int J. Biol. Macromol.* **245**, 125527 (2023).
30. Keenan, J., Dooley, M., Pearson, D. & Clynes, M. Recombinant Human Albumin in Cell Culture: Evaluation of Growth-Promoting Potential for NRK and SCC-9 Cells In Vitro. *Cytotechnology* **24**, 243–252 (1997).
31. Deguchi, M. et al. Establishment and optimization of a hemp (Cannabis sativa L.) agroinfiltration system for gene expression and silencing studies. *Sci. Rep.* **10**, 3504 (2020).
32. Heidari-Japelaghi, R., Valizadeh, M., Haddad, R., Dorani-Uliaie, E. & Jalali-Javaran, M. Production of bioactive human IFN-γ protein by agroinfiltration in tobacco. *Protein Expr. Purif.* **173**, 105616 (2020).
33. Charnsatabut, C. et al. Optimized expression of human interleukin-15 in Nicotiana benthamiana and in vitro assessment of its activity on human keratinocytes. *Biotechnol. Rep.* **46**, e00889 (2025).
34. Li, F. et al. Evaluation of parameters affecting Agrobacterium-mediated transient expression in citrus. *J. Integr. Agriculture* **16**, 572–579 (2017).
35. Mandal, M. K., Ahvari, H., Schillberg, S. & Schiermeyer, A. Tackling Unwanted Proteolysis in Plant Production Hosts Used for Molecular Farming. *Front. Plant Sci.* **7**, 2016 https://doi.org/10.3389/fpls.2016.00267 (2016).
36. Nguyen, M. T. et al. Bacterial overexpression and purification of soluble recombinant human serum albumin using maltose-binding protein and protein disulphide isomerase. *Protein Expr. Purif.* **167**, 105530 (2020).
37. Fernández-San Millán, A., Mingo-Castel, A., Miller, M. & Daniell, H. A chloroplast transgenic approach to hyper-express and purify Human Serum Albumin, a protein highly susceptible to proteolytic degradation. *Plant Biotechnol. J.* **1**, 71–79 (2003).
38. Mirzaee, M. et al. Seed plastids: A novel platform for recombinant protein expression. *Plant Biotechnol. J.* **22**, 2575–2577 (2024).
39. Privalov, P. L. Cold Denaturation of Protein. *Crit. Rev. Biochem. Mol. Biol.* **25**, 281–306 (1990).
40. An, Q. et al. Thermochemical valorization of tobacco wastes into biofuels and carbon materials: A comprehensive review. *Chem. Eng. J.* **505**, 159544 (2025).
41. Sillman, J. et al. Bacterial protein for food and feed generated via renewable energy and direct air capture of CO2: Can it reduce land and water use?. *Glob. Food Security* **22**, 25–32 (2019).
42. Kobayashi, Y. et al. Life-cycle assessment of yeast-based single-cell protein production with oat processing side-stream. *Sci. Total Environ.* **873**, 162318 (2023).
43. https://patents.google.com/patent/EP0510678A2/en, E. P. O. P. o. r. h. s. a. i. P. p. E. A. I. c. M. A. f.
44. Chaudhary, S., Ali, Z. & Mahfouz, M. Molecular farming for sustainable production of clinical-grade antimicrobial peptides. *Plant Biotechnol. J.* **22**, 2282–2300 (2024).
45. Whittinghill, K. *Life Cycle Assessment*, https://bio.libretexts.org/Courses/University_of_Pittsburgh/Environmental_Science_(Whittinghill)/11%3A_Solid_Waste_Management/11.03%3A_Life_Cycle_Assessment (2024).
46. Hartmann, T. From waste products to ecochemicals: Fifty years research of plant secondary metabolism. *Phytochemistry* **68**, 2831–2846 (2007).
47. Baranska, M., Roman, M., Dobrowolski, J., Schulz, H. & Baranski, R. Recent Advances in Raman Analysis of Plants: Alkaloids, Carotenoids, and Polyacetylenes. *Curr. Anal. Chem.* **9**, 108–127 (2013).
48. Sheen, S. J. Biomass and Chemical Composition of Tobacco Plants Under High Density Growth. *Contributions Tob. Nicotine Res.* **12**, 35–42 (1983).
49. Willis, J. D., Mazarei, M. & Stewart, C. N. Transgenic Plant-Produced Hydrolytic Enzymes and the Potential of Insect Gut-Derived Hydrolases for Biofuels. *Front. Plant Sci.* **7**, https://doi.org/10.3389/fpls.2016.00675 (2016).
50. Naim, F., Shrestha, P., Singh, S. P., Waterhouse, P. M. & Wood, C. C. Stable expression of silencing-suppressor protein enhances the performance and longevity of an engineered metabolic pathway. *Plant Biotechnol. J.* **14**, 1418–1426 (2016).
51. Cabrera, A. et al. The sound of silence: Transgene silencing in mammalian cell engineering. *Cell Syst.* **13**, 950–973 (2022).
52. Planinc, A., Bones, J., Dejaegher, B., Van Antwerpen, P. & Delporte, C. Glycan characterization of biopharmaceuticals: Updates and perspectives. *Analytica Chim. Acta* **921**, 13–27 (2016).
53. Rogstad, S. et al. A Retrospective Evaluation of the Use of Mass Spectrometry in FDA Biologics License Applications. *J. Am. Soc. Mass Spectrom.* **28**, 786–794 (2017).
54. Fekete, S., Beck, A., Veuthey, J.-L. & Guillarme, D. Theory and practice of size exclusion chromatography for the analysis of protein aggregates. *J. Pharm. Biomed. Anal.* **101**, 161–173 (2014).
55. Brusotti, G. et al. Advances on Size Exclusion Chromatography and Applications on the Analysis of Protein Biopharmaceuticals and Protein Aggregates: A Mini Review. *Chromatographia* **81**, 3–23 (2018).
56. Liu, H. & Timko, M. P. Improving Protein Quantity and Quality—The Next Level of Plant Molecular Farming. *Int. J. Mol. Sci.* **23**, 1326 (2022).
57. Naji-Talakar, S. Plant-derived biopharmaceuticals: Overview and success of agroinfiltration. *Trends Capstone* **2**, 12 (2017).
58. Chen, Q., He, J., Phoolcharoen, W. & Mason, H. S. Geminiviral vectors based on bean yellow dwarf virus for production of vaccine antigens and monoclonal antibodies in plants. *Hum. Vaccin* **7**, 331–338 (2011).
59. Jay, F., Brioudes, F. & Voinnet, O. A contemporary reassessment of the enhanced transient expression system based on the tombusviral silencing suppressor protein P19. *Plant J.: cell Mol. Biol.* **113**, 186–204 (2023).
60. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).

61. Jimenez-Gonzalez, C., Ponder, C. S., Broxterman, Q. B. & Manley, J. B. Using the Right Green Yardstick: Why Process Mass Intensity Is Used in the Pharmaceutical Industry To Drive More Sustainable Processes. *Org. Process Res. Dev.* **15**, 912–917 (2011).

Acknowledgements

This project was funded by the National Research Council of Thailand (NRCT) and Chulalongkorn University (No. N42A670577) and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphisek Somphot Endowment Fund) from Chulalongkorn University. The authors express their gratitude to Assoc. Prof. Supaart Sirikantaramas for providing the N. benthamiana seeds. We thank Assoc. Prof. Jittima Luckanagul for the kind supply of NIH3T3 mouse fibroblast cells.

Author contributions

I.S.: Writing – review & editing, Writing – original draft, Methodology. U.N.: Writing – original draft, Methodology. P.S.: Writing – original draft, Methodology. K.R.: Formal analysis, Investigation, Methodology. K.J.: Formal analysis, Investigation. P.A.: Writing – original draft, Methodology. P.R.: Conceptualization, Writing – original draft, Writing – review & editing. C.J.I.B.: Writing – review & editing, Writing – original draft, Methodology, Formal analysis. W.P.: Conceptualization, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing.

Competing interests

Waranyoo Phoolcharoen is a co-founder and shareholder of Baiya Phytopharm Co., Ltd. Utapin Ngaokrajang, Pipob Suwanchaikasem, Kaewta Rattanapisit, Kitti Jirananon, and Christine Joy I. Bulaon are employees of Baiya Phytopharm Co., Ltd. The remaining authors declare no conflicts of interest.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s44383-025-00005-5.

Correspondence and requests for materials should be addressed to Christine Joy I. Bulaon or Waranyoo Phoolcharoen.