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Rapid transient expression of human granulocyte-macrophage colony-stimulating factor in two industrial cultivars of tobacco (*Nicotiana tabacum* L.) by agroinfiltration

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ABSTRACT

We report the production of hGM-CSF cytokine in leaves of industrial tobacco cultivars DH-17 and DH-27 by using *Agrobacterium*-mediated transient expression. We prove the concept that very high biomass industrial tobacco plants are suitable platforms for rapid, low cost production of foreign proteins. Successful transient expression of the GM-CSF was achieved in less than three months, opening the possibility for future applications of this approach in rapid response production of various proteins of non-plant origin in industrial tobacco.

1. Introduction

The concept of utilizing plants for production of valuable pharmaceuticals, such as vaccines and recombinant proteins, has been introduced over twenty years ago, however, its implementation as an established technology is quite novel. Initial reliance on stably transformed transgenic plants has been shifted toward transient transformation approaches, enabling fast and efficient production of desired proteins. In the cases of vaccine production, such systems have proven superior in terms of speed and cost over the traditional methodologies. Further, transient plant expression techniques are scalable and relatively uncomplicated, thus enabling, e.g., production of rapid response vaccines. The method of vacuum-agroinfiltration has been used for rapid large-scale production of recombinant antibodies [1–3] and to evaluate the expression of their different forms [4–7]. Unlike bacterial expression systems, plant cells are able to express and correctly process complex foreign proteins, with low or minimal cross-contamination with adventitious agents. Genetically engineered plants therefore offer viable production alternatives, with downstream processing of potentially low complexity. Various plant species have so far been utilized as expression platforms, nevertheless the plants with large biomass are more cost-effective. Hitherto, research has been done on potato, clover, soybean, lettuce, tomato, maize, *Arabidopsis*, and several other species. However, tobacco (*Nicotiana tabacum* L.) has proven to be the most frequently used due to its relatively simple genetic transformation and regeneration procedures [8,9]. The main disadvantages of tobacco are relatively high concentrations of nicotine and other alkaloids in leaves, consequently requiring elaborate recombinant protein purification procedures. This obstacle can be avoided if young plants in pre-nicotine phase are used. Further, it has been shown that the accumulation of foreign proteins varies substantially in different cultivars of tobacco, and that just the selection of another cultivar can dramatically increase yields [10].

Expression of various mammalian proteins in plants is a developing area of biotechnology and the production of numerous pharmaceutically relevant factors has so far been attempted. Granulocyte macrophage colony-stimulating factor (GM-CSF) is a cytokine that acts as a white blood cell growth factor. T-cells, B-cells, macrophages, mast cells, endothelial cells, fibroblasts, and

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adipocytes, in response to cytokine or inflammatory stimuli, produce GM-CSF. Th1-biased immune response, angiogenesis, allergic inflammation, and the development of autoimmunity are all promoted by the GM-CSF, thus this cytokine is a part of elaborate immune/inflammatory cascades. GM-CSF exhibits clinical effectiveness in ameliorating chemotherapy-induced neutropenia, while GM-CSF transfected tumor cells are used as cancer vaccines. The 22 kDa glycosylated GM-CSF, similar to IL-3 and IL-5 proteins, is a polypeptide with a core of four bundled alpha helices. The active form of the protein is found extracellulary as a homodimer. To date, production of GM-CSF in plant-based expression systems has been achieved in cell cultures of tobacco [11–16] and rice [17]. Further, whole tobacco plants transfected with viral vectors have also been employed [18].

In this work, we report successful production of the hGM-CSF in the leaves of two industrial cultivars of tobacco. We used *Agrobacterium*-mediated transient expression procedure, preceded by the fast Gateway-based cloning steps. Two binary vectors for both transient and stable transformation were evaluated. The accumulation of recombinant hGM-CSF was dependent on the presence of the wetting agent supplemented during vacuum-agroinfiltration, duration of vacuum-agroinfiltration, and the age of the leaves. We demonstrate that industrial tobacco cultivars are suitable platforms for rapid, low cost, and efficient production of the hGM-CSF, and probably other recombinant proteins. Our long-term goal is to use transgenic plant technology to produce large quantities of foreign proteins in order to meet potential demands and cost requirements.

2. Materials and methods

2.1. Clones and vectors

Human GM-CSF cDNA gene clone (Granulocyte macrophage colony-stimulating factor, GenBank acc. no. NM_000758) was obtained from Sino Biological Inc., Beijing, China. The gene GM-CSF (413 bp) was amplified by PCR using primers: GM-CSFFor 5′-CACCATGTGGCTGCAGAGCCTGCTGCTCTTGGGC-3′, GM-CSFHisRev 5′-TCA**GTGGTGGTGGTGGTGGTG**CTCCTGCACTGGCTCCCAGCAGTCA AAGGG-3′ and GM-CSFHisHARev 5′-TCAAGCGTAATCTGGAACATCG TATGGGTATAACTTAGC**GTGGTGGTGGTGGTGGTG**CTCCTGCACTGG-3′, specifically designed to enable cloning of the gene into the Gateway pENTR/SD/D-TOPO vector, as well as the C-terminal tagging of GM-CSF protein with the 6 × histidine (His) (marked in bold) and hemagglutinin (HA) (underlined) peptide epitopes, for the purpose of specific immunochemical detection of the recombinant protein in total protein extracts. Between the His- and HA-tags, an Ala-Lys-Leu linker (shaded) was inserted. Resulting GM-CSF-His-HA construct was cloned into the pENTR/SD/D-TOPO entry vector (Invitrogen Gateway System, Life Technologies Corporation, Carlsbad, USA), according to the protocol recommended by the producer. Insert was subsequently transferred into two binary destination vectors: pH7WG2.0, for stable plant transformation, and p2GW7.0, suitable for transient expression in plants. In both vectors, expression of the inserted gene is driven by the constitutive CaMV-35S promoter. All cloning steps, including insert sequences and their orientation in vectors, were checked by DNA sequencing. As a control, the pH7WG2.0 destination vector harboring chloroplast protein kinase CSK (gene *At1g67840* [19]) tagged with the HA- and FLAG-tags has been used.

2.2. Plant material and growth conditions

Tobacco plants var. Virginia, cultivars DH-17 and DH-27, were grown on Stender B400 substrate in green house until the age of 1 month. Plantlets were subsequently transferred to the phytotron and grown under 12/12 h light/dark regime, 100 $\mu mol_{PHOTONS}\ m^{-2}\ s^{-1}$, at 23 °C and 65% humidity during light, and 18 °C with 75% humidity during dark.

2.3. Transformation

One hundred μl of chemically competent cells of the *Agrobacterium tumefaciens* strain EHA105 were transformed with 500 ng of GM-CSF-His-HA/p2GW7.0, GM-CSF-His-HA/pH7WG2.0, or CSK-HA-FLAG/ pH7WG2.0 constructs and grown at 28.5 °C over night on YEB plates supplemented with 125 mg/ml rifampicin and 100 mg/ml ampicillin (p2GW7.0) or 40 mg/ml spectinomycin (pH7WG2.0). An individual colony of each sample was inoculated into the liquid medium of the same composition, supplemented with 2 μmol/ml $MgSO_4$. Liquid agrobacterial cultures were grown at 28.5 °C under 300 rpm agitation until the $O.D._{600}$ reached 1.7–2.0. Overnight cultures were centrifuged at 5000 × g for 10 min at 4 °C and pellet was resuspended in the infiltration medium (1/2x MS salts (Sigma–Aldrich, St.Louis, USA), 5% sucrose, pH 5.8, 1 × Gamborg's vitamin solution (Sigma–Aldrich), and 10 μg/l 6-benzylaminopurine (BAP) (Sigma–Aldrich)) to the $O.D._{600}$ of approx. 0.1.

2.4. Vacuum infiltration

Tobacco leaves of 40-day-old plants were vacuum infiltrated with transformed agrobacterial suspensions. Agrobacterial pellets from 300 ml overnight cultures were individually resuspended in 1.5 l of infiltration medium containing 0.03% (v/v) of the mild surfactant Silwet L-77 (Momentive Performance Materials GmbH & Co KG, Leverkusen, Germany) to lower surface tension. Plants were submerged in this medium by inverting the pots upside down into the 2.5 l laboratory glass. Prior the pots were covered with the aluminium foil to prevent contamination of the medium with soil debris. Pot and glass were sealed in the large exicator connected to the laboratory vacuum pump of medium strength. Vacuum was applied for 5, 10, 15, 20 or 25 min periods. Following infiltration, plants were laid down, covered with the plastic hood, and kept in the dark for additional 12 h in the phytotron. On the next day, plants were raised up, watered, and grown further under 12/12 h light/dark regime. The infiltration procedure was repeated once again after 10 days, for 10–20 min. in the case of the CSK construct, or 5–10 min. for the GM-CSF constructs.

2.5. Harvesting of infiltrated leaves and preparation of protein extracts

Ten days after second infiltration for the CSK construct, or 3–7 days after second infiltration for the GM-CSF constructs, bottom leaves were collected and frozen at −80 °C until further use. Total proteins were isolated from 1 g of leaves ground in liquid nitrogen to a fine powder with a mortar and pestle. Proteins were extracted by using 2 ml of extraction buffer (Laemmli [20]) per gram of leaf material, samples were vortexed and incubated at 80 °C for 10 min. Cell debris was removed by centrifugation at 15 000 × g for 15 min, 4 °C and protein concentrations were estimated according to Bradford [21].

2.6. Detection of GM-CSF expression

Samples were analysed by SDS-PAGE followed by immunochemical detection with the α-HA-tag high affinity rat monoclonal antibody (1:1000, Roche, Basel, Switzerland). As the secondary antibody, anti-rat peroxidase conjugate was applied (1:5000, Sigma) and the results were visualised by enhanced chemiluminescence (ECL).

3. Results

Vacuum infiltration of tobacco leaves was optimized by using agrobacteria harboring pH7WG2.0 vector containing the *Arabidopsis thaliana* chloroplast sensor protein kinase CSK, C-terminally labeled with the HA- and FLAG- epitopes. In *Arabidopsis*, CSK is translocated into chloroplasts with no apparent cleavable transit sequence [19]. Plants were infiltrated twice, with a 10-day interval. The first infiltration was performed in the absence of the surfactant Silwet L-77, resulting in the lack of recombinant CSK accumulation, as determined by the immunochemical analyses of the total leaf protein extracts (Fig. 1, Lanes 1–4). Ten days later, the infiltration was repeated for the purpose of enhanced agrobacterial permeation and subsequently more effective transformation. In the second infiltration step, Silwet L-77 was added, which resulted in recombinant CSK accumulation (Fig. 1, Lanes 9–13). As an additional control, we have infiltrated several plants only once as specified, but in the presence of Silwet L-77 (Fig. 1, Lanes 6–7). Thus, even a single infiltration step in the presence of Silwet L-77 renders plants transiently expressing recombinant *Arabidopsis* CSK.

Further, infiltration times of 5, 10, 15, 20, and 25 min. have been evaluated (Fig. 1, Lanes 9–13). Optimal duration between 5 and 10 min was determined to be the most effective, simultaneously causing the least mechanical damage to the leaves (Fig. 2). Longer exposure to vacuum caused tissue trauma, resulting in leaf yellowing and senescence within a few days post infiltration (Fig. 2). Following second infiltration, transient expression of recombinant CSK proceeded for 14 days, after which the leaves were collected and stored at −80 °C. Total proteins were extracted and immunochemically analysed using high specificity monoclonal α-HA antibodies. In all Western analyses, recombinant CSK-HA-FLAG was reproducibly visualized, in case its overexpression was initiated in infiltrated leaf. In summary, a single infiltration step lasting for 5–10 min. in the presence of Silwet L-77 was chosen as the optimal transformation protocol. In all subsequent experiments, infiltration/expression of chloroplast protein CSK-HA-FLAG was repeated and used as a positive control.

Since we have established a straightforward infiltration protocol, defined vacuum-exposure times, and successfully tested the specificity of our immunochemical detection system, we have now turned our attention to successful overexpression of the recombinant human GM-CSF-His-HA. To this end, optimized conditions for the expression of the CSK-HA-FLAG have been used for vacuum infiltration experiments with the GM-CSF-His-HA construct. Since the *Arabidopsis* CSK was cloned into the binary vector pH7WG2.0 designed for stable illegitimate recombination into recipient plant genome, we have decided to simultaneously test the overexpression of GM-CSF-His-HA in the same vector, or when in the p2GW7.0 vector, designed for transient transformation of dicotyledonous species. Although preliminary experiments demonstrated that a single infiltration in the presence of the surfactant was enough for successful transformation and expression, selected plants were infiltrated for the second time after 10 days, for 5–10 min., respectively. The leaves were collected 10 days after first infiltration, and 3–7 days after second infiltration. For both infiltrations Silwet L-77 has been supplemented into the agrobacterial suspensions, since we have previously demonstrated that this particular surfactant is essential for successful transformation. Accordingly, described conditions were applied for both vector/construct combinations. Western-transfer and immunochemical detection with highly specific monoclonal anti-HA antibodies were again employed for the determination of the recombinant protein fusion GM-CSF-His-HA, as well as of the control recombinant CSK-HA-FLAG accumulation.

Ten days after first infiltration, we have successfully determined that the recombinant human GM-CSF-His-HA, overexpressed from the p2GW7.0 genetic cassette, accumulates in DH-17 tobacco plants (Fig. 3a, Lane 7). The accumulation of this recombinant cytokine was confirmed 3–7 days after second infiltration, when the pH7WG2.0 vector was used in combination with the DH-17 cultivar (Fig. 3a, Lanes 11, 17). In the case of the another industrial tobacco cultivar, DH-27, recombinant GM-CSF-His-HA was expressed from pH7WG2.0 vector 3 days after the second infiltration that lasted for 10 min., and 7 days after second 5 min.-long infiltration step (Fig. 3b, Lanes 11, 16). When we evaluated the p2GW7.0 vector, the recombinant GM-CSF-His-HA accumulated only after 7 days, following the second 10 min.-long infiltration step (Fig. 3b, Lane 19). Finally, we have determined that the accumulation of the recombinant proteins and their subsequent detection was highly dependent on the health of particular plants, irrespective of the chosen cultivar.

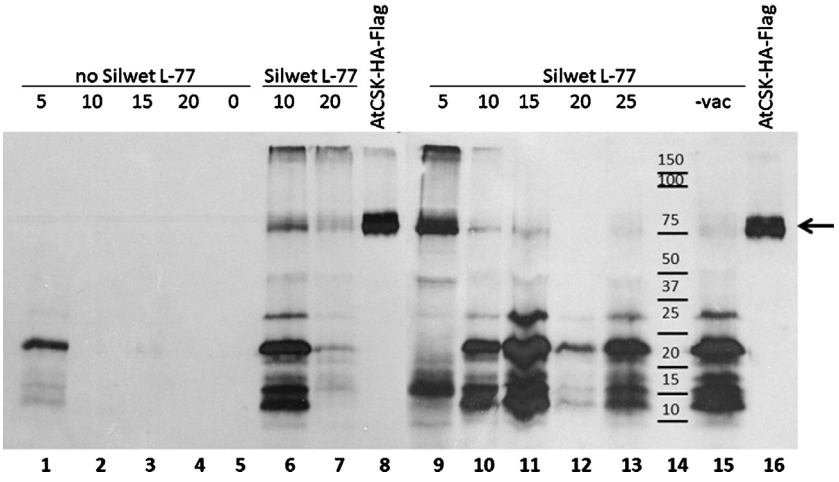

Fig. 1. Immuno-blot using anti-HA antibodies of a control protein construct CSK-HA-Flag in transformed tobacco leaves. Plants were infiltrated once (Lanes 1–7) or twice (Lanes 9–13) with agrobacteria comprising CSK-HA-Flag/pH7WG2.0 construct. 15 μg of total soluble protein (corresponding to 30 mg leaf tissue) was loaded per lane. Lanes 1–4: Vacuum infiltration 5–20 min in the absence of Silwet L-77. Lane 5: Control of non-infiltrated leaves. Lanes 6–7: Transient expression of CSK-HA-Flag in 10 and 20 min. infiltrated leaves in the presence of Silwet L-77, 12 days after infiltration. Lanes 8 and 16: AtCSK-HA-Flag expressed in *A. thaliana*, as a marker of expected protein size. Lanes 9–13: Transient expression of CSK-HA-Flag after different infiltration duration (5–25 min.) in the presence of Silwet L-77, 12 days after second infiltration. Lane 14: Protein standard. Lane 15: control of infiltration without the application of vacuum.

Fig. 2. Phenotype of the experimental tobacco plants. 40 days old tobacco plants before vacuum infiltration (a), one week after 1st infiltration of 5 min. (b), one week after 2nd infiltration of 5 min. (c), one week after 1st infiltration of 20 min. (d), one week after 2nd infiltration of 20 min. (e). Yellowing of the leaves and tissue necrosis were visible after all infiltrations, however more pronounced following longer infiltration times.

4. Discussion

Plants are becoming important hosts for manufacturing commercially interesting pharmaceutical proteins, with high scalability, safety, speed, and cost saving benefits. Recently, the first plant-made human product has been approved by the US Food and Drug Administration [22], indicating the onset of a new era for plant-made pharmaceuticals.

Transient expression has become the leading technique among transformation systems since it is a fast method with possible high protein production yields. Further, there is no stable genome integration of foreign DNA elements, thus subsequent concerns about transgene escape and contamination of food/feed chain are avoided. Novel expression vectors have been developed that have significantly enhanced the accumulation levels of pharmaceutical proteins, giving the plants an advantage in the competition with mammalian expression systems. Agroinfiltration based on syringe and vacuum infiltration provide an efficient, robust, and scalable gene-delivery technology for the transient expression of recombinant proteins in plants [23]. With respect to the downstream analyses of the recombinant proteins, transient gene expression systems offer further advantages over stable expressions. For example, initiation of gene expression and subsequent synthesis of the recombinant protein can be analysed within a very short period of time and expression is not affected by positional effects [24]. The results are obtained within a few days and can be evaluated before initiating stable transformation of plants, if desired. Because the system can be up scaled and larger quantities of transiently expressed proteins can be purified, a more elaborate analyses can be carried out. When compared to other transient systems, such as microinjection, particle bombardment, or electroporation, transient plant agroinfiltration experimental procedures do not require sophisticated equipment and are relatively inexpensive [5].

The aim of this research was to engineer and express human protein GM-CSF in tobacco leaves, using *Agrobacterium*-mediated transient expression [24]. GM-CSF has been easily expressed in plants using virus vector system and *Agrobacterium*-mediated vacuum infiltration within relatively short time periods. In this work, we wanted to investigate whether high yielding industrial cultivars of tobacco are capable of transiently expressing human protein without codon usage optimization. Our aim was not to achieve the production of the GM-CSF with higher degree of efficiency than previously reported, but to prove the concept that widely available, very high biomass, cultivars of tobacco are suitable platforms for foreign protein expression. Further, we have demonstrated that the expression can be achieved in less than

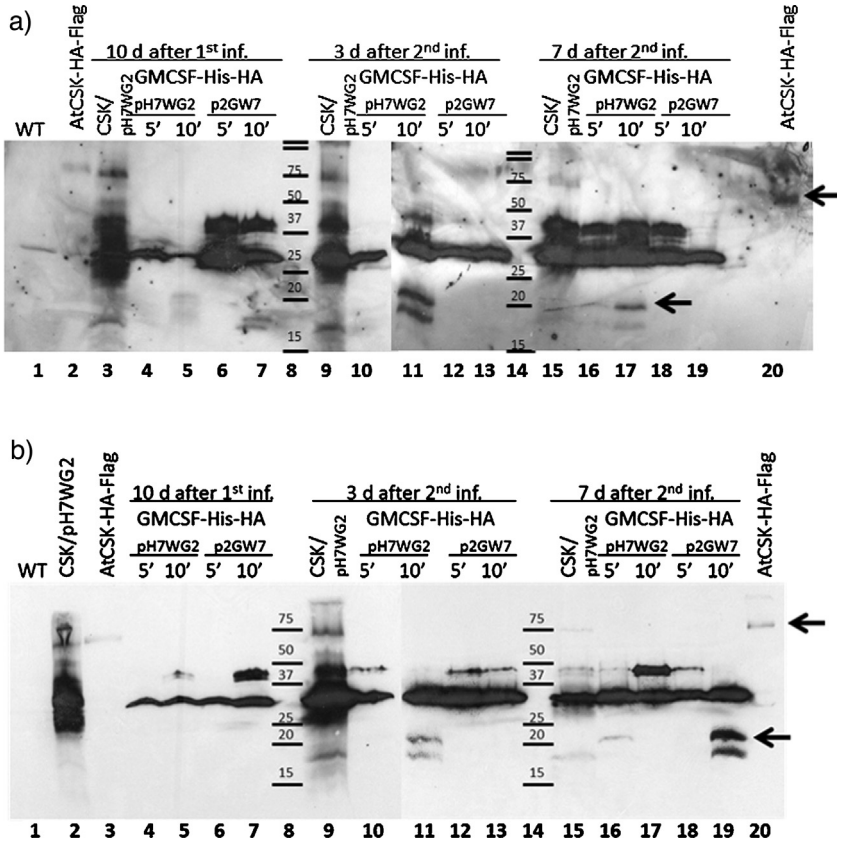

Fig. 3. Immuno-blot using anti-HA antibodies of recombinant hGM-CSF-His-HA in transformed tobacco leaves. Two *N. tabacum* cultivars: DH-17 (a) and DH-27 (b) have been transformed. 15 μg of total soluble protein (corresponding to 30 mg leaf tissue) was loaded per lane. Lane 1: Non-infiltrated leaves. Lanes 2–3: AtCSK-HA-Flag expressed in *A. thaliana*, as a marker of expected protein size and CSK/pH7WG2 from test expression, as controls. Lanes 4–7: GMCSF in two different expression vectors, pH7WG2 and p2GW7, after 5–10 min. of vacuum infiltration, samples collected 10 days after 1st infiltration. Lanes 9–13: The same samples collected 3 days after 2nd infiltration. Lanes 15–19: The same samples collected 7 days after 2nd infiltration. Lane 20: AtCSK-HA-Flag expressed in *A. thaliana*, as a marker of expected size. Lanes 8 and 14: Protein standard. All leaves were infiltrated in the presence of Silwet L-77. Arrows indicate CSK-HA-Flag (calculated size 69.7 kDa) and GMCSF-His-HA (calculated size 17.9 kDa).

three months after the human gene was acquired. Our results demonstrate that *Agrobacterium*-mediated transient expression in tobacco leaves is a suitable method for efficient and rapid expression of foreign protein in commercial Virginia tobacco cultivars DH-17 and DH-27. Due to the natural variability and health of infiltrated plants, GM-CSF expression was neither even nor optimal in all tested samples at the same time point after infiltration. Therefore, for the more accurate detection of the protein expression, whole leaves were harvested, grinded, and aliquots of the whole-leaf extract were used for protein detection. For the purpose of further optimization of uniformity and efficacy of the expression, leaves should be collected at multiple time-points to determine the peak of GM-CSF expression. One of the future tasks should be the assessment of tobacco plants in earlier developmental stages. Expression at these stages could be more evenly distributed among plants/leaves. Further, the apparent size of the expressed recombinant hGM-CSF, as judged from our SDS gel system, is about 20 kDa. It has been reported that the molecular size of GM-CSF can vary; due to its N-terminal glycosylation (calculated size is 17.9 kDa) [25]. It is conceivable to assume that the observed size difference originates form this type of post-translational modification, which might occur in our plant-based expression system. Also, numerous plant secondary metabolites may interact, in an unspecific manner, with overexpressed protein [26]. Such interactions may occur during and after homogenization, but before separation has taken place. According to the literature, six-week old tobacco plants represent the optimal leaf material that balances the need for biomass yield, protein accumulation, space requirements, and the ease of agroinfiltration [23]. In future experiments, we will determine conditions for maximal expression of GM-CSF in transiently transgenic tobacco plants. As we are dealing with the heterologous expression system, for further optimization and attainment of maximal yield of GM-CSF protein, plant codon optimization might be used.

Tobacco plants produce high levels of toxic alkaloids and phenolic substances that are released during grinding and protein extraction and can interfere with downstream processing [27,28]. To avoid some of these problems, we used the plants in pre-nicotinic phase, up to 40 days old, that are suitable for agroinfiltration. For infiltration $O.D._{600} = 0.12$ of *A. tumefaciens* strain was used, since it was shown to be the optimal density of transformed bacteria that enables maximum transgene delivery, without the risks of causing tissue necrosis and senescence [29,30].

Efficiency of agroinfiltration is highly dependent on the ability of bacteria to penetrate leaf tissues [27]. Prolonged infiltration times increase the possibility for agrobacteria to pass the epidermis barrier and infect the neighbouring cells. While standardizing for maximum expression level at different infiltration times, we have established that after 5–10 min. of vacuum infiltration the highest protein expression was achieved. Therefore, 10-min. vacuum exposure and infiltration were used in subsequent experimental setups. Additionally, this time interval was shown to be optimal for thorough infiltration without significant leaf damage (Fig. 2). Prolonged exposure to vacuum could decrease the temperature of bacterial suspension that may additionally reduce the expression [31]. Infiltration was tested in the presence or absence of the surfactant Silwet L-77, since this surfactant decreases leaf surface tension, thus enabling better infiltration of

bacterial suspension. We conclude that the inclusion of Silwet L-77 is essential for successful infiltration and final recombinant protein accumulation (Fig. 1).

5. Conclusions

The aim of this study was to prove the concept that the human protein could be expressed in industrial tobacco cultivars, by using relatively low cost straightforward procedure that could be used for rapid, large-scale production of various other proteins of non-plant origin. Further, commercial tobacco cultivars are widely accessible and easy to grow and maintain. As transient expression systems do not generate stable transgenic lines, there are no potential risks for food/feed contamination or undesired transgene outflow from genetically modified plants to environment. This greatly reduces the regulatory process and possible conflicting public acceptance issues for this technology [23].

Conflict of interest

The authors declare no conflict of interest.

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