Biotechnology Reports 33 (2022) e00699

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Research Article

Transient recombinant expression of highly immunogenic *CagA, VacA* and *NapA* in *Nicotiana benthamiana*

Rambod Barzigar [a,*], Nanjundappa Haraprasad [b], Basaralu Yadurappa Sathish Kumar [a,c], Mohammad Javad Mehran, please put this name as a second author and others like before. [a], Bashasab Fakrudin [d]

[a] JSS Research Foundation, SJCE Technical Campus, Mysore 570006 India
[b] JSS Science and Technology University, SJCE, Technical Campus, Mysore 570006 India
[c] Postgraduate Department of Biotechnology, JSS College, Ooty Road, Mysore 570025 India
[d] Department of Biotechnology and Crop Improvement, College of Horticulture, University of Horticulture Sciences Campus, GKVK Post, Bengaluru 560065 India

ARTICLE INFO

Keywords:
Helicobacter pylori
CagA
VacA
NapA
Nicotiana bethamiana
qRT-PCR
ELISA

ABSTRACT

Interest in the plant-based transient production of recombinant immunogenic antigens has tremendously progressed because plants are cost-effective, easily selectable, free of mammalian contamination, and support complex post-translational modifications. *Nicotiana benthamiana* is a convenient system for transient expression of recombinant antigens. The present study documented a platform for rapid production of *Helicobacter pylori CagA, VacA* and *NapA* antigens three days (first harvest, FH) and six days (second harvest, SH) after agro-infiltration using a syringe. In this study, *CagA, VacA* and *NapA* antigen genes from *Helicobacter pylori* were cloned into the binary vector pBI121 and transformed into *Nicotiana benthamiana* by the *Agrobacterium*-mediated process. Leaves of four to five weeks old *Nicotiana benthamiana* plants were agroinfiltrated with EHA105 subtype of *Agrobacterium tumefaciens* strain containing cloned *CagA* (pBI121-CagA), *VacA* (pBI121-VacA) and *NapA* (pBI121-NapA) constructs. The transient expression and accumulation of the recombinant genes containing *CagA, VacA* and *NapA* expression cassettes were confirmed using qRT-PCR by comparing the relative expression at FH and SH post-infiltration with the non-infiltrated (control) samples and using ELISA at 1/5 and 1/10 dilution ratios. The qRT-PCR findings showed that Agrobacterium-mediated syringe infiltration of leaves of four to five weeks old *Nicotiana benthamiana* plants produced significantly higher transcript levels of *CagA* (about 8-fold and 7-fold), *VacA* (38-fold and 24-fold) and *NapA* (7-fold and 5-fold) genes at FH and SH compared to the control sample. Besides, the maximum amount of *CagA, VacA* and *NapA* antigens were detected at the FH stage compared to the SH stage, when the antibody concentrations of the agro-infiltrated leaf extracts containing these recombinant antigens were diluted in a 1/5 ratio. This study has developed evidence to support that recombinant *CagA, VacA* and *NapA* can be transiently produced in *Nicotiana benthamiana* plants.

1. Introduction

Helicobacter pylori bacterium is the most prevalent cause of chronic gastroduodenal ulcer, infecting nearly 50% of human beings, globally [1]. The occurrence of the infection in adults varies as per geographical regions, where more than three-fourth of the populations in Latin American, Asian, and African regions are infected by this bacterium [2]. This chronic bacterial infection may develop into a gastroduodenal ulcer in nearly 10% – 15% of the infected individuals and into severe disease outcomes like gastric cancer in nearly 1% – 3% of the infected individuals [3]. Except for gastric cancer, all gastroduodenal infections of this bacterium can be cured by immunization [4]. An effective vaccine could overcome the challenges of treatment related to *Helicobacter pylori* infection and thereby prevent the linked complications.

In-silico identification of membrane proteins for outer membrane localization in 53 *Helicobacter pylori* immunogenic strains reported 826 conserved proteins as potential vaccines and therapeutic targets [5]. Highly immunogenic subtypes encode 128–145 KDa the cytotoxin-associated gene (*CagA*) antigen, which resides within the variable region Cag pathogenicity island (PAI). The CagPAI contains

* Corresponding author at: JSS Research Foundation, SJCE Technical Campus, Mysore 570006, Karnataka, India, ORCID ID:0000-0002-9494-2853.
E-mail address: rambod2500@yahoo.com (R. Barzigar).

https://doi.org/10.1016/j.btre.2021.e00699
Received 30 July 2021; Received in revised form 22 December 2021; Accepted 28 December 2021
Available online 29 December 2021

genes that synthesize the type 4 (T4) secretion system (SS), where the chromosomal transfer of *CagA* into the epithelial lining of the host cells is implicit [6]. *CagA* triggers the NF-κB transcription factor in epithelial cells of the gastric gland and induces antiapoptotic gene expression [7]. Besides, other genes relevant for colonization and damage to the gastric mucosa include vacuolating cytotoxin A (*VacA*) and neutrophil-activating gene A (*NapA*) antigens. *VacA* encodes an 88 kDa multimeric secretory protein that reduces immune responses and induces immune tolerance in host cells [8]. The immunogenic antigens, namely *CagA* and *VacA* resisted the bacterial *Helicobacter pylori* infection in mice [9]. *NapA* encodes a 150 kDa quaternary protein, which has a structure similar to that of bacterioferritin (Bfr) and represents a major virulence factor in response against *Helicobacter pylori* antigens in humans [10]. Clinical trial testing revealed *Helicobacter pylori NapA*as a potential vaccine antigen [11]. There is a link between the severity of this bacterium-induced infection outcome and the neutrophil activation by the *NapA* gene [12]. Immunization with the *Lactococcus lactis NapA* strain in mice demonstrated slightly lower levels of *Helicobacter pylori* infection in contrast to individual administration of *Lactococcus lactis* vector [13].

Identification of bacterial infection and evaluation of immunization efficiency plays an important role in controlling severe infections and eventual outcomes. Immunization with vaccines containing *Helicobacter pylori CagA, VacA* and *NapA* is recognized to be one of the most suitable ways to resist infection against this bacterium [14]. The presence of these antigens is known to facilitate the qualitative and quantitative understanding of the process associated with the immune response against *Helicobacter pylori* infection [15]. Previous studies have reported that immunization with recombinantly expressed *CagA, VacA* and *NapA* confers protection in humans and animal models [16]. With modern advances in plant genetic engineering technology, transient production of recombinant proteins in plants is receiving increasing attention in the medical field, as it serves as a novel alternative to the conventional cell-based expression systems like mammalian cultures, *E. coli*, insects, whole animals, yeast, or filamentous fungi, as each system has its strength and limitations for expression of foreign genes [17, 18, 19]. Generally, for the transient production of foreign genes in plant-based systems, agro-infiltration is the most preferred method [20, 21].

Although several studies reported the application of plant-based expression systems for recombinant protein production, identifying the most suitable plant model is crucial for therapeutic purposes [22]. The selection of the host plant is equally important because recombinant protein production is influenced by the distinctive biological attributes of each species [23]. For instance, the organized head structure of *Lactuca sativa* (lettuce) allows for more effective transient transformation than the unorganized leaves of *Nicotiana tabacum* (tobacco) [24]. Also, *Mucuna bracteata* leaves allow for a higher level of transient expression of recombinant proteins than *Nicotiana benthamiana* leaves [25]. However, the advantage of *Nicotiana* spp. lies in its ability to alleviate biosafety, environmental and ethical issues since it is an industrial and feed plant.

Nicotiana benthamiana is a suitable plant host for agro-infiltrated transient expression of recombinant proteins since it is well studied and facilitates high levels of heterologous gene expression [26]. Scholarly information regarding genetic modification in plants and particularly in tobacco to yield beneficial compounds is in plenty [27,28]. However, the utilization of other plant-based systems to improve the production of existing or new recombinant proteins for therapeutic purposes has not been well-reported in the existing literature [29]. Thus, this concept of plant-based recombinant protein production is gaining increasing scholarly attention [30]. An increased understanding of this concept in plants will help boost the acceptance of this approach and strengthen its therapeutic applications. The present study aimed to assess the production of recombinant *CagA, VacA* and *NapA* immunogenic antigens of *Helicobacter pylori* in *Nicotiana benthamiana* as an expression host. The study attempted to develop a transformation practice for *Nicotiana benthamiana* via a syringe-aided agro-infiltration method to transiently express *CagA, VacA* and *NapA* immunogenic genes on the third day of harvest. This would also be the first-ever information on the rapid expression of the recombinant *Helicobacter pylori CagA, VacA* and *NapA* antigens in *Nicotiana benthamiana*.

2. Methodology

2.1. Plant sample and culture environment

Nicotiana benthamiana seeds were surface disinfected with 100% ethanol for 1 min as mentioned by Clemente (2006), thoroughly cleaned with water followed by disinfection with 50% (v/v) clorox solution containing 3% sodium hypochlorite (NaOCl) for 15 min. Seeds were subsequently washed with sterile water thrice or up to five times. A total of 15 seeds were germinated in a propagation tray containing peat pellets and water, which were maintained at 25 ± 1 °C with 84% humidity under a 16 h and 8 h light-dark daily illumination. Two weeks after sowing, seedlings were transferred to potting soil in 10 cm^2 plastic pots (one seedling per pot) following a 16/8 h light-dark daily illumination which was maintained at 24–26 °C during the light period and 20–22 °C during the dark period at 225 μmol/m^2/s light intensity with 60% humidity. Following three weeks of transplantation at an optimal developmental stage where the plants had five completely true leaves without any noticeable flower buds, the young plant leaves were used for agro-infiltration (4–5 weeks old).

2.2. Cloning and sequencing of CagA, VacA and NapA genes

Primer pairs, each for *CagA* (5′-ATATCAGGATCCATGGATTTACTT-GATGAAAGGGGT-3′ and 5′-TATCGAGAGCTCTTATTTTTGAGCTT GTTGAGCCAATTGC-3′), *VacA* (5′-ATATCAGGATCCATGAATAAAACC CCAGATAAACCCGA-3′ and 5′-TATCGAGAGCTC TTAAGCGTTAGCC-CAAACATTGGTAG-3′) and *NapA* (5′- TATCTGGATCCATGAAAA-CATTTGAAATTCTAAAAC-3′ and 5′-ATACTGGATCCTTAAGCCAAAT GGGCTTGCAGCATC-3′) were designed based on the *Helicobacter pylori* gene sequence. The primer pairs were reported to generate DNA sequences of approximately 1700 bp (*CagA*), 2877 bp (*VacA*) and 434 bp (*NapA*). PCRs were performed in a reaction tube containing total genomic DNA (50 ng), PCR buffer (1X), dNTPs (0.2 mM), $MgCl_2$(3 mM), primers (0.1 μM each for forward and reverse pairs), Taq DNA polymerase (1 U) (Bangalore GeNei, India) and distilled water making up to a total volume of 25 μL for optimization of annealing conditions. The PCR amplification protocols were as follows: ***CagA*:** 10 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min with a final extension of 72 °C for 10 min; ***VacA*:** 5 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 3 min with a final extension of 72 °C for 7 min; ***NapA*:** 5 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min with a final extension of 72 °C for 7 min. PCR amplicons were detected on 1% agarose gel in TAE buffer (1X) by staining with ethidium bromide (EtBr; 1 μg/ml) and photographed using a UV transilluminator (UVITEC Cambridge, UK). Molecular weights of the PCR products were estimated by comparing them with 500 bp, 1000 bp and 100 bp DNA ladder for *CagA, VacA* and *NapA*, respectively. The PCR products (Fig. 1) were purified using a Genei clean purification system (Gel Extraction Kit, Genei), digested with *SacI/Bam*HI (for *CagA* and *VacA* genes) and with *Bam*HI (for *NapA* gene), and ligated into the pBI121vector (for *CagA* and *VacA* genes) and pRT100 (for *NapA* gene) digested with *SacI/Bam*HI and *Bam*HI, giving rise to pBI121-CagA, pBI121-VacA and pRT100-NapA clones. Positive clones were confirmed by insert release and DNA sequencing. Sequences were made available at NCBI (National Center for Biotechnology Information). Triplicates of PCR products and sequencing reactions were conducted to ensure no errors during the experiments.

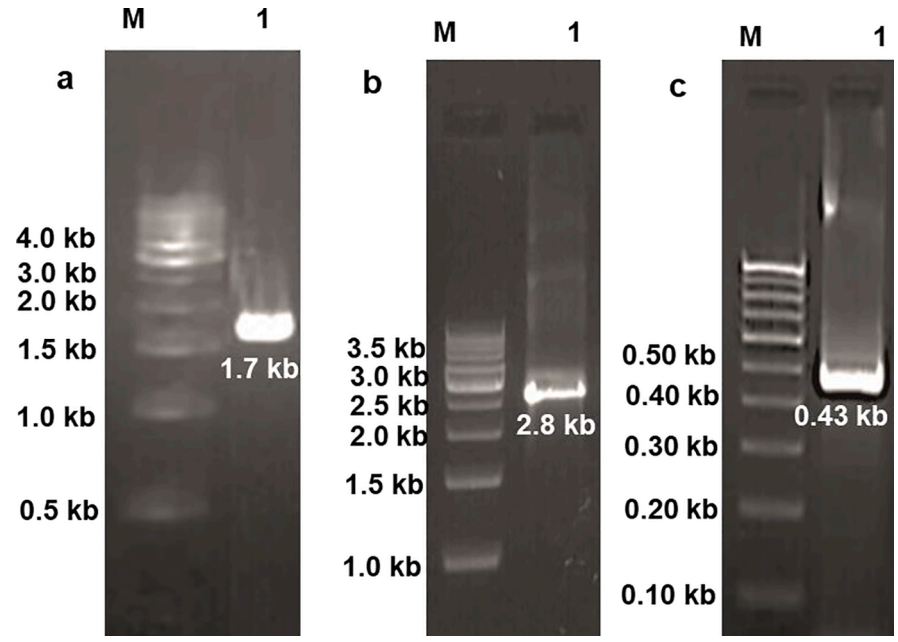

Fig. 1. PCR amplification of *CagA* (a) *VacA* (b) and *NapA* (c) genes . M: DNA ladder; lane 1: Desired PCR amplicons.

2.3. Construction of plant expression vector pBI121-CagA, pBI121-VacA and pBI121-NapA

The pBI121-CagA and pBI121-VacA clones were digested with restriction enzymes *SacI and Bam*HI to release *CagA* and *VacA* genes, and then the inserts were transferred into the multiple *SacI and Bam*HI *sites* of binary vector pBI121. *NapA* was inserted into pRT100 with *Bam*HI restriction digestion for cloning in the *E. coli* DH5α and then the pRT100-NapA clone was cut at specific sites and used for sub-cloning in pBI121 binary vector with *HindIII* restriction enzyme. The constructed plasmids (denominated as pBI121-CagA, pBI121-VacA and pBI121-NapA) (Fig. 2) were then exposed to transformation using DH5α competent *E. coli* cells. The expression cassette containing the *CagA, VacA* and *NapA* genes cloned into binary vector pBI121 with added *Bam*HI and *SacI* restriction sites were transformed into the competent cells of *Agrobacterium tumefaciens* strain EHA105.

2.4. Transformation of Agrobacterium tumefaciens with pBI121-CagA, pBI121-VacA and pBI121-NapA vectors

The EHA105 subtype of *Agrobacterium tumefaciens* was used to activate binary vectors pBI121-CagA, pBI121-VacA and pBI121-NapA for recombination by electroporation [31]. About, 50 µl of competent *Agrobacterium tumefaciens* subtype EHA105 was mixed separately with 1 µl of pBI121-CagA, pBI121-VacA and pBI121-NapA in three pre-chilled 2 mm electroporation cuvettes. The mixtures were pulsed for 5 ms at 2800 V, followed by immediate inoculation in 1 ml Luria-Bertani (LB) medium and kept under shaking condition at 28 °C for 2 h at 200 rpm. The cultures were inoculated in an LB plate containing kanamycin antibiotic and kept undisturbed at 28 °C for 72 h. Transformation of pBI121-CagA, pBI121-VacA and pBI121-NapA in *Agrobacterium tumefaciens* were verified by colony PCR using primers for *CagA, VacA* and *NapA*, respectively.

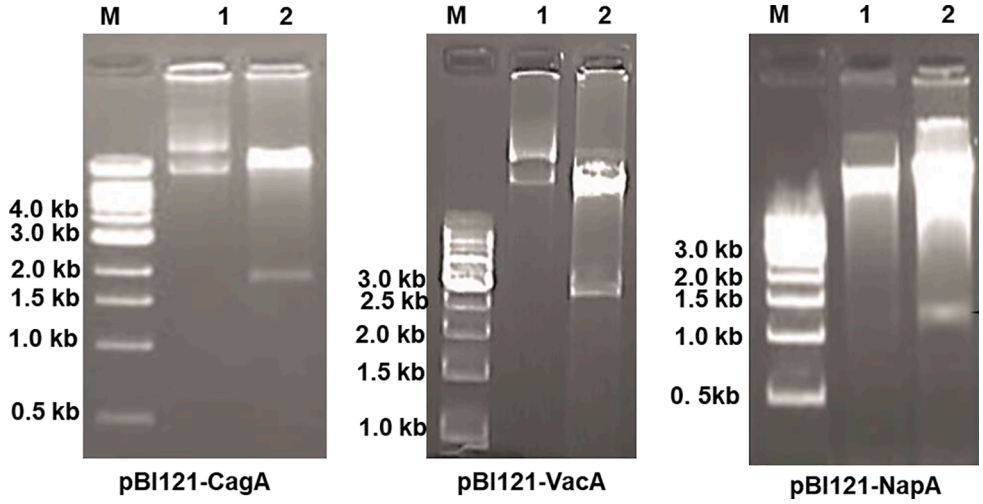

Fig. 2. Plasmid construction by restriction digestion of the gene constructs. M: DNA ladder; lane 1: undigested gene construct; lane 2: digested gene construct.

2.5. Agro-infiltration of Nicotiana benthamiana leaves

Recombinant pBI121-CagA, pBI121-VacA and pBI121-NapA were prepared for agro-infiltration-mediated transfer into *Nicotiana benthamiana* using syringe agro-infiltration [32]. Four representative leaves each three plants of *Nicotiana benthamiana* (biological replicates), twelve leaves each for FH and SH were agro-infiltrated using four injections with a syringe for each leaf as below:

1 Briefly, a colony of *Agrobacterium tumefaciens* EHA105 containing pBI121-CagA, pBI121-VacA and pBI121-NapA recombinant antigens was inoculated into 5 ml of sterile LB medium (tryptone, yeast extract and NaCl (10 g/L, 5 g/L and 10 g/L), pH 7.0) containing kanamycin (20 μg/mL).
2 Cultures were kept under shaking until the next day at 28 °C. Following this, 1 ml of the culture was inoculated again in 25 ml LB sterile broth and left undisturbed at 28 °C until the next day.
3 The mixture was centrifuged at 5000 rpm for 15 min and the bacteria pellet was dissolved in resuspension solution (10 mM MES pH 5.8, 150 μM acetosyringone and 10 mM $MgSO_4$; optical density (OD) at $A_{600} = 0.4$).
4 Cultures were then kept for two to four hours at room temperature (RT) with gentle shaking. Pots of *Nicotiana benthamiana* plantlets (approximately four to five weeks old; Fig. 3a) were placed on the bench at RT for two to three hours (or until the next day) before infiltration.
5 The leaf samples were infiltrated with each vector using a 5 ml blunt-tipped needleless plastic syringe by pressing the syringe on the underside of the leaves and exerting pressure in the reverse direction with a finger on the other side (Fig. 3b).
6 Four to five weeks old agro-injected leaf samples with *Agrobacterium tumefaciens* subtype EHA105 containing pBI121-CagA, pBI121-VacA and pBI121-NapA expression cassettes indicated intake of the *Agrobacterium tumefaciens* as noticed as a 'wetting' area on the leaves.
7 Following this, the plants were kept at 25 °C conditions with a 16 h and 8 h light-dark daily illumination and 55% – 65% humidity until harvest (duration of 3 – 6 days).
8 The successful infiltration of pBI121-CagA, pBI121-VacA and pBI121-NapA containing *Agrobacterium tumefaciens* strain EHA105 and expression of *CagA, VacA* and *NapA* genes were further confirmed by studying the expression of these genes.

Post-infiltration of *Nicotiana benthamiana* plantlets, leaves were harvested at 3 days (first harvest; FH) and 6 days (second harvest; SH), for in vivo gene expression, frozen in liquid nitrogen at −80 °C for extraction of RNA using the RNeasy Plant Mini Kit (Genei) and treated with DNase (Genei), as per the manufacturer's instruction. The yield and purity of RNA were assessed based on the $A_{260/280}$ and $A_{260/230}$ ratios in a NanoDrop (ND)−1000 spectrophotometer (Thermo Fisher Scientific, USA). First-strand synthesis of complementary DNA (cDNA) was done from RNA (1 μg) using M-MuLV RT-PCR Kit (Genei) and oligo $(dT)_{12–18}$ primers (Genei) in a 20 μL total volume as per the manufacturer's instruction.

2.6. Quantitative real-time PCR (qRT-PCR) amplification procedures and data analysis

Expression for each of the genes was assessed by quantitative real-time PCR (qRT-PCR) using SYBR Green master mix (Biorad, USA). The experiment was done in a reaction tube containing 1μL of cDNA and making the volume up to 20 μL. The reactions were conducted in an RT PCR cycler (Eppendorf, Germany) with the following conditions: 94 °C (pre-incubation) for 10 min, 35 cycles at 94 °C (denaturation) for 30 s, 55 °C (annealing) for 30 s, and 72 °C (elongation) for 30 s (signal acquiring stage). The primers used for qRT-PCR were designed based on mRNA sequences deposited in GenBank using the Primer3plus software (version 4.13). The sequences of the primer pairs and the length of amplicons obtained are provided in Table 1. In the present study, *β*-Actin (ACT) was used as a reference gene to normalize the variation in the concentration of template that may have occurred in each replicate. The forward and reverse sequences for the primer used for the *β*-Actin gene are also listed (Table 1). The expression of genes was measured using the ΔΔCT method [33]. For each gene, the Ct (threshold cycle for target amplification, that is, the corresponding FH and SH) value for the target

Fig. 3. Syringe-assisted agro-infiltration of *Nicotiana benthamiana*. (a) Four to five weeks old *Nicotiana benthamiana* plant. (b) Agro-infiltrated *Nicotiana benthamiana* leaflet.

Table 1
Primer pairs with amplicon lengths used for expression analysis.

Gene name	Forward DNA sequence (5′−3′)	Reverse DNA sequence (5′−3′)	Amplicon length (bp)
CagA	ATTGTATGCGGGCAATGGTG	CCCTTTCTCACCACCTGCTA	151
VacA	GCGGGTTATGCCAGACAAAT	GGTGTGCCTTCTGGAGAGAT	190
NapA	GCGGATGCGATCGTGTTATT	TCCTTTCAGCGAGATCGTCA	138
Actin	TCAGGTGTCCAGAGGTGTTGTA	ATGGTTGTGCCTCCTGAAAGTA	150

gene was deducted from the Ct value of ACT (ΔCT1). In control replicates (non-infiltrated plants), the Ct value for the target gene was deducted from the Ct value of ACT (ΔCT2). ΔΔCT was determined by deducting the ΔCT of control replicates from the ΔCT of all individual replications for each gene (ΔCT1-ΔCT2). Relative expression was determined using the $2^{-(\Delta\Delta CT)}$ equation. Relative quantification was carried out by constructing standard curves for each target gene and the endogenous control by making a 10-fold dilution series of an RNA sample to check whether the target genes and ACT had similar efficiency of amplification. The melt curve generated after the amplification showed that a single amplicon was produced in each reaction. For each gene, the ΔΔCT value for the control sample was 0 and the expression level was assigned an induction value of 1-fold after calculation ($2^{-\Delta\Delta CT} = 2^0 = 1$). Final expression values denote the fold change in relation to the control. The reactions were conducted in duplicate for each biological replicate.

2.7. Protein extraction

Initially, the agro-infiltrated *Nicotiana benthamiana* leaves were lyophilized and 500 mg of these leaves were crushed to a fine powder using liquid nitrogen. This was followed by suspension of 50 mg of the powder in extraction buffer (pH 7.2) containing 0.1 mM sodium phosphate, 2 mM dithiothreitol (DTT) and 3% polyvinylpyrrolidone (PVP). The solution was centrifuged at 15,000 rpm for 20 min at 4 °C and the supernatant was collected which was frozen in liquid nitrogen then kept at −80 °C for experimental analysis. Total protein samples were prepared from inoculated leaves with the use of 1:5 (w/v) coating buffer (0.1 M carbonate/bicarbonate buffer, pH 9.6). After standing for 20 min, the supernatant was recovered and quantified for total soluble protein using Bradford colorimetric assay. The concentration in every sample was adjusted to approximately 4.5–5 mg/mL.

2.8. ELISA

The leaves extracts of the FH containing antigens,*i.e., CagA, VacA*, and *NapA* were diluted at 1:5 and 1:10 in sodium bicarbonate (pH 9.8) and coated to the wells of two separate microtiter plates, by adding 100 μl of antigen solution. The plates were maintained at 37 °C for 2 hrs and the wells were washed thrice with washing buffer (phosphate-buffered saline-Tween 20 (PBS-T; 1 X) for 5 min. The non-specific antigen binding was prevented by adding 0.05% Tween 20 (200 μl) in 3% bovine serum albumin (BSA) in each well and maintained at 4 °C overnight. This was followed by washing and 100 μl of polyclonal antibody-biotin of *Helicobacter pylori* (1:500) (Thermo Fisher Scientific Inc., USA) was added to each well and the plates were maintained for 1 hr at RT. Post-incubation, wells were washed again with the washing solution and 100 μl per well of streptavidin-HRP conjugate (1:10,000) was added and incubation was continued at RT for 30 min. After washing the wells with the washing buffer, each well was treated with 100 μl of substrate O-phenylenediamine (OPD)/H_2O_2 and left undisturbed at RT for 30 min. The reaction was terminated using 1 N sulfuric acid (H_2SO_4) and the absorbance was read at 490 nm in an ELISA reader (Bio-Rad). Further, the experiment was also repeated for the leaves of SH done after 6 days. All experiments were performed in duplicates.

2.9. Statistical analysis

Experimental results represent the means values ± standard deviation (SD) of experiments, each replicated twice. SD of the means was calculated using Microsoft Excel and is represented as vertical bars in the figures. Paired sample *t*-test and analysis of variance (ANOVA) were conducted to analyze the statistical significance of differences in the level of expression between the target samples and the control. The statistical part was conducted using Statistical Package for the Social Sciences (SPSS) statistics software v24. Here, P-values < 0.05, < 0.01 and 0.001 were considered statistically significant, more statistically significant and extremely statistically significant.

3. Results

3.1. Analysis of cloned inserts

The *CagA* gene (1.7 kb) of *Helicobacter pylori* subtype 26,695 was amplified through PCR and cloned into pBI121vector. The clones containing the recombinant plasmid DNA were sequenced and compared with other *Helicobacter pylori* subtypes reported in NCBI (*Helicobacter pylori* straindRdM2addM2, *Helicobacter pylori* strain 26,695-dR, *Helicobacter pylori* strain 26,695-dRdM2, *Helicobacter pylori* strain dRdM1, *Helicobacter pylori* strain 26,695–1MET) by Basic Local Alignment Search Tool (BLAST; Supplementary file 1). The sequence analysis revealed that the *CagA* gene from the 26,695 subtypes had similarities with those of other subtypes, ranging from 92.6% for CP051292-ASHA-003 (USA) to 96.6% for CP026515-dRdM2addM2 (USA) at the DNA level (Supplementary Figures S1 and S2). Similarly, the *VacA* gene (2.87 kb) of *Helicobacter pylori* subtype 26,695 was amplified through PCR and cloned into pBI121vector. BLAST analysis of the sequences revealed a similarity ranging from 96% for CP002980-Puno120 (USA) to 99% for CP026515-dRdM2addM2 (USA) at the DNA level (Supplementary Figures S3 and S4). Further, the *NapA* gene (0.4 kb) of *Helicobacter pylori* subtype 26,695 was amplified through PCR and cloned into a pRT100 vector. The sequence analysis of the clones containing the recombinant plasmid DNA revealed similarities with other *Helicobacter pylori* subtypes ranging from 97.2% for AB045143-NAP gene for neutrophil-activating protein (Japan) to 100% for HQ831507-NapA gene (Iran) (Supplementary Figures S5 and S6).

3.2. Recombinant vector construction and transformation

To express *CagA, VacA and NapA* in *Nicotiana benthamiana* plants, recombinant binary vectors were constructed as pBI121-CagA, pBI121-VacA and pBI121-NapA (Fig. 4a). The transformants of *Agrobacterium tumefaciens* subtype EHA105 verified by colony PCR technique revealed a successful amplification of the *CagA, VacA* and *NapA* genes with *CagA, VacA* and *NapA* gene-specific primers. Electrophoresis of amplified product indicated an efficient intake of engineered pBI121-CagA, pBI121-VacA and pBI121-NapA constructs (Fig. 4b), which was further confirmed by agro-infiltration in *Nicotiana benthamiana* leaflets and screening for the gene product.

3.3. Transient CagA, VacA and NapA expression post-infiltration

First, the expression of the recombinant genes, *CagA* (pBI121-CagA),

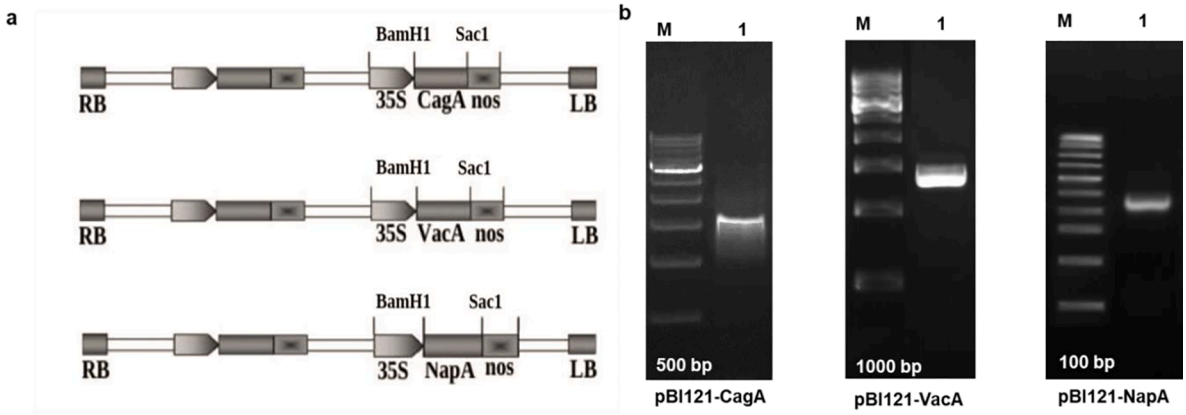

Fig. 4. Presence of engineered pBI121-CagA, pBI121-VacA and pBI121-NapA constructs. (a) t-DNA sections of the pBI121-CagA, pBI121-VacA and pBI121-NapA vectors; LB: left border and RB: right border of t-DNA; nos: nopaline synthase terminator from Agrobacterium; 35S: promoter of the cauliflower mosaic virus RNA. (b) Colony PCR of *Agrobacterium tumefaciens* containing pBI121-CagA, pBI121-VacA and pBI121-NapA plasmids; M: DNA ladder and lane 1: Desired PCR amplicons.

VacA (pBI121-VacA) and *NapA* (pBI121-NapA) after agro-infiltration was compared with the control (non-infiltrated) sample. These three recombinant genes followed a similar pattern of expression, where the transcript levels of the pBI121-CagA gene at FH and SH were significantly higher by 8-fold and 7-fold to that of the non-infiltrated plants (P-values < 0.05 and < 0.001; Fig. 5). Similarly, the transcript levels of the *VacA* gene at FH and SH were upregulated by about 38-fold and 24-fold post-infiltration compared to the control sample. These changes were significant at P-values < 0.05 and < 0.001. Furthermore, the transcript levels of the *NapA* gene at FH and SH (upregulated by about 7-fold and 5-fold) post-infiltration were significantly higher compared to the control sample ($P < 0.01$; Fig. 5). For *CagA, VacA* and *NapA* genes, there is a significant difference of 1 fold, more than 10 fold and 2 fold between FH and SH, respectively. For all three genes, the average relative expression at the FH was significantly higher compared to the SH. The transcript level of the *VacA* gene was observed to be significantly highest at FH followed by *CagA* and *NapA*.

3.4. ELISA analysis

The spectrophotometric analysis of the antibody concentrations of the diluted samples was plotted on a graph (Fig. 6). It was observed that the maximum amount of *CagA, VacA* and *NapA* antigens were detected in 1/5 sample dilution after the FH, being significant at $P < 0.001$. No antigens were detected in the control sample. The mean OD of the samples expressing the recombinant *CagA* antigens in 1/5 dilution after first and SH was approximately more than 4-–5-folds greater than that of the negative control; more than 2- – 6-folds compared to negative control for *VacA* and more than 3- – 4-folds greater than that of the negative control for *NapA*. These changes were statistically significant (P-values < 0.05 and < 0.001).

4. Discussion

The use of transient plant expression platforms offers some discrete advantages over other platforms and has been considered a promising alternative for addressing various infectious diseases, by producing recombinant antigens within a few weeks [34, 35]. The exploitation of plants over mammalian cell lines and other transgenic systems like yeast or bacteria for the rapid expression of therapeutic proteins against several diseases has gained importance owing to economical, practical and safe factors [36]. Syringe-assisted agro-infiltration is a vital transformation technique widely used in plant genetic engineering for the rapid expression of recombinant antigens for immunization and therapeutic purposes [37, 38]. To increase transient levels of expression, researchers have looked for improving the process, such as utilizing expression hosts that are vulnerable to pathogenesis and support the accumulation of a large quantity of recombinant antigens. Besides, this

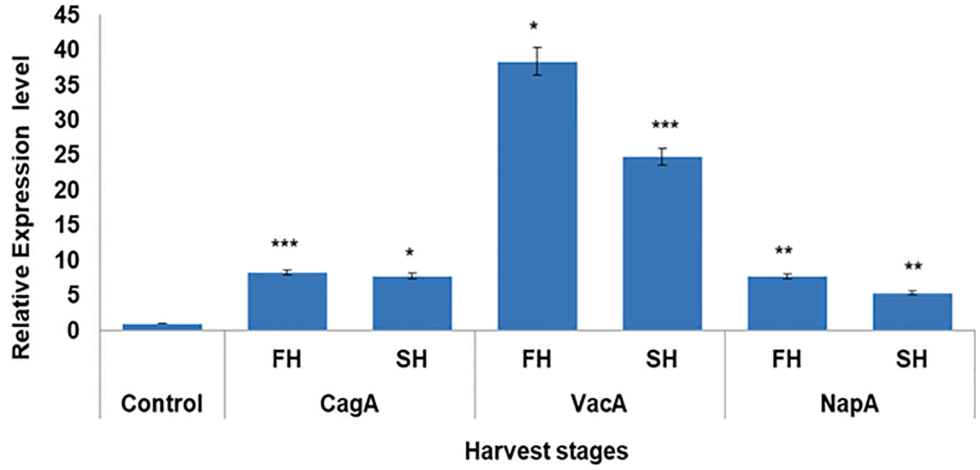

Fig. 5. Relative gene expression of the pBI121-CagA, pBI121-VacA and pBI121-NapA constructs in agro-infiltrated and non-infiltrated (control) *Nicotiana bethamiana* leaves at first and second harvest post-infiltration; FH: first harvest; SH: second harvest; y-axis: fold change analyzed by qRT-PCR; x-axis: harvest stages; the bars indicate the mean ± SD of the experiments, statistical significance was represented by single, double and triple asterisks ($P < 0.05$, < 0.01 and < 0.001).

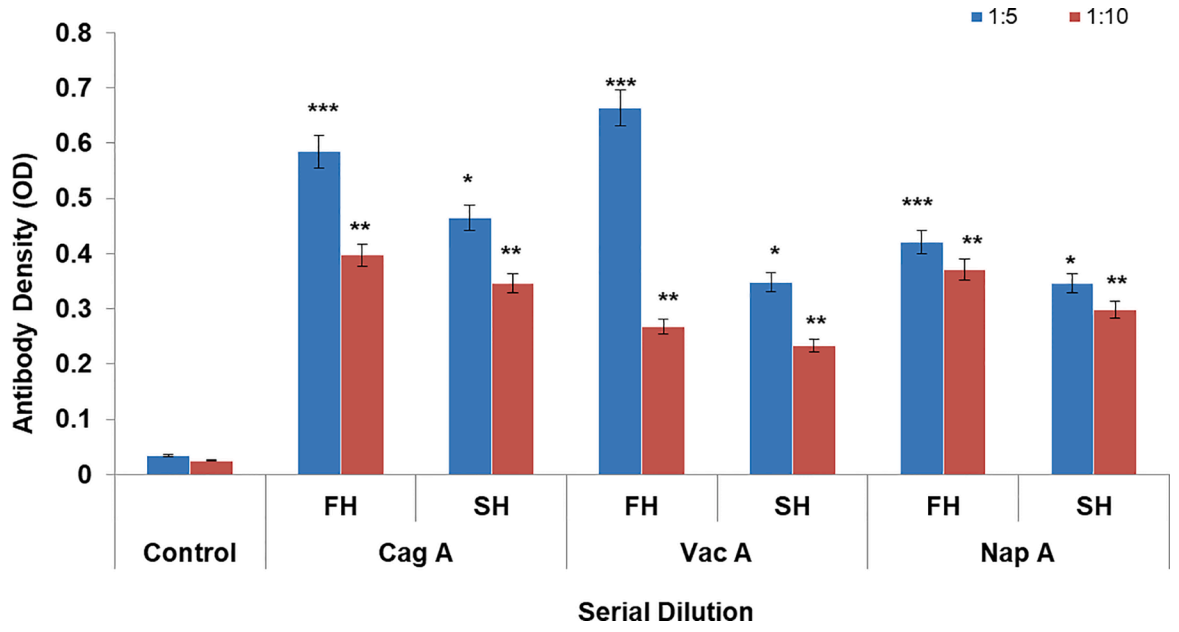

Fig. 6. ELISA assay with respect to 1/5 and 1/10 dilution ratios of recombinant *CagA, VacA* and *NapA* in agro-infiltrated and control *Nicotiana bethamiana* leaves after first and second harvests. FH: first harvest; SH: second harvest; OD: optical density; y-axis: antibody density; x-axis: serial dilution ratios; the bars indicate the mean ± SD of the experiments, statistical significance was represented by single, double and triple asterisks ($P < 0.05$, < 0.01 and < 0.001). Expression of highly immunogenic *CagA, VacA* and *NapA* antigens of *Helicobacter pylori* recombinantly in *Nicotiana benthamiana*.

method is experimentally versatile since it requires a minimal quantity of samples to penetrate *Agrobacterium* suspensions that may harbor diverse constructs on diverse sections of a leaf, allowing several experiments to be performed on one leaf. This was concurrent with the successful attempt to syringe infiltrate leaf samples of *Nicotiana benthamiana* in this study, where during the application of pressure with extreme caution, the infiltrated area expanded beyond the syringe nozzle part, indicating no barriers to the infiltration method through the mesophyll cells [39]. This could be attributed to the structure of the leaf epidermis [40]. Similar results for *Nicotiana benthamiana* leaves were reported [41, 42]. The present experiment on *Agrobacterium*-mediated needleless syringe infiltration of *Nicotiana benthamiana* leaves is promising as denoted by the presence of wetting areas. Thus, the present study used syringe agro-infiltration in *Nicotiana benthamiana* leaves with the pBI121 vector system [43, 44] for rapid expression of recombinant *Helicobacter pylori CagA, VacA* and *NapA* genes.

In the present study, *CagA, VacA* and *NapA* genes were successfully cloned from *Helicobacter pylori* subtype 26,695 and these have been expressed as immunogenic antigens in *Nicotiana benthamiana* plants. A similar agro-infiltration-assisted method was established to transiently express GUS (*β*-glucuronidase) in *Nicotiana benthamiana* using the pEAQ-HT vector system to improve agro-infiltration efficiency for the accumulation of higher levels of protein [42]. Vaccination has been established to be a promising way to alleviate severe disease outcomes due to *Helicobacter pylori* infection. With the advances of genetic modification techniques, the recombinant *CagA, VacA* and *NapA* antigens production for resisting *Helicobacter pylori* infection have been produced in animal systems [45, 46] and *E. coli* [47, 14].

Based on RT-PCR analysis, it seems that *CagA, VacA* and *NapA* genes at FH and SH post-agro-infiltration were rapidly expressed and a sufficient number of antigens were produced for detection (Fig. 5a). Successful recombinant expression of virulent genes of *Helicobacter pylori* was reported in humans [48, 49] and *E. coli* BL21 [14, 50]. The present study observed a considerable difference in the transcript levels of *CagA, VacA* and *NapA* genes between infiltrated plants at FH and SH and control plants. This could be due to the effect of syringe injection on the absorption of leaves since the pressure could not be controlled as a vacuum device. When using vacuum, for all samples the same pressure is used with determined time but when using syringe, the same pressure cannot be controlled with determined time.

Also, this accumulation might result from the activation of proteins that participate in the plant cell response to incubation time after infiltration [40]. However, transcript levels of the *CagA, VacA* and *NapA* genes at the FH were significantly higher than the SH. This might be associated with the silencing of an active endogenous RNA and protease-mediated hydrolysis of peptide bonds in plant cells. This finding is consistent with previous findings where transient expression of proteins decreased significantly after 2 – 3 days post-infiltration in rose petals [51], tobacco leaves [52] and onion epidermis [53]. However, it is different from a previous finding [39] where transient protein expression in grapevine significantly increased after 6 days post-infiltration, which could be due to the simultaneous expression of RNA silencing suppressor gene.

The plant extract transiently expressing *CagA, Vac*A and *NapA* were quantitated using ELISA. The production of recombinant *CagA, VacA* and *NapA* antigens in agro-infiltrated *Nicotiana benthamiana* leaves was detected in the FH and SH stages. The majority of the infiltrated regions expressed the recombinant antigens and the highest level of expression was detected at FH, that is, three days after infiltration. The highest quantity of recombinant antigens in 1/5 dilution ratio reached approximately 2.5, 4.5 and 1.5 folds higher than that of the SH for *CagA, VacA* and *NapA* genes, respectively. This higher peak in 1/5 dilution ratio could be due to fast protein synthesis in plant cells. High protein concentration in the diluted samples could lead to non-specific binding of the antibodies to antigens other than *CagA, VacA* and *NapA*; specifically, the dissolved peptides compete with the peptides bound to receptors for antibodies. Thus, a diluted concentration of antigens should be used to obtain an accurate result [54, 55]. However, the high transcript levels of *CagA, VacA* and *NapA* did not completely correspond to the protein yields, which could be because that the quantity of mRNA levels does not restrict translation and that post-translation modification can lead to the lysis of proteins [56]. A similar result was reported in a recent study [57], where the higher transcript levels of green fluorescent protein (GFP) and *Discosoma* sp. red fluorescent protein (DsRed) transiently expressed in tobacco BY-2 plant cell packs did not necessarily correspond to the protein yield. Thus, the present findings demonstrated that

significantly high-level of *Helicobacter pylori CagA, VacA* and *NapA* recombinant antigens were expressed in *Nicotiana benthamiana*, suggesting the suitability of the host as a significant platform for recombinant antigens production and that an expression platform from plants may be a promising method to develop *Helicobacter pylori* vaccines. Whether the *CagA, VacA and NapA* recombinant antigens are safe and immunogenic when tested and evaluated in animal models needs to be examined.

5. Conclusion

A platform for rapid expression of genes through agro-infiltration is a suitable alternative to the laborious process of generating stable transgenic lines for producing recombinant immunogenic antigens in plants. This study has successfully developed and demonstrated a reproducible transformation protocol for rapid expression of *Helicobacter pylori* recombinant *CagA, VacA* and *NapA* antigens in *Nicotiana benthamiana*. This is the first study of recombinant expression *CagA, VacA* and *NapA* gene of *Helicobacter pylori* in *Nicotiana benthamiana* via syringe-assisted *Agrobacterium* infiltration. A syringe infiltration of a four to five weeks old *Nicotiana benthamiana* plant with *Agrobacterium tumefaciens* subtype EHA105 was optimal to produce the highest expression of *CagA, VacA* and *NapA* on third day post- infiltration in leaf.

6. Author contributions

Rambod Barzigar & Mohammad Javad Mehran conceived of and designed the study. Rambod Barzigar & Mohammad Javad Mehran performed experiments. Rambod Barzigar & Mohammad Javad Mehran & Nanjundappa Haraprasad, BasaraluYadurappa Sathish Kumar & Bashasab Fakrudin analyzed data. Rambod Barzigar wrote the manuscript. All authors reviewed the manuscript. Corresponding by Rambod Barzigar.

7. Availability of data and material

All the data and supplementary materials are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare that there are no competing interests.

Acknowledgments

The authors thank the Postgraduate Department of Biotechnology, JSS College, Ooty Road, Mysore −570025 and Genei Laboratories Private Limited – Biotechnology Company, Peenya, Bangalore and Department of Biotechnology and Crop Improvement, College of Horticulture, University of Horticulture Sciences Campus, GKVK Post, Bengaluru 560065 for their support.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2021.e00699.

References

[1] J.K. Hooi, W.Y. Lai, W.K. Ng, M.M. Suen, F.E. Underwood, D. Tanyingoh, S.C. Ng, Global prevalence of Helicobacter pylori infection: systematic review and meta-analysis, Gastroenterology 153 (2017) 420–429, https://doi.org/10.1053/j.gastro.2017.04.022.

[2] C. Porras, J. Nodora, R. Sexton, C. Ferreccio, S. Jimenez, R.L. Dominguez, R. Herrero, Epidemiology of Helicobacter pylori infection in six Latin American countries (SWOG Trial S0701), Cancer Causes Control 24 (2013) 209–215, https://doi.org/10.1007/s10552-012-0117-5.

[3] R.M. PeekJr, J.E. Crabtree, Helicobacter infection and gastric neoplasia, J. Pathol. 208 (2006) 233–248, https://doi.org/10.1002/path.1868.

[4] P. Malfertheiner, F. Megraud, C.A. O'morain, J.P. Gisbert, E.J. Kuipers, A.T. Axon, E.M. El-Omar, Management of Helicobacter pylori infection—the Maastricht V/Florence consensus report, Gut 66 (2017) 6–30, https://doi.org/10.1136/gutjnl-2016-312288.

[5] C. Pasala, C.S.R. Chilamakuri, S.K. Katari, R.M. Nalamolu, A.R. Bitla, A. Umamaheswari, An in silico study: novel targets for potential drug and vaccine design against drug resistant H. pylori, Microb. Pathog. 122 (2018) 156–161, https://doi.org/10.1016/j.micpath.2018.05.037.

[6] F. Fahimi, M.R. Tohidkia, M. Fouladi, R. Aghabeygi, N. Samadi, Y. Omidi, Pleiotropic cytotoxicity of VacA toxin in host cells and its impact on immunotherapy, BioImpacts 7 (2017) 59–71, https://doi.org/10.15171/bi.2017.08.

[7] A. Gall, R.G. Gaudet, S.D. Gray-Owen, N.R. Salama, TIFA signaling in gastric epithelial cells initiates the cag type 4 secretion system-dependent innate immune response to Helicobacter pylori infection, MBio 8 (2017), https://doi.org/10.1128/mBio.01168-17 e01168-17.

[8] M.Bauer Altobelli, K. Velez, T.L. Cover, A. Müller, Helicobacter pylori VacA targets myeloid cells in the gastric lamina propria to promote peripherally induced regulatory T-cell differentiation and persistent infection, MBio 10 (2019) 1–13, https://doi.org/10.1128/mBio.00261-19.

[9] L.E. Wroblewski, R.M. Peek, K.T. Wilson, Helicobacter pylori and gastric cancer: factors that modulate disease risk, Clin.Microbiol.Rev 23 (2010) 713–739, https://doi.org/10.1128/CMR.00011-10.

[10] A. Amedei, A. Cappon, G. Codolo, A. Cabrelle, A. Polenghi, M. Benagiano, E. Tasca, A. Azzurri, M.M. D'Elios, G. Del Prete, M. De Bernard, The neutrophil-activating protein of Helicobacter pylori promotes Th1 immune responses, J. Clin. Investig. 116 (2006) 1092–1101, https://doi.org/10.1172/JCI27177.

[11] P. Malfertheiner, M. Selgrad, T. Wex, B. Romi, E. Borgogni, F. Spensieri, L. Zedda, P. Ruggiero, L. Pancotto, S. Censini, E. Palla, Efficacy, immunogenicity, and safety of a parenteral vaccine against Helicobacter pylori in healthy volunteers challenged with a Cag-positive strain: a randomised, placebo-controlled phase 1/2 study, Lancet. Gastroenterol. Hepatol. 3 (2018) 698–707, https://doi.org/10.1016/S2468-1253(18)30125-0.

[12] G. Wang, Y. Hong, A. Olczak, S.E. Maier, R.J. Maier, Dual roles of Helicobacter pylori NapA in inducing and combating oxidative stress, Infect. Immun 74 (2006) 6839–6846, https://doi.org/10.1128/IAI.00991-06.

[13] X. Peng, R. Zhang, G. Duan, C. Wang, N. Sun, L. Zhang, S. Chen, Q. Fan, Y. Xi, Production and delivery of Helicobacter pylori NapA in Lactococcuslactis and its protective efficacy and immune modulatory activity, Sci. Rep. 8 (2018) 1–12, https://doi.org/10.1038/s41598-018-24879-x.

[14] A.S. Moghaddam, S. Mansouri, A. Neshani, F. Firoozeh, A. Matinpur, A. Khaledi, M. Ghazalibina, Construction, cloning, and expression of caga recombinant protein of Helicobacter pylori, Avicenna J. Med. Biotechnol. 12 (2020) 135–138. PMID: 32431799.

[15] N.A. Sheikh, Delivery systems for molecular vaccination, Curr.Opin. Mol. Ther. 2 (2000) 37–54. PMID: 11249651.

[16] P. Malfertheiner, V. Schultze, B. Rosenkranz, S.H. Kaufmann, T. Ulrichs, D. Novicki, F. Norelli, M. Contorni, S. Peppoloni, D. Berti, D. Tornese, Safety and immunogenicity of an intramuscular Helicobacter pylori vaccine in noninfected volunteers: a phase I study, Gastroenterology 135 (2008) 787–795, https://doi.org/10.1053/j.gastro.2008.05.054.

[17] J.K.C. Ma, P. Christou, R. Chikwamba, H. Haydon, M. Paul, M.P. Ferrer, S. Ramalingam, E. Rech, E. Rybicki, A. Wigdorowitz, D.C. Yang, Realising the value of plant molecular pharming to benefit the poor in developing countries and emerging economies, Plant Biotechnol. J. 11 (2013) 1029–1033, https://doi.org/10.1111/pbi.12127.

[18] J.K. Ma, P.M. Drake, P. Christou, The production of recombinant pharmaceutical proteins in plants, Nat. Rev. Genet. 4 (2003) 794–805, https://doi.org/10.1038/nrg1177.

[19] R.A. Rader, Expression systems for process and product improvement, Bioprocess. Int. 6 (2008) 4–9.

[20] E.L. Rech, G.R. Vianna, F.J. Aragao, High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants, Nat. Protoc. 3 (2008) 410–418, https://doi.org/10.1038/nprot.2008.9.

[21] E.L. Rech, Seeds, recombinant DNA and biodiversity, Seed Sci. Res. 22 (2012), https://doi.org/10.1017/S0960258511000341. S36-S34.

[22] A. Jamal, K. Ko, H.S. Kim, Y.K. Choo, H. Joung, K. Ko, Role of genetic factors and environmental conditions in recombinant protein production for molecular farming, Biotechnol. Adv 27 (2009) 914–923, https://doi.org/10.1016/j.biotechadv.2009.07.004.

[23] E. Rech, G. Vianna, A. Murad, N. Cunha, C. Lacorte, A. Araujo, M. Brigido, W. Michael, A. Fontes, O. Barry, S. Andrew, C. Otavia, Recombinant proteins in plants, BMC Proceed 8 (2014) 1–2, https://doi.org/10.1186/1753-6561-8-S4-O1.

[24] V. Negrouk, G. Eisner, H.I. Lee, K. Han, D. Taylor, H.C. Wong, Highly efficient transient expression of functional recombinant antibodies in lettuce, Plant Sci 169 (2005) 433–438, https://doi.org/10.1016/j.plantsci.2005.03.031.

[25] N. Abd-Aziz, B.C. Tan, N.A. Rejab, R.Y. Othman, N. Khalid, A new plant expression system for producing pharmaceutical proteins, Mol. Biotechnol. 62 (2020) 240–251, https://doi.org/10.1007/s12033-020-00242-2.

[26] M.M. Goodin, D. Zaitlin, R.A. Naidu, S.A. Lommel, Nicotiana benthamiana: its history and future as a model for plant-pathogen interactions, Mol. Plant Microbe Interact. 21 (2008) 1015–1026, https://doi.org/10.1094/MPMI-21-8-1015.

[27] I.K. Vasil, A history of plant biotechnology: from the cell theory of Schleiden and Schwann to biotech crops, Plant cell Rep 27 (2008) 1423–1440, https://doi.org/10.1007/s00299-008-0571-4.

[28] J. Zhang, Y. Li, J. Jin, Q. Chen, X. Xie, Z. Wang, Z. Luo, J. Yang, Recent advances in tobacco chloroplast genetic engineering, YancaoKeji 50 (2017) 88–98, https://doi.org/10.16135/j.issn1002-0861.2017.0035.
[29] M.J.B. Burnett, A.C. Burnett, Therapeutic recombinant protein production in plants: challenges and opportunities, Plants People Planet 2 (2019) 1–12, https://doi.org/10.1002/ppp3.10073.
[30] Y. Tekoah, A. Shulman, T. Kizhner, I. Ruderfer, L. Fux, Y. Nataf, D. Bartfeld, T. Ariel, S. Gingis–Velitski, U. Hanania, Y. Shaaltiel, Large-scale production of pharmaceutical proteins in plant cell culture-the protalix experience, Plant Biotechnol.J 13 (2015) 1199–1208, https://doi.org/10.1111/pbi.12428.
[31] W.J. Dower, J.F. Miller, C.W. Ragsdale, High-efficiency transformation of E. coli by high voltage electroporation, Nucleic Acids Res 16 (1988) 6127, https://doi.org/10.1093/nar/16.13.6127.
[32] R.H. Japelaghi, R. Haddad, M. Valizadeh, E. DoraniUliaie, M. JalaliJavaran, High-efficiency Agrobacterium-mediated transformation of tobacco (Nicotiana tabacum), J. Plant. Mol. Breed. 6 (2018) 38–50, https://doi.org/10.22058/JPMB.2019.92266.1170.
[33] L.Overbergh A.Giulietti, D. Valckx, B. Decallonne, R. Bouillon, C. Mathieu, An overview of real-time quantitative PCR: applications to quantify cytokine gene expression, Methods 25 (2001) 386–401, https://doi.org/10.1006/meth.2001.1261.
[34] J. Yao, Y. Weng, A. Dickey, K.Y. Wang, Plants as factories for human pharmaceuticals: applications and challenges, Int. J. Mol. Sci. 16 (2015) 28549–28565, https://doi.org/10.3390/ijms161226122.
[35] E.P. Rybicki, Plant-made vaccines and reagents for the one health initiative, Hum. Vaccines Immunother 13 (2017) 2912–2917, https://doi.org/10.1080/21645515.2017.1356497.
[36] B.M. Shanmugaraj, S. Ramalingam, Plant expression platform for the production of recombinant pharmaceutical proteins, Austin J. Biotechnol. Bioeng. 1 (2014) 2378–3036, 14, ISSN.
[37] N. Bechtold, D. Bouchez, Agrobacterium-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration, Gene transfer to plants, Springer 82 (1995) 19–23, https://doi.org/10.1007/978-3-642-79247-2_3.
[38] F. Sainsbury, G.P. Lomonossoff, Extremely high-level and rapid transient protein production in plants without the use of viral replication, Plant Physiol 148 (2008) 1212–1218, https://doi.org/10.1104/pp.108.126284.
[39] A. Ben-Amar, P. Cobanov, G. Buchholz, A. Mliki, G. Reustle, In planta agro-infiltration system for transient gene expression in grapevine (Vitis spp.), Acta Physiol.Plant 35 (2013) 3147–3156, https://doi.org/10.1007/s11738-013-1348-0.
[40] Q. Chen, H. Lai, J. Hurtado, J. Stahnke, K. Leuzinger, M. Dent, Agro-infiltration is an effective and scalable strategy of gene delivery for the production of pharmaceutical proteins, Adv.Tech.Biol. Med. 1 (2013) 1–21, https://doi.org/10.4172/atbm.1000103.
[41] H. Zhao, Z. Tan, X. Wen, Y. Wang, An improved syringe agro-infiltration protocol to enhance transformation efficiency by combinative use of 5-azacytidine, ascorbate acid and tween-20, Plants 6 (2017) 1–9, https://doi.org/10.3390/plants6010009.
[42] K. Norkunas, R. Harding, J. Dale, B. Dugdale, Improving agro-infiltration-based transient gene expression in Nicotiana benthamiana, Plant Met 14 (2018) 1–14, https://doi.org/10.1186/s13007-018-0343-2.
[43] P.F. Liu, Y. Wang, R.G. Ulrich, C.W. Simmons, J.S. VanderGheynst, R.L. Gallo, C. M. Huang, Leaf-encapsulated vaccines: agro-infiltration and transient expression of the antigen Staphylococcal endotoxin B in radish leaves, J. Immunol.Res. (2018) 1–9, https://doi.org/10.1155/2018/3710961.
[44] A.A. Tyurin, A.V. Suhorukova, K.V. Kabardaeva, I.V. Goldenkova-Pavlova, Transient gene expression is an effective experimental tool for the research into the fine mechanisms of plant gene function: advantages, limitations, and solutions, Plants 9 (2020) 1–19, https://doi.org/10.3390/plants9091187.
[45] M. Stein, P. Ruggiero, R. Rappuoli, F. Bagnoli, Helicobacter pylori CagA: from pathogenic mechanisms to its use as an anti-cancer vaccine, Front.Immunol 4 (2013) 328, https://doi.org/10.3389/fimmu.2013.00328.
[46] A. Nami, V.B. Azzawri, H. Ucar, Acar, Recombinant Helicobacter pylori CagA protein induces endoplasmic reticulum stress and autophagy in human cells, BioRxiv (2020) 1–20, https://doi.org/10.1101/2020.06.26.174615.
[47] V. Farjadi, H. Abtahi, M.R. Zolfaghari, S. Soufian, L. Hasanzadeh, Production of recombinant CagA protein of Helicobacter pylori, J. Arak Uni. Med. Sci 16 (2013) 35–44, https://doi.org/10.5578/mb.7642.
[48] F. Avilés-Jiménez, A. Reyes-Leon, E. Nieto-Patlán, L.M. Hansen, J. Burgueño, I. P. Ramos, M. Camorlinga-Ponce, H. Bermúdez, J.M. Blancas, L. Cabrera, R. M. Ribas-Aparicio, J.V. Solnick, J. Torres-López, In vivo expression of Helicobacter pylori virulence genes in patients with gastritis, ulcer, and gastric cancer, Infect. Immun. 80 (2012) 594–601, https://doi.org/10.1128/IAI.05845-11.
[49] A. Szkaradkiewicz, T.M. Karpiński, K. Linke, P. Majewski, D. Rożkiewicz, O. Goślińska-Kuźniarek, Expression of cagA, virB/D complex and/or vacA genes in Helicobacter pylori strains originating from patients with gastric diseases, PLoS ONE 11 (2016) e0148936, 10.1371/journal.pone.0148936.
[50] L.Z. Yuan, X. Shi, D. Tang, S.P. Zheng, Z.M. Xiao, F. Wang, Construction and preservation of a stable and highly expressed recombinant Helicobacter pylori vacuolating cytotoxin A with apoptotic activity, BMC Microbiol 21 (2021) 1–15, https://doi.org/10.1186/s12866-021-02262-7.
[51] A. Yasmin, T. Debener, Transient gene expression in rose petals via Agrobacterium infiltration, Plant Cell Tiss. Org. Cul. 102 (2010) 245–250, https://doi.org/10.5578/mb.7642.
[52] L. Zheng, G. Liu, X. Meng, Y. Li, Y. Wang, A versatile Agrobacterium-mediated transient gene expression system for herbaceous plants and trees, Biochem.Genet 50 (2012) 761–769, https://doi.org/10.1007/s10528-012-9518-0.
[53] K. Xu, X. Huang, M. Wu, Y. Wang, Y. Chang, K. Liu, J. Zhang, Y. Zhang, F. Zhang, L. Yi, T. Li, A rapid, highly efficient and economical method of Agrobacterium-mediated in planta transient transformation in living onion epidermis, PLoS ONE 9 (2014), https://doi.org/10.1371/journal.pone.0083556 e83556.
[54] O. Braitbard, H. Glickstein, J. Bishara-Shieban, U. Pace, W.D. Stein, Competition between bound and free peptides in an ELISA-based procedure that assays peptides derived from protein digests, Proteome Sci 4 (2006) 1–14, https://doi.org/10.1186/1477-5956-4-12.
[55] J. Wojciak, J. Fleming, Competition binding between a monoclonal antibody and serum albumin for several lysophosphatidic acid isoforms in solution, FASEB J 29 (2015), https://doi.org/10.1194/jlr.D068866, 886-18.
[56] J. Jansing, J.F. Buyel, The correlation between DsRed mRNA levels and transient dsred protein expression in plants depends on leaf age and the 5′ untranslated region, Biotechnol. J. 14 (2019), 1800075, https://doi.org/10.1002/biot.201800075.
[57] Z. Poborilova, H. Plchova, N. Cerovska, C.J. Gunter, I.I. Hitzeroth, E.P. Rybicki, T. Moravec, Transient protein expression in tobacco BY-2 plant cell packs using single and multi-cassette replicating vectors, Plant Cell Rep. 39 (2020) 1115–1127, https://doi.org/10.1007/s00299-020-02544-w.