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GREEN PRODUCTION AND CHARACTERISATION OF HIGH-VALUE THERAPEUTIC PROTEINS IN EDIBLE PLANTS

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Abstract

This work explores plant-based strategies for the sustainable production of proteinatious pharmaceuticals and other high-value proteins, using transient and stable transformation. The first approach focuses on engineering safe-to-eat plants free from various bacterial pathogens via introducing genes encoding antibacterial proteins, such as colicin M. The second strategy substitutes plant sources to produce unique proteins in climate-resilient crops, exemplified by the thaumatin II, a natural low-calorie sweetener protein. Finally, the usage of plants as green bioreactors for the pharmaceuticals production, such as human interferon α2b, an essential medicine for antiviral and anticancer treatments.

Key words: *plant-based protein production, sustainable production, Agrobacterium-mediated transformation, transgenic plants, plant bioreactors, Colicin M, Thaumatin II, Human interferon α2b.*

Plant-based protein production systems offer key advantages over traditional platforms such as bacterial, yeast, or mammalian cells, making them attractive for pharmaceutical and nutraceutical applications. Plants provide a safe production environment, generally free from antibiotics, toxins, and human pathogens [1]. The natural encapsulation of proteins by the plant cell wall provides protection against proteolytic enzymes, enabling oral immunisation and targeted delivery through the gastrointestinal tract [2].

From an environmental perspective, plant-produced proteins can be stored in plant dry biomass, eliminating the need for cold chain logistics and lowering the carbon footprint. Moreover, plants allow for overcoming the need for large-scale fermenters, aseptic conditions and high-energy inputs. These align with several of the UN's Sustainable Development Goals (SDGs), particularly SDG 3 (good health and well-being), SDG 9 (industry, innovation and infrastructure), and SDG 13 (climate action).

For these reasons, we developed both transient and stable expression plant systems, using the *Agrobacterium*-mediated transformation, targeting several goals: improving food safety, replacing

the environmentally damaging extraction of natural compounds, and enabling cost-effective pharmaceutical protein production.

The first strategy focused on obtaining safe-to-eat leafy vegetables resistant to bacterial contamination. Green leafy vegetables, such as lettuce, are often linked to *Escherichia coli* outbreaks, which pose serious public health concerns due to numerous reports of severe disease and fatalities. Moreover, *E. coli* strains causing escherichiosis have shown increasing resistance to major antibiotics and are classified by WHO as "critical priority pathogens" [3].

To address this, the gene encoding colicin M (ColM) - a toxin effective against pathogenic *E. coli*, was introduced into lettuce and broccoli through *Agrobacterium*-mediated transformation (picture 1.a-b). All selected transgenic plant lines expressing ColM performed antibacterial activity (picture 1.c). Lettuce extracts successfully inhibited the growth of major pathogenic strains (including EHEC O157:H7 and O104:H4), demonstrating potential as an antibacterial food and feed supplement. Additionally, in dried plant material, ColM retained biological activity with no significant activity loss up to 3 months at room temperature, supporting the feasibility of cold chain-independent storage [4].

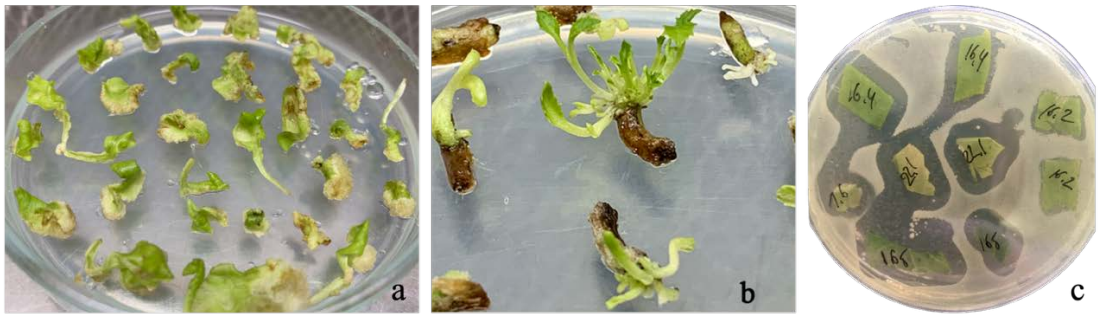

Fig.1. *ColM production in plants. (a) In vitro culture of transgenic lettuce expressing ColM protein on B5 medium Kin 3 mg/l, NAA 0.2 mg/l, PPT 5 mg/l; (b) in vitro culture of transgenic broccoli expressing ColM protein on MS medium BAP 3,5 mg/l, NAA 0.02 mg/l, PPT 5 mg/l; (c) antibacterial activity of explants of different transgenic lines of T1 generation against a model laboratory strain of E. coli XL1-Blue*

In a second application, we aimed to produce thaumatin II, a sweet-tasting protein from *Thaumatococcus danielli*, which is up to 100,000 times sweeter than sugar [5]. However, natural extraction is geographically limited and ecologically harmful. In contrast, sugar production uses more than 31 million hectares of agricultural land worldwide. Significant ecological effects of the widespread production of sugar crops include soil degradation, deforestation, high water and energy consumption, and chemical inputs [6].

To create a more suitable alternative, the thaumatin II gene was inserted into lettuce and tobacco genomes. However, lettuce was found to be an unsuitable host due to thaumatin's toxic effect on this plant. We hypothesise that thaumatin's original function as a stress-response protein [7], and its stable overexpression, may trigger defence pathways in lettuce, exhausting metabolic resources and inhibiting development. Meanwhile, tobacco plants maintained a healthy phenotype, detectable foreign protein expression, and produced a sweeter taste profile, indicating their potential as a host for sweet protein production in temperate climates.

Lastly, plants were used as bioreactors for the human interferon α2b (IFNα2b) production, an essential cytokine used in antiviral and anticancer therapies [8]. While production in traditional platforms is expensive, here we used *Nicotiana benthamiana* and transgenic broccoli, with broccoli offering the added benefit of inherent anticancer properties. This dual approach may enhance

antiproliferative activity by creating synergy between the host plant and the therapeutic protein. For antiviral application, oral administration of IFNα2b, unlike injectable forms, activates systemic immunity at doses approximately 10^4-fold times lower [9], making edible plants an advantageous delivery system due to the natural encapsulation by the plant cell wall.

Initial expression of IFNα2b showed that it not only has a short blood serum half-life [10] but is also quickly degraded in the plant apoplast, leading to a loss of functional protein (picture 2). To produce IFNα2b with a longer window of action and enhanced storage stability, we tested several strategies, including protein modifications and co-expression with protease inhibitors and helper enzymes in *Nicotiana benthamiana*. We co-expressed IFNα2b with several pathogen-triggered immunity suppressors from *Phytophthora infestans* (e.g., PiTG_04314, PiTG_22926, PiTG_02860, AVR2-SP, GR103-1) [11] alongside the tomato protease inhibitor *Sl* CYS8 [12]. However, this combination did not show any positive effect on IFNα2b accumulation, but developed protocols could be adapted for the expression of other proteins of interest. Further optimisation studies are in progress; however, specific details remain confidential at this stage.

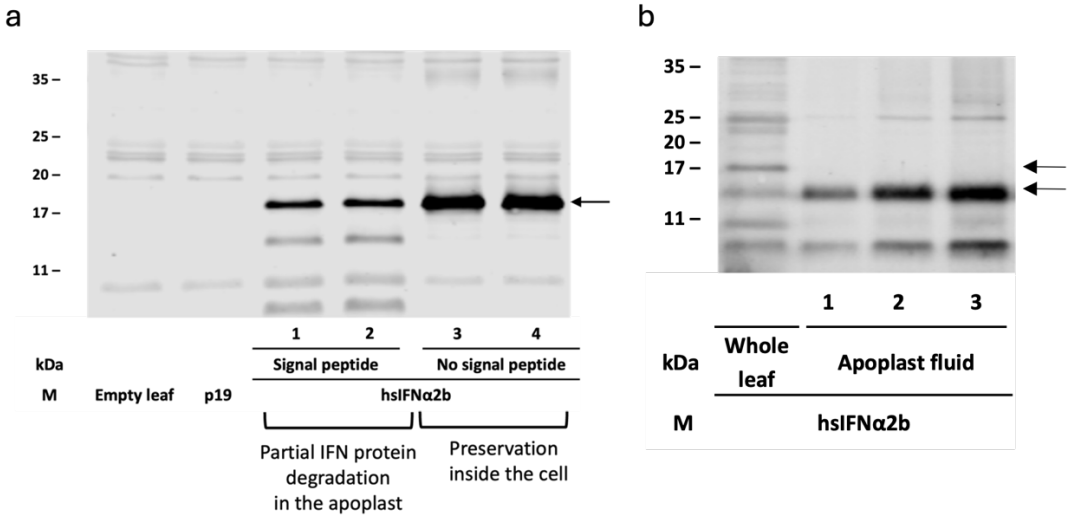

Fig. 2. *Expression of IFNα2b in N. benthamiana was analysed via immunoblot with polyclonal IFN antibody.* ***(a)*** *Pattern of IFNα2b with and without signal peptide: M – NSR molecular weight marker; p19 – negative control, suppressor of RNA silencing; 1,2 - IFNα2b construct with calreticulin SP; 3,4 - IFNα2b construct without calreticulin SP. Results are presented in 2 technical repeats.* ***(b)*** *Comparative analysis of IFNα2b extracts derived from the whole leaf tissue versus apoplastic fluid. Expected size of IFNα2b – 19 kDa, in case of partial IFN protein degradation – 7, 15 kDa fragments were observed*

Together, all mentioned approaches demonstrated the flexibility and potential of plant systems for sustainable, cost-effective and safe biomanufacturing. Further work will focus on enhancing the activity and stability of therapeutic proteins and developing a universal edible production system.

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«ЗЕЛЕНЕ» ВИРОБНИЦТВО ТА АНАЛІЗ ЦІННИХ ТЕРАПЕВТИЧНИХ БІЛКІВ У ЇСТІВНИХ РОСЛИНАХ

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Анотація

У цій роботі досліджуються стратегії сталого виробництва фармацевтичних білків та інших цінних біомолекул із використанням транзієнтної й стабільної генетичної експресії в рослинних системах. Перший підхід передбачає створення безпечних для споживання рослин, резистентних до бактеріальних патогенів, шляхом інтеграції генів, що кодують антибактеріальні білки, зокрема коліцин М. Друга стратегія спрямована на використання альтернативних, кліматостійких культур як джерела унікальних білків, таких як тауматин II – природний низькокалорійний білок-цукрозамінник. І також, розглядається використання потенціалу їстівних рослин як «зелених» біореакторів для виробництва білків фармацевтичного призначення, зокрема людського інтерферону α2b – ключового агенту з противірусною та протипухлинною активністю.

Ключові слова: рослинна система білкового синтезу, стале виробництво, *Agrobacterium*-опосередкована трансформація, генетично модифіковані рослини, рослинні біореактори, коліцин М, тауматин II, рекомбінантний людський інтерферон α2b.