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The Nicotiana Benthamiana Biofactory

Production of the High Value Pharmaceutical Target, Human Granulocyte-Colony Stimulating Factor, in *Nicotiana benthamiana* Is Improved by Co-Expression of the Transcriptional Regulator of the Unfolded Protein Response, bZIP60

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Nicotiana benthamiana is particularly attractive for production of high-value pharmaceuticals that are recalcitrant to cost effective production in microbial or mammalian cell platforms. A standout example is human granulocyte-colony stimulating factor (hG-CSF), a primary regulator of granulopoiesis and neutrophil functions, used to treat chemotherapy-induced neutropenia. Clinically approved hG-CSF analogs include Filgrastim and Lenograstim, produced in *Escherichia coli* and Chinese hamster ovary (CHO) cells, respectively, both of which show efficacy equivalent to native hG-CSF (Lipiäinen et al. 2015). However, the absence of glycosylation in *E. coli*-derived hG-CSF necessitates PEG-conjugation to enhance serum half-life, increasing production complexity and cost (Wang et al. 2025), while production in CHO cells remains inherently expensive (Fischer et al. 2015). Previous reports in plants have shown low hG-CSF expression levels, ranging from 0.1 to 17 mg/mL in BY-2 tobacco suspension cells (Hong et al. 2002), of 2.5 mg/g total soluble protein in transgenic tobacco plants (Tian and Sun 2011) and 100 mg/kg leaf fresh weight (LFW) of affinity chromatography purified product estimated by western blot analysis from *N. benthamiana* (Zvereva et al. 2009), highlighting the need for strategies to enhance yield and quality at scale in plants, which can be rapidly tested in *N. benthamiana*.

Expression of hG-CSF in *N. benthamiana* (Appendix S1) resulted in visibly severe leaf tissue damage 2–4 days post-infiltration (dpi), with reduced turgor pressure, leaf discoloration, rapid wilting by 4 dpi, elevated malondialdehyde (MDA) levels (an indicator of membrane damage resulting from lipid peroxidation), a 20% aerial biomass loss and low hG-CSF yields (Figure 1A,B). These effects are similar to those previously seen with another high-value target, human erythropoietin (hEPO), and are indicative of improper protein folding (Wagner et al. 2024). Therefore, we pursued a strategy to enhance protein folding and quality control in the endoplasmic reticulum (ER) by overexpressing transcription factors known to regulate the unfolded protein response (UPR).

Accumulation of misfolded proteins in the ER induces the UPR, a conserved signalling cascade that restores homeostasis. In plants, the UPR is primarily mediated via three transcription factors: basic leucine zipper (bZIP)17, bZIP28 and bZIP60 (Kim et al. 2018; Zhang et al. 2015). Upon detection of unfolded proteins in the ER, inositol-requiring enzyme 1 (IRE1) unconventionally splices bZIP60 mRNA to yield a transcriptionally active form, bZIP60s, which translocates to the nucleus and induces ER stress response genes such as the 78 kDa glucose regulated protein (GRP78 or BiP), calreticulin and protein disulfide isomerase, thus mitigating ER stress (Kim et al. 2018; Zhang et al. 2015). By contrast, under non-stressed conditions, unspliced bZIP60 (bZIP60u) remains cytosolic and inactive (Zhang et al. 2015).

The effect of over expressing bZIP17 or bZIP28, each derived from *Arabidopsis thaliana*, or unspliced versus spliced bZIP60, derived from *Nicotiana tabacum*, alongside hG-CSF

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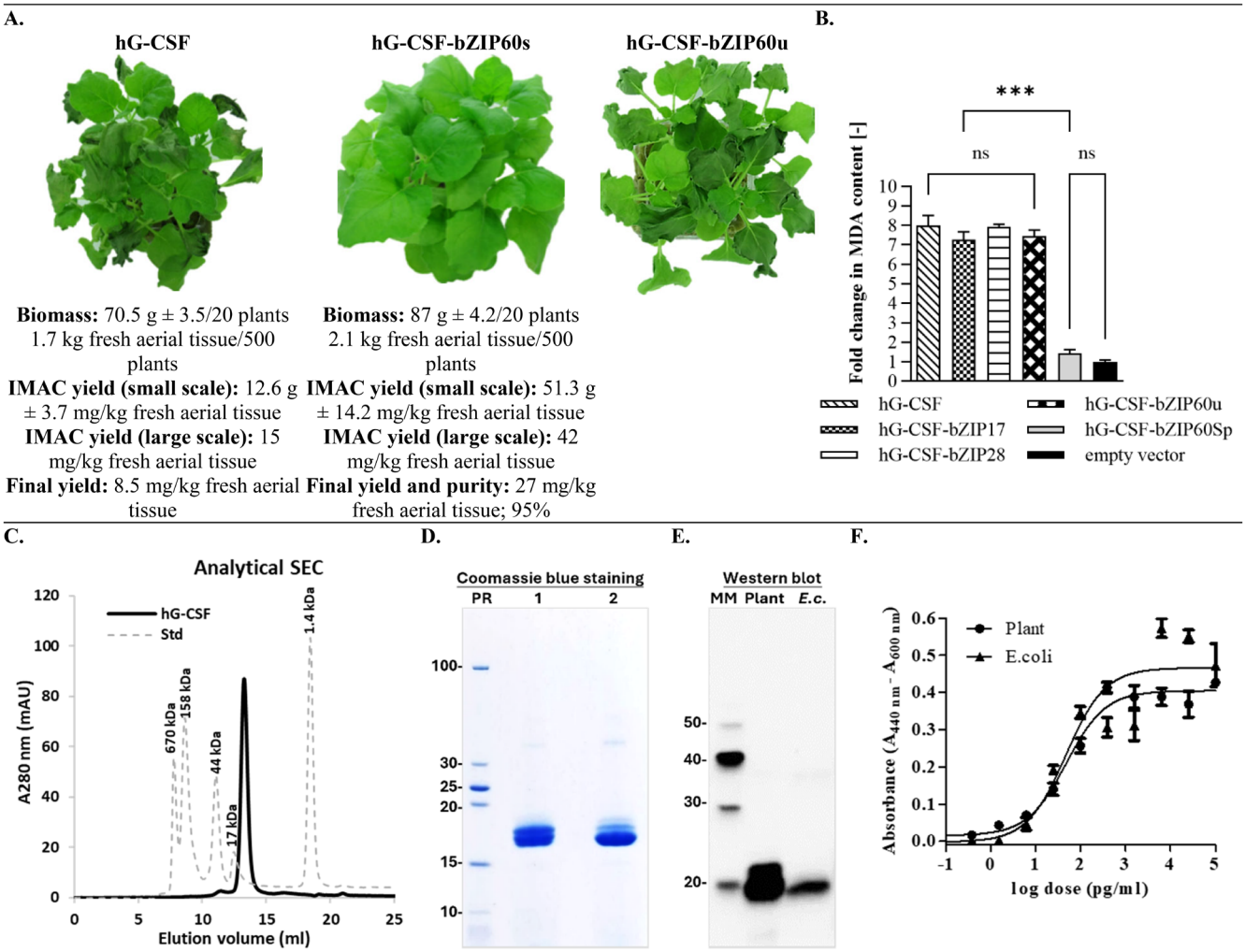


FIGURE 1 | Production and characterization of hG-CSF expressed in *N. benthamiana* with bZIP transcription activators. (A) Visual and (B) biochemical (MDA content) characterization of plants infiltrated with an empty vector or vectors over expressing hG-CSF with unspliced bZIP60u or spliced bZIP60s at 4 dpi. Yields for hG-CSF and hG-CSF-bZIP60s plants are presented. Error bars represent the standard deviation of biological replicates from the mean ($n = 3$) (C) Analytical SEC of plant produced hG-CSF (bold black line) compared to BioRad standards (grey dash line). (D) SDS-PAGE analysis of purified plant produced hG-CSF under 1-reducing and 2-non-reducing conditions. PR, Page Ruler. (E) Western blot analysis using anti-hG-CSF monoclonal antibody. MM, Magic Mark XP; Plant, plant produced hG-CSF; *E.c.*, *E. coli* produced hG-CSF. (F) Proliferation of M-NFS-60 cells in response to increased doses of plant- or *E. coli*-produced hG-CSF. Values represent the mean ± SD of three independent experiments. EC50 values were calculated from nonlinear regression analysis using least squared algorithms (GraphPad Prism).

was investigated (Appendix S1). Over expression of bZIP60s alongside hG-CSF prevented gross tissue damage, biomass loss and elevated MDA levels and led to a threefold increase in hG-CSF yield relative to hG-CSF expression alone, whereas over expression of bZIP60u had no such effect (Figure 1A,B). These results indicate that hG-CSF expression in *N. benthamiana* induces stress by surpassing the protein processing capacity of the ER. However, over expression of the active cytosolic domains of bZIP17 or bZIP28 alongside hG-CSF did not prevent tissue damage, prevent elevated MDA levels or improve yield (Figure S1, Figure 1B). This is in contrast to expressing hEPO in *N. benthamiana*, in which case all three transcription factors can mitigate tissue damage and enhance yield (Wagner et al. 2024). This suggests that some recombinant proteins have more specific requirements than others to alleviate improper folding.

Following extraction in sodium acetate buffer (pH 4) to selectively precipitate most host-cell proteins (Figure S2, TP vs. IL) and nucleic acids, hG-CSF, co-expressed with bZIP60s, was purified by immobilized metal ion affinity chromatography and ion exchange chromatography, for a final yield of 27 mg/kg fresh aerial tissue and over 95% purity (Figure 1A, Figures S2 and S3). Analytical size exclusion chromatography (SEC) revealed that the plant-expressed hG-CSF is monomeric, eluting just after the 17 kDa standard (Figure 1C). Reducing and non-reducing SDS-PAGE confirmed a molecular weight of 18–19 kDa (Figure 1D), consistent with the theoretical value of 18.8 kDa (Link 2022), and Western blot analysis with an anti-hG-CSF monoclonal antibody verified its identity (Figure 1E). hG-CSF has a single predicted glycosylation site at threonine 133 (Link 2022), and Pro-Q Emerald 300 staining confirmed that the plant-produced protein is glycosylated (Figure S4). Biological activity, assessed

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by a WST-1 proliferation assay, demonstrated that the plant-produced hG-CSF has equivalent potency to commercial *E. coli*-derived hG-CSF (Figure 1F).

In conclusion, targeted manipulation of the UPR pathway in *N. benthamiana* through bZIP60s overexpression efficiently alleviates ER stress and significantly enhances the yield and quality of hG-CSF. Modulating UPR signalling thus represents a powerful means to enhance the versatility of plant-based production systems. Future research will explore the broader applicability of this approach to other recombinant proteins, so advancing the potential of plant biotechnology for affordable and scalable drug manufacturing.

Author Contributions

Nazgul Wagner conceptualization, data collection, data analysis and manuscript preparation. Konstantin Musiychuk and Hong Bi upstream processing data analysis, Stephen Tottey downstream processing data analysis, editing. Jukka Kervinen downstream processing data analysis, Stephen J. Streatfield conceptualization, manuscript preparation and editing, Rainer Fischer editing and Vidadi Yusibov conceptualization and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the S1 of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Appendix S1:** pbi70652-sup-0001-Appendix1.docx. **Figure S1:** Visual characterization of plants infiltrated with an empty vector or vectors over expressing hG-CSF with bZIP17 or bZIP28 at 4 dpi. **Figure S2:** Coomassie Brilliant Blue stained SDS-PAGE gel analysis of hG-CSF purification samples from *N. benthamiana* over expressing bZIP60s alongside hG-CSF. BM, BenchMark; TP, total protein of the plant tissue homogenate (pH 4); IL, IMAC load; CL, IMAC eluate after dialysis against IEX binding buffer, same as IEX load; C1-C6, 100-fold concentrated IEX elution fractions (except C4, which is not concentrated). Arrow indicates plant-produced hG-CSF, with identity confirmed by Western blot analysis using an anti-hG-CSF monoclonal antibody. **Figure S3:** Chromatogram depicting hG-CSF purification from *N. benthamiana* co-expressing bZIP60s using cation exchange chromatography. FT, flow through fraction; C1-C6, IEX elution fractions at increasing conductivity. **Figure S4:** In-gel characterization of purified plant produced hG-CSF. Pro-Q Emerald 300 stained SDS-PAGE gel. M, molecular weight standard; 1, hG-CSF in reducing buffer; 2, hG-CSF in a non-reducing buffer.

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