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Potential of plant chloroplast-based recombinant expression systems to express bacterial membrane proteins as vaccine candidates for *Salmonella* in poultry



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Zoonotic diseases pose significant public health risks, with *Salmonella* being a major pathogen responsible for gastroenteritis, often transmitted through contaminated poultry products. Effective control of *Salmonella* in poultry is critical, but current methods face limitations due to the stealthy nature of the bacteria, which can persist in asymptomatic birds and spread during slaughter and processing. In this study, we investigated the potential of *Nicotiana tabacum* chloroplasts to produce TonB-dependent outer membrane transporters (TBDTs), including CirA, FepA, FhuA and IroN from *Salmonella* Enteritidis. These membrane proteins are critical for bacterial iron uptake and represent promising vaccine targets. Transplastomic tobacco plants successfully produced full-length recombinant CirA, FepA, and IroN proteins. Immunization of mice with chloroplast extracts containing these proteins elicited significant antibody responses, with statistical significance observed for CirA and FepA ($p < 0.05$). Fluorescence microscopy demonstrated specific antibody binding to *Salmonella* cells, confirming immune recognition. These findings highlight the feasibility of using chloroplasts to produce complex bacterial membrane proteins and support the development of plant-based vaccines targeting iron acquisition systems to combat *Salmonella* infections in poultry.

Membrane proteins are highly valuable in the field of biopharmaceuticals^{1,2}. These proteins play essential roles in various biological processes and are often associated with disease mechanisms³. Targeting and modulating the activity of membrane proteins opens up possibilities for the development of more effective and selective drugs. Membrane proteins are also vital for cellular communication and signaling processes⁴. By targeting specific membrane receptors or ion channels, it becomes possible to regulate intracellular signaling cascades, presenting innovative opportunities for therapeutic interventions^{5–7}.

The extraction and purification of membrane proteins while preserving their native conformation pose significant challenges⁸. These proteins often require specific detergents or lipids to maintain solubility, but finding

the right composition that preserves both their structure and function remains a significant obstacle⁹. Overcoming these challenges necessitates the development of advanced purification techniques and innovative approaches to stabilize and fold membrane proteins effectively^{10,11}.

Expression of recombinant membrane proteins also presents substantial obstacles¹². One major challenge stems from the hydrophobic nature of these proteins, as they are embedded within lipid bilayers. This hydrophobicity often leads to protein aggregation, misfolding, and degradation during expression. Maintaining the native (or functional) conformation and stability of membrane proteins throughout the expression process proves to be a considerable hurdle¹¹. Additionally, overexpressing certain membrane proteins can be toxic, resulting in reduced cell viability and protein yields¹³.

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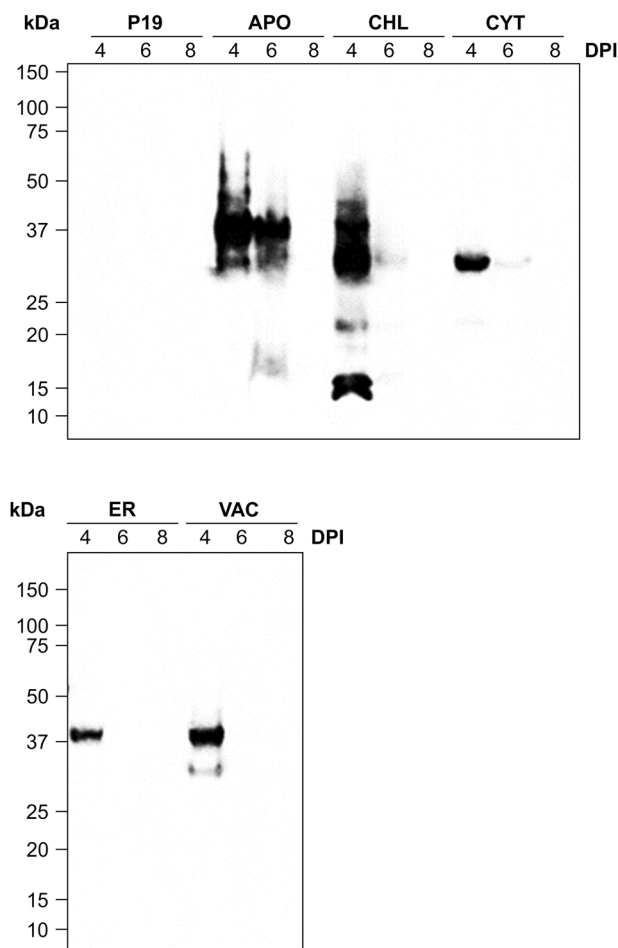


Fig. 1 | Transient expression of Fep A results in a truncated protein. FepA was transiently expressed in 5 different compartments of *N. benthamiana*. FepA was transiently expressed in 5 different compartments of *N. benthamiana*, including APO (apoplast), CHL (chloroplast), CYT (cytosol), ER (endoplasmic reticulum), and VAC (vacuole). Leaf tissue was collected at 4-, 6-, and 8-days post-infiltration (DPI). A volume of 20 μ l of crude extract was loaded into each well. An anti-HA antibody (1:5,000) was used to detect the protein. Transient expression of FepA resulted in a truncated protein which was degraded by 6 days post infiltration. The expected size of FepA is about 82 kDa. Negative controls (p19) consisted of plants infiltrated solely with the p19 expression vector.

The overexpression of these proteins disrupts normal cellular processes, membrane integrity, or ion homeostasis, making it challenging to achieve high expression levels¹⁴.

Membrane proteins frequently exhibit complex structures, comprising multiple transmembrane domains and intricate tertiary structures, accompanied by specific post-translational modifications. Consequently, recombinant expression systems must accurately reproduce these features¹⁵. Chloroplasts offer a promising alternative for expressing membrane proteins due to their endogenous lipid bilayer and chaperones, which facilitate native (or functional) protein folding¹⁶. Being of prokaryotic origin and possessing their own genetic material¹⁷, chloroplasts can effectively express membrane proteins, particularly those originating from bacteria¹⁸. Transplastomic plants are generated by introducing foreign genes into the chloroplast genome, resulting in the expression of recombinant proteins within the chloroplasts¹⁹. Transplastomic plants offer several advantages as expression systems for recombinant protein production²⁰. One key advantage lies in the high protein yield that can be achieved within chloroplasts, which is due to the lack of gene silencing and the high copy number (up to 10,000) of the chloroplast genome in every cell²¹.

Poultry products serve as a prominent reservoir of *Salmonella*, a pathogenic bacterium that poses a substantial risk of infection in humans. One approach to controlling *Salmonella* infection in the poultry industry is with vaccines^{22–27}. *Salmonella* relies on TonB-dependent outer membrane transporters (TBDTs) for iron acquisition, which is an essential process for its survival and virulence. Each TBDT specializes in transporting different iron-bound siderophores: FepA and IroN handle catecholate types²⁸, CirA facilitates the transport of ferric iron-catecholate complexes, and FhuA mediates ferrichrome (a hydroxamate siderophore) uptake^{29,30}. TBDTs hold potential as antigen candidates for vaccine development. However, studies show that disrupting individual TBDTs only partially affects iron uptake due to functional redundancy³¹. Therefore, targeting all four TBDTs in vaccine development aims to significantly hinder iron acquisition, thereby limiting *Salmonella*'s growth and survival.

In this study, transplastomic *Nicotiana tabacum* plants carrying *Salmonella cirA*, *fepA* and *iroN* genes were generated. The results obtained contribute to the development of plant-based systems for efficient production of recombinant membrane proteins and provide evidence supporting the potential use of chloroplasts derived from transplastomic plants as a viable platform for drug delivery.

Results

Transplastomic *Nicotiana tabacum* plants carrying *cirA*, *fepA*, *fhuA* and *iroN* genes

The *cirA*, *fepA*, *fhuA*, and *iroN* genes were transiently expressed and targeted to five distinct compartments of *Nicotiana benthamiana* including apoplast, chloroplast, cytosol, endoplasmic reticulum, and vacuole. The accumulation of recombinant proteins was quantified at 4-, 6-, and 8 days post-infiltration. The expected size of CirA, FepA, FhuA, and IroN are approximately 74 kDa, 82 kDa, 81 kDa, and 80 kDa, respectively. Western blots, specifically detecting the HA tag, revealed that, with the exception of the FepA construct, none of the target genes exhibited transient expression. The FepA construct resulted in the synthesis of a truncated protein (~37 kDa) which was detected on day 4 post infiltration (dpi) and was undetectable or barely detectable in the chloroplast, cytosol, ER, and vacuole by 6 dpi. It was detected on 6 dpi but not on 8 dpi in the apoplast (Fig. 1).

The lack of success with transient expression led to the implementation of a transplastomic expression approach. Transplastomic expression involves the integration of the target genes into the chloroplast genome, facilitating stable and high-level expression (Fig. 2). Transplastomic FhuA, IroN, FepA and CirA tobacco plants were generated. FepA and CirA plants exhibited high transformation frequencies of 8–12 independent transplastomic clones from the bombardment of five sterile-grown tobacco leaves. Homoplastomy of representative T0 plants was confirmed by Southern blot analysis (Fig. 3). The analysis revealed distinct band patterns corresponding to the expected sizes of *RsrII*-digested fragments. The wild-type *N. tabacum* plastome served as a control for expected band patterns without transgene integration, with an expected fragment size of 1.054 kbp. In contrast, the FepA transgenic plants exhibited a fragment size of 4.783 kbp. For CirA and IroN, *RsrII* digestion produced two distinct fragments: CirA transplastomic plants yielded fragments of 2.358 kbp and 2.193 kbp, while IroN transplastomic plants showed fragments of 3.506 kbp and 1.180 kbp. This is because there is an *RsrII* restriction site internal to both of these genes. These results confirm the integration of the *cirA*, *fepA*, and *iroN* transgenes into the chloroplast genome of the respective transplastomic plants, and demonstrate that the generated lines are homoplastomic due to the absence of the wild type product.

For the initial analysis of recombinant protein production in T0 clones transformed with *cirA*, *fepA*, *fhuA* and *iroN*, samples were taken from young, well-developed leaves of the same size (~30 cm long, ~third-fourth leaf from top), thus minimizing possible developmental variations in recombinant expression of each target gene among the clones. Equal amounts of extracted leaf tissue were analyzed by western blotting with an anti-HA antibody. In the extracts obtained from CirA, FepA and IroN plants, products of the expected sizes were detected (Fig. 4). However, with

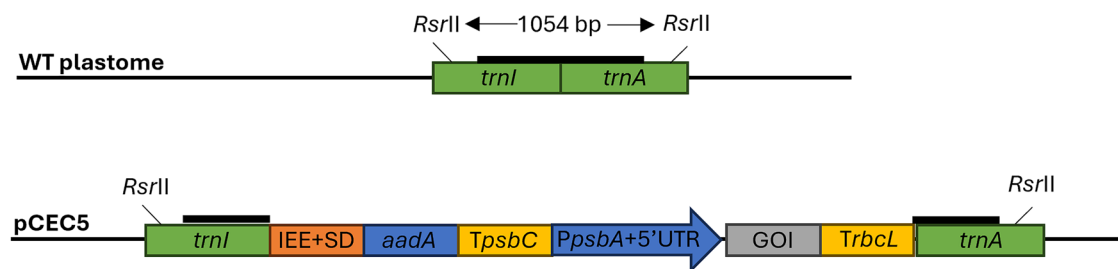


Fig. 2 | The wild-type *N. tabacum* plastome is presented alongside the modified plasmid vector CEC5. The wild-type (WT) *N. tabacum* plastome is depicted alongside the CEC5 (pCEC5), which is utilized for expressing target genes in wild-type *N. tabacum*. The sequences of target genes, including *CirA*, *FepA*, *FhuA*, and *IroN*, were cloned into the Gene of Interest (GOI) position of pCEC5, which features various cis-acting regulatory elements designed for integration into the transcriptionally active spacer region between the *trnI* and *trnA* genes of the tobacco

plastome. Key components of pCEC5 include the Intercistronic Expression Element (IEE) containing the Shine-Dalgarno sequence (SD), the *aadA* gene for antibiotic resistance, and 3' UTRs from the white poplar plastome (*TpsbC* and *TrbcL*), as well as the tobacco *psbA* gene promoter (*PpsbA*). Thick black lines indicate hybridization sites for probes used in Southern blot analyses. The WT plastome is illustrated with the expected sizes of *RsrII*-digested fragments.

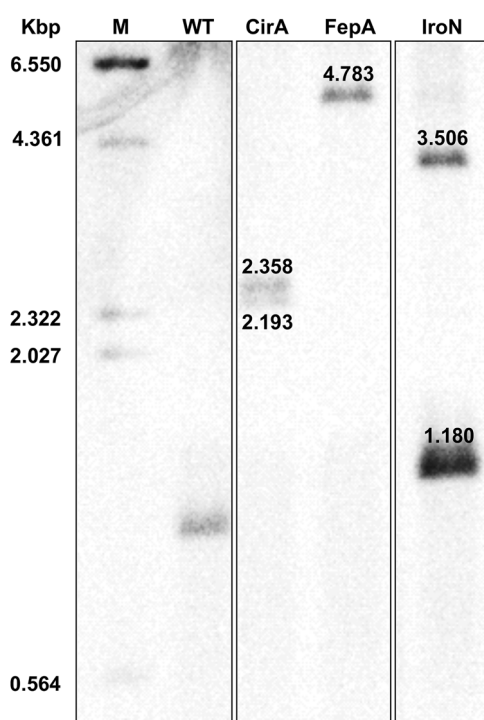


Fig. 3 | Southern blot analysis of *CirA*, *FepA*, and *IroN* transplastomic plants confirming transgene integration and homoplasmy in the transplastomic lines. Chloroplast DNA was digested with *RsrII* and separated by electrophoresis. Homoplasmy of the three representative *CirA*, *FepA*, and *IroN* transplastomic plants was confirmed by the absence of a WT band. In the case of *CirA* and *IroN*, *RsrII* cleaves at sites within the genes and their flanking sequences, resulting in two distinct fragments.

FhuA, only a faint and truncated product of about 37 kDa was detected, significantly smaller than the predicted molecular weight of 81 kDa (Fig. 4).

Recombinant expression of *CirA*, *FhuA*, *FepA*, and *IroN* affects transplastomic plant growth and phenotype

The sustainability of a plant-based, recombinant protein production system relies on a delicate balance between the plant's ability to generate abundant biomass and achieve sufficient levels of accumulated recombinant protein. Simply having a well-expressed gene and substantial protein production per fresh leaf weight is not enough if the plant exhibits stunted growth and yields minimal biomass. To delve into the growth rate and accumulation of recombinant protein in T1 plants derived from self-pollinated T0 transformant plants for each

target gene, T1 seeds were germinated simultaneously with wild-type control seeds. Within as little as two weeks post-germination, noticeable differences in growth rate and phenotype emerged between the T1 seedlings and the WT. These differences became more pronounced as the T1 plants matured.

By growing wild-type *N. tabacum* plants as controls alongside transplastomic plants, we observed that the production of *FepA* and *FhuA* had a detrimental impact on the growth rate and phenotype of the transplastomic plants (Fig. 5). *FhuA* caused severe growth retardation and resulted in bleached and light green leaves. *FhuA* T0 transplastomic plants were maintained in the greenhouse and after approximately 10 months, a small side stem produced flower buds. However, the seed pods were mostly empty, with very few seeds that were viable. *FepA* expression resulted in leaf distortion in T0 *N. tabacum* plants, however, these plants were still able to produce flower buds that successfully set seeds. The T1 progeny also exhibited the same leaf distortion phenotype as the T0 plants. Conversely, *CirA* and *IroN* did not significantly affect the growth and phenotype of the plants. *CirA* and *IroN* transplastomic plants were also able to produce flower buds that successfully set seeds.

FepA and *CirA* are embedded in the chloroplast envelope

FepA and *CirA* are membrane proteins and therefore are expected to embed within membrane structures. However, their specific localization within the chloroplast membranes could not be predicted prior to experimental investigation. To investigate the presence of *FepA* and *CirA* within the chloroplast's various sub-compartments, intact chloroplasts were separated from other organelles and cell debris via Percoll gradient centrifugation. The intact chloroplasts were then ruptured using osmotic shock and loaded on a sucrose gradient to separate the stroma, the envelope, and the thylakoid membrane. Fractions corresponding to the stroma, envelope, and thylakoids were collected from the sucrose gradient as described by Bouchnak et al. 2018³². Immunoblot detection of *FepA* and *CirA* indicated that both were primarily embedded in the envelope of the chloroplast in of transplastomic plants and were not found in the thylakoid membrane (Fig. 6). While our study confirms that *FepA* and *CirA* localize to the envelope, it does not distinguish between the inner and outer membranes that make up the chloroplast envelope.

Antibodies produced in mice immunized with transplastomic chloroplasts target *Salmonella* Enteritidis

Groups of six mice were immunized with chloroplasts purified from individual transplastomic plants (*CirA*, *FepA*, and *IroN*). Doses contained 30 µg of each recombinant protein or a combination of chloroplasts expressing and containing 10 µg of each of these three proteins. Chloroplasts isolated from wild-type plants served as the negative control. Subsequently, mouse sera were subjected to analysis through an indirect ELISA where plates were coated with *S. Enteritidis* to assess the presence of specific *Salmonella* antibodies in the collected sera samples.

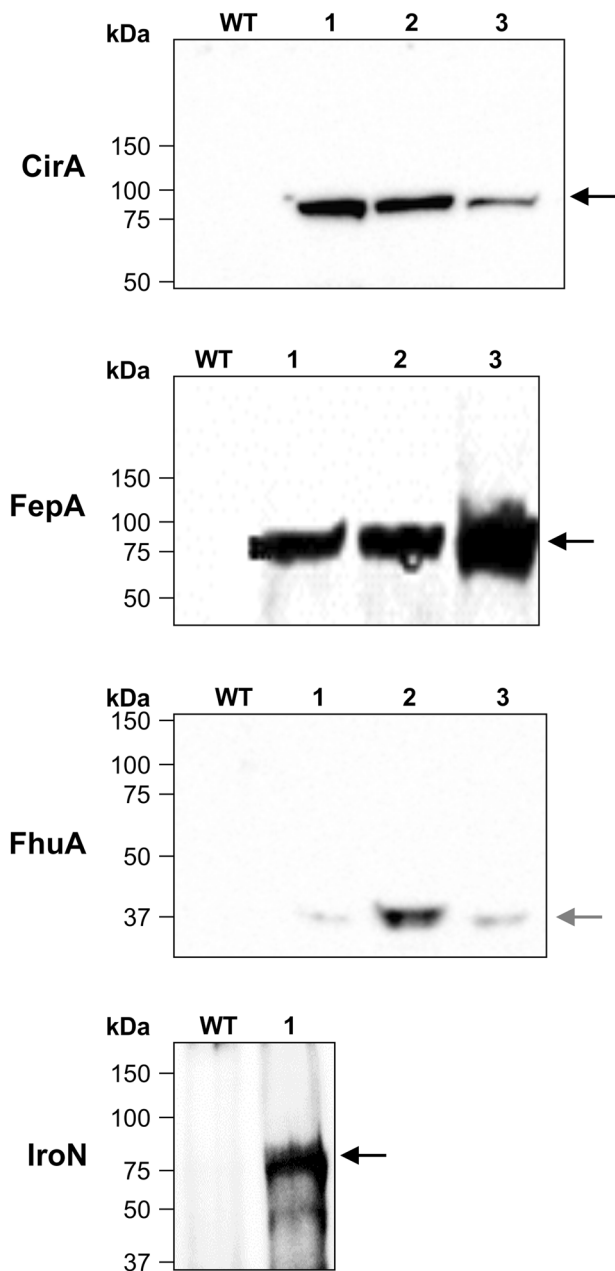


Fig. 4 | CirA, FepA, and IroN full length proteins are produced in transplastomic plants. A volume of 20 μ l of crude extract from independent transplastomic plants was loaded into each well. An anti HA antibody (1:5,000) was used to detect the protein. Protein extracted from a wild-type (WT) plant was used as negative control. In extracts from CirA, FepA, and IroN plants, full-length products of the expected sizes were detected. With FhuA, only a faint and truncated product (pointed with a red arrow) was detected. Black arrows indicate the expected size of a full-size protein. The expected size of CirA, FepA, FhuA, and IroN are about 74 kDa, 82 kDa, 81 kDa, and 80 kDa, respectively.

Statistically significant differences were identified between the pre-immune and final bleed sera for FepA ($p = 0.027$) and CirA ($p = 0.043$) treatments, as well as between the final bleeds of both FepA ($p = 0.022$) and CirA ($p = 0.031$) compared to the wild-type negative control (Fig. 7). However, no significant differences between the pre-immune and final bleed sera for wild-type, IroN, and the mix, or between the wild-type, IroN, and the mix were observed (Fig. 7). This variability in antibody responses suggests unique immunogenic reactions associated with CirA and FepA compared to IroN. An alternative explanation is that an increased dose of more than 30 μ g IroN may be required to stimulate a statistically significant

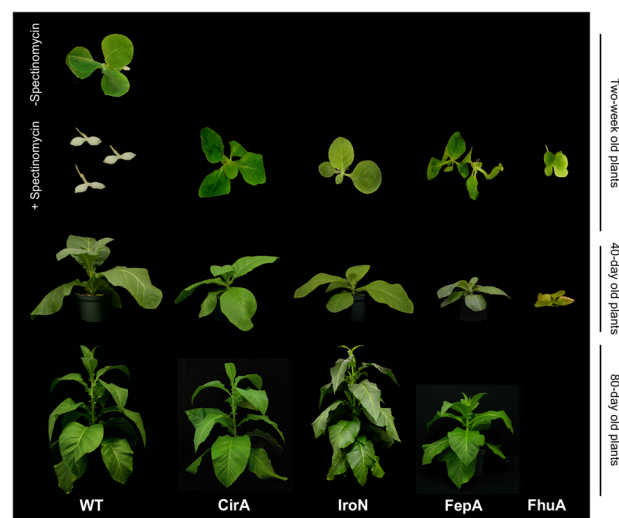


Fig. 5 | Synchronized germination and growth of wild-type *N. tabacum* and transplastomic plants. T1 transplastomic *N. tabacum* plants carrying CirA, FepA, FhuA, and IroN genes were grown synchronously with wild-type *N. tabacum* (WT). Compared to wild-type controls, FepA and FhuA caused a negative impact on growth rate and phenotype of the transplastomic plants. FepA caused leaf distortion and growth retardation, while FhuA caused severe growth retardation and leaf bleaching. CirA and IroN did not remarkably affect plant growth and phenotype. Wild-type *N. tabacum* did not grow on selective medium containing 500 mg/ml spectinomycin (second row).

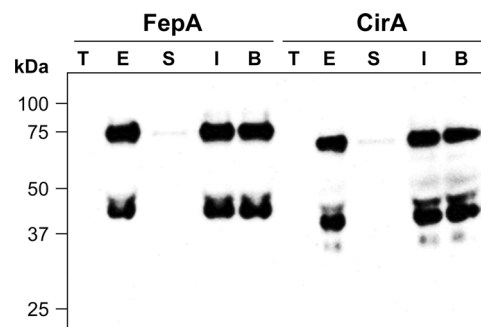


Fig. 6 | FepA and CirA are embedded in the chloroplast envelope. Intact chloroplasts were isolated from other organelles, such as broken chloroplasts, mitochondria, or nuclei and cell debris using continuous Percoll gradient. The intact chloroplasts were then ruptured using an osmotic shock and loaded on a discontinuous sucrose gradient to separate the stroma, the envelope, and the thylakoid membrane (Bouchnak et al., 2018). Recombinant proteins localized to each sub-compartment were detected via western blotting, using an anti-HA antibody (1:5,000). The expected size of CirA and FepA are about 74 kDa and 82 kDa, respectively. T: Thylakoid, E: Envelope, S: Stroma, I: Intact Chloroplasts, B: Broken Chloroplasts.

immune response. The lack of statistically significant differences between the mix and WT control, in comparison to individual immunizations with FepA and CirA, can be attributed to the lower concentration of CirA and FepA in the mix group. The mix contained only a third of the amount of each protein (10 μ g) compared to the individual immunizations (30 μ g). This reduced concentration likely resulted in a decreased magnitude of the antibody response relative to their individual immunizations.

To visualize antibody binding to *S. Enteritidis*, bacterial cells were incubated with sera from immunized mice and visualized using a secondary fluorescent antibody. Bacteria were visualized by staining with DAPI. The confocal images showed consistent co-localization of Alexa Fluor® 594 signal in *Salmonella* treated with sera from mice immunized with

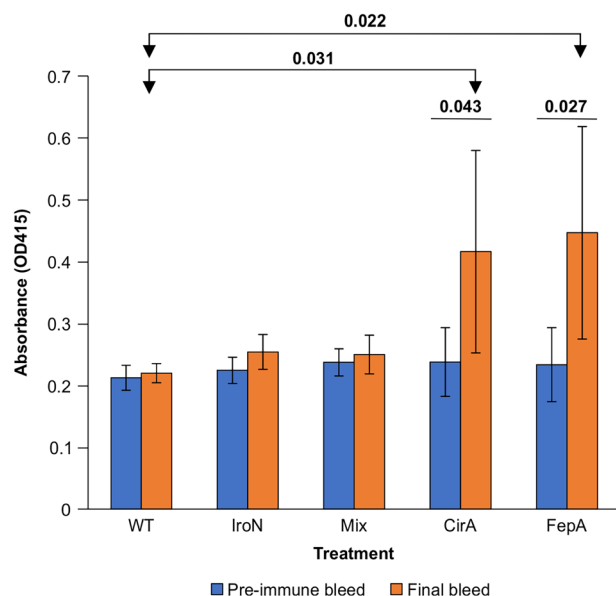


Fig. 7 | Mice immunized with chloroplast extracts from FepA and CirA transplastomic plants exhibited statistically significant antibody responses compared to the wild-type control group and their respective pre-immune bleed samples.

Antibody responses were assessed after immunizing mice ($n = 6$ per group) with chloroplasts isolated from each of the CirA, FepA, or IroN transplastomic plants, or a combination of chloroplasts isolated from CirA, FepA, and IroN transplastomic plants. Chloroplasts isolated from wild-type plants (WT) were used as a control. The graph displays the mean and standard deviation from independent experiments (6 mice per group). Mouse sera samples collected during the final bleed were diluted to 8000-fold and tested for antibodies against *S. Enteritidis*. The y-axis shows absorbance readings at 415 nm. p-values are presented on the figure.

transplastomic chloroplasts expressing all three TBDTs and the mix of TBDTs (Fig. 8). Sera from mice immunized with wild-type chloroplasts were used as a control to ensure that the signal we observed was specific to the TBDTs in transplastomic plants. No binding was observed in the control treatment, indicating that the immune responses are specific to the *Salmonella* TBDTs.

Discussion

The surface of *Salmonella* bacteria features TonB-dependent outer membrane transporters (TBDTs), which are crucial for iron acquisition—an essential process for bacterial survival and virulence^{33,34}. In the host-pathogen tug-of-war over iron, host cells restrict iron availability to suppress bacterial growth, while pathogens like *Salmonella* deploy siderophores to capture iron from host reservoirs³⁵.

Siderophores are small, high-affinity iron-chelating molecules that are classified into catecholate, hydroxamate, α -hydroxycarboxylate, and mixed types^{36,37}. Iron-regulated TBDTs transport iron-loaded siderophores across the bacterial outer membrane, a process dependent on the TonB protein and the proton motive force³⁵.

Due to their crucial role in iron acquisition, TBDTs represent promising candidates for vaccine development³⁸. Given the redundancy of TBDTs and their diverse roles in iron uptake, it is plausible that blocking only specific group of TBDTs might inadequately hinder the overall iron acquisition essential for *Salmonella*'s growth and survival³¹. Therefore, this study strategically targeted four transporters, CirA, FepA, FhuA, and IroN, as antigens for vaccine development. FepA and IroN facilitate the transport of catecholate-type siderophores, specifically enterobactin and its glucosylated derivative, salmochelin²⁸, respectively, across the outer membrane. CirA mediates the transport of various ferric iron-catecholate complexes, including monomers, dimers, and linear trimers of 2,3-dihydroxybenzoylserine, as well as colicins and microcins²⁹. Additionally, FhuA is involved in the uptake of ferric iron bound to the hydroxamate siderophore ferrichrome³⁰.

Initially, the FepA, FhuA, and IroN genes were transiently expressed in different compartments of *N. benthamiana*. However, only FepA showed detectable expression, and even then, the protein was truncated and degraded by six days post-infiltration. The lack of accumulation for the other proteins may be attributed to their mislocalization or entrapment within membrane systems during trafficking, either during import into the chloroplast or transit through the secretory pathway. Membrane proteins are rarely found freely in the cytosol due to their hydrophobic transmembrane domains, which require integration into lipid bilayers for stability and function^{39,40}. Their failure to accumulate in the cytosol likely reflects degradation due to misfolding or lack of appropriate membrane anchoring⁴¹. Considering the bacterial origin of chloroplasts and their specialized machinery for expressing and folding complex proteins^{42–44}, we explored the feasibility of expressing TonB-dependent transporters (TBDTs) via transplastomic transformation. Chloroplasts offer a unique expression environment, including isolation from cytosolic proteases, a prokaryotic-like translation system, and membrane-rich architecture conducive to proper folding of membrane proteins^{45,46}. Moreover, chloroplasts support high-level protein expression due to several intrinsic advantages: each cell contains multiple chloroplasts, each with hundreds to thousands of genome copies, and plastid genomes are not subject to gene silencing mechanisms that commonly affect nuclear transgenes^{45,47,48}. These features collectively enabled successful expression of CirA, FepA and IroN in transplastomic lines. By embedding within the chloroplast envelope, CirA and FepA likely benefited from the surrounding lipid bilayers and protein complexes that contributed to their proper folding and stable accumulation. These findings highlight the superiority of plastid-based systems for producing structurally complex membrane proteins.

FhuA caused severe growth retardation, suggesting potential toxicity of the full-size protein or challenges related to high transcription levels, resulting in the accumulation of a truncated protein. This utilization of chloroplast energy and machinery could compromise photosynthetic functions and energy provision to plant cells, impacting their survival. Similarly, to drive HPV L1 expression in chloroplasts, Legen et al. (2023)³⁹ established tobacco lines that express two variants of the HPV L1 protein, including the HPV18 L1 protein and an HPV16B Enterotoxin::L1 fusion protein, under the control of the target site of the PPR protein CHLORORESPIRATORY REDUCTION2 (CRR2). Their results showed that the homoplasmic line carrying the HPV16B Enterotoxin::L1 fusion protein exhibited severe growth retardation and pigment loss, suggesting that the fusion protein was toxic to the chloroplasts⁴⁹.

Plants are increasingly recognized for their potential as oral delivery systems due to several advantages including cost-effective production, scalability, and the ability to induce mucosal immunity^{50–52}. In a toxicology study, Srinivasan et al. (2021) established the first lettuce transplastomic lines expressing coagulation factor IX (FIX), whose deficiency causes haemophilia B, fused with the transmucosal carrier CTB (cholera toxin subunit B) without antibiotic resistance genes⁵³. Oral administration of CTB-FIX to Sprague Dawley rats and Beagle dogs showed no adverse effects or toxicity⁵³. In addition, the recent FDA approval of a plant-based oral immunotherapy drug for inducing tolerance to peanut allergy marks a significant milestone, paving the way for the evaluation and regulatory approval of orally delivered, plant-produced products⁵⁴. However, the plant-made recombinant protein industry is largely based on *Nicotiana* species owing to their well-established genetic engineering protocols and high biomass yield^{55–58}.

Most recombinant proteins produced in *Nicotiana* species are intended for human therapeutic applications, where purification of the final product is a critical step requiring rigorous downstream processing to ensure dosage accuracy and safety^{59–61}. In contrast, our objective focuses on animal health, specifically oral administration in poultry, where whole-cell delivery systems offer distinct advantages. The findings from Fragoso et al. (2017)³⁸ offer compelling evidence to support the feasibility of oral vaccine administration using lyophilized plant tissues. In their study, transgenic papaya callus expressing the protective peptides of the S3Pvac anti-cysticercosis vaccine elicited significant immune responses in both mice and

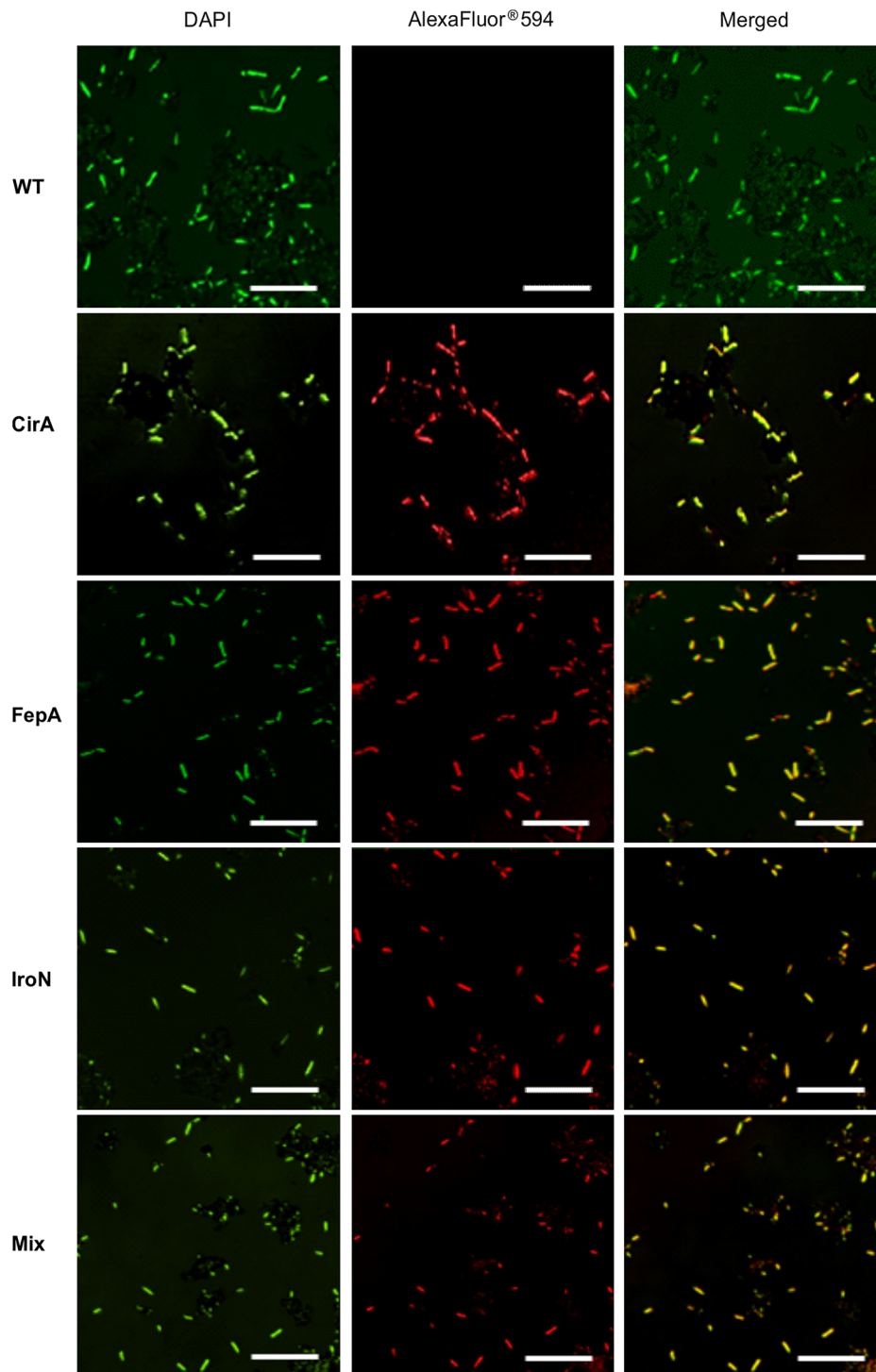


Fig. 8 | Mice immunized with transplastomic chloroplasts developed antibodies that bind *Salmonella* Enteritidis. Final bleed sera were tested for antibodies capable of binding to *Salmonella* Enteritidis. Binding is visualized by DAPI (Green) that stains bacterial cells' nuclei and Alexa Fluor® 594 goat anti-mouse IgG (H + L) (Red)

that binds to mouse IgG. A merged image (yellow) shows an overlay of the green and red channels used to visualize DAPI and Alexa Fluor® 594, respectively, to assess co-localization. Size bar = 10 μ m.

pigs across a broad dose range. In mice, doses ranging from 0.56 ng to 56 ng of vaccine peptides per animal produced protective immunity, while in pigs, oral administration of 1, 10, or 100 μ g per animal resulted in notable antibody production and lymphocyte proliferation. These results demonstrate that oral delivery of plant-derived antigens can achieve immunogenicity without requiring adjuvants or invasive administration. This dose flexibility strengthens the rationale for oral immunization strategies, particularly those utilizing lyophilized leaves, which offer enhanced stability, simplified

logistics, and needle-free delivery⁶². However, the presence of nicotine and other alkaloids in *Nicotiana* poses challenges for oral applications due to their potential toxicity upon ingestion⁶³. To address these concerns, this study employed the low-alkaloid tobacco cultivar 81V9, which enables scalable production of recombinant antigens while minimizing toxicity risks. Previous research demonstrated that mice consuming a diet containing 30% lyophilized 81V9 leaves experienced no adverse effects, validating its safety for oral administration⁶⁴. This plant-based strategy supports

the development of cost-effective oral immunization tools targeting zoonotic pathogens such as *Salmonella enterica*, with the long-term goal of mitigating bacterial shedding and environmental contamination. Each mouse was immunized with 30 µg of chloroplast-expressed recombinant protein, based on the maximum yield attainable after chloroplast extraction and processing in this study. While this standardized dose elicited significant antibody responses for FepA and CirA, IroN failed to produce a statistically significant response, suggesting that dose limitations may have hindered its immunogenic potential. In the “mix” group, which contained 10 µg of each protein, the reduced concentration may have further impacted overall responsiveness. It is therefore plausible that IroN requires higher antigenic input to trigger robust adaptive immunity and future studies could explore dose optimization strategies or adjuvant refinement to enhance its efficacy. Several animal studies have demonstrated that insufficient antigen dosage can result in suboptimal or absent immune responses, whereas increasing the dose can amplify immunogenicity. For instance, Billeskov et al. (2017)⁴¹ showed that mice vaccinated with low antigen doses failed to mount a detectable immune response unless paired with a potent adjuvant (CAF09); however, when the antigen dose was increased, robust CD4⁺ T cell responses with enhanced functional avidity were observed, leading to improved antiviral protection⁶⁵. Similarly, Yam et al. (2015)⁴² investigated AS03-adjuvanted influenza vaccines in BALB/c mice and found that very low antigen doses (as low as 0.003 µg) elicited immune responses comparable to unadjuvanted high-dose vaccines (3 µg), but only when adjuvants were present, highlighting that higher antigen doses were necessary to compensate for the absence of adjuvant and achieve comparable antibody titers and T cell activation⁶⁶. Moreover, Garg et al. (2021)⁴³ used a stochastic simulation model of germinal center reactions to predict that low-dose priming without sufficient antigen availability failed to induce meaningful B cell selection, whereas increasing the antigen dose or delaying the booster dose significantly enhanced antibody affinity and overall vaccine efficacy in murine models⁶⁷.

Whole-cell *Salmonella* ELISA and confocal microscopy indicate that antibody responses in mice immunized with transplastomic chloroplasts target *S. Enteritidis*. Although the IroN final bleed sera did not yield a statistically significant difference in antibody level when compared to IroN pre-immune sera and wild-type pre-immune and final bleeds, the *Salmonella* binding assay demonstrated the presence of *Salmonella*-specific antibodies in the IroN-immunized group. This suggests that while specific antibodies to IroN were produced, their concentrations may not have been high enough to result in a statistically significant difference when quantified by ELISA. A similar pattern is observed for the mix group. Further investigations will be crucial to determine whether these antibodies neutralize the bacteria and prevent colonization of chickens.

In summary, the expression of membrane proteins remains challenging due to their hydrophobic transmembrane domains, structural complexity, and dependence on lipid environments for stability⁶⁸. These characteristics often result in misfolding, aggregation, or degradation when expressed in heterologous systems. In this study, transient nuclear-based expression in *N. benthamiana* failed to produce full-length membrane proteins, likely due to trafficking limitations or proteolytic degradation. Even within plastids, where expression systems resemble prokaryotic environments, only three out of four TBDTs accumulated successfully. These findings highlight both the technical and biological hurdles associated with recombinant membrane protein production, especially for structurally complex antigens intended for vaccine development.

Despite these challenges, our results demonstrate that chloroplasts have significant potential for expressing complex membrane proteins. The chloroplast-expressed TBDT proteins exhibited immunostimulatory properties, eliciting specific antibody responses in mice that recognised *S. Enteritidis*. Future studies are needed to evaluate the protective efficacy of these vaccine candidates in live challenge models, including their ability to reduce *Salmonella* colonisation and shedding. Ultimately, this approach holds promise for minimising *Salmonella* contamination in food products and limiting its transmission within the environment.

Materials and Methods

Construct design and cloning

The nucleotide sequences of CirA, FepA, FhuA, and IroN derived from *Salmonella enterica* subspecies *enterica* (subsp. I) serotype Enteritidis were retrieved from GenBank (Accession Number AM933172.1). To facilitate detection of the recombinant proteins, a hemagglutinin (HA) tag was added to the 5' end and a c-Myc tag was added to the 3' end of the genes. The genes were then synthesized by BioBasic Inc. (Mississauga, ON), subjected to *NheI* and *NcoI* digestion, and subsequently introduced into the chloroplast expression cassette vector (pCEC5)⁶⁹ through direct cloning at the corresponding restriction sites. The vector contains the promoter and 5' UTR of the tobacco *psbA* gene, along with the 3' UTRs of *psbC* and *rbcL* derived from the white poplar plastome, to control gene expression. Integration of the target genes into the tobacco plastome occurred between the *trnI* (tRNA-Ile) and *trnA* (tRNA-Ala) genes (Fig. 2).

Generation of transplastomic plants

Transplastomic tobacco plants (cv. 81V9)⁷⁰ were obtained by the biolistic method⁶⁹. For bombardment of leaf tissue, plants were aseptically grown from seed on Murashige-Skoog (MS) medium containing 3% (w/v) sucrose and 0.7% (w/v) agar. Leaves were placed abaxial side up on RMOP medium in a Petri dish. The RMOP medium consists of MS salts, N6-benzyladenine (1 mg/liter), 1-naphthaleneacetic acid (0.1 mg/liter), thiamine (1 mg/liter), myo-inositol (100 mg/liter), agar (7 g/liter) at pH 5.8, and sucrose (30 g/liter). Leaf tissue bombardment was conducted using the PDS-1000/He Biolistic particle delivery system (BioRad). Two days following bombardment, the leaves were carefully cut into sections measuring 5 mm×5 mm. These leaf sections were then transferred to RMOP medium supplemented with 500 µg/ml of spectinomycin dihydrochloride. On the bleached leaves, green calli emerged which were subcultured onto the same selective medium. Following three rounds of regeneration on selective medium, transplastomic plants were transferred to soil for seed production. Homoplasmy of all the clones was confirmed by Southern blot analysis as previously described⁶⁹. DNA samples from CirA, FepA, and IroN transplastomic plants, along with wild-type *N. tabacum* serving as a control for expected band patterns without transgene integration, were extracted and digested with the *RsrII* restriction enzyme. The digested DNA was separated on a 0.8% agarose gel. A DIG-labeled probe synthesized by Kolotilin et al (2013) was used for hybridization⁶⁹. The sequences of the PCR primers used to generate the DIG-labeled probes were Probe-F (5'-caccagcgctctcttctctg-3') and Probe-R (5'-ttctacgggggtggagatgatgg-3'), which target the intergenic spacer region between the *trnI* and *trnA* genes in the tobacco plastome.

Recombinant protein extraction and detection

Leaf tissue samples weighing approximately 0.05 g were flash-frozen in liquid nitrogen and then pulverized in 2 ml microcentrifuge tubes containing 3 silica beads (Bio Spec Products Inc., Bartlesville, OK, USA) using a TissueLyser II (Qiagen, Venlo, Netherlands) for 2 minutes. Subsequently, 300 µl of 2x Laemmli sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, and 0.01% bromophenol blue) was added to the tubes. The samples were vortexed for 30 seconds and sonicated with a 30 second burst at 30% amplitude (Fisherbrand™ Model 120 Sonic Dismembrator, Fisher Scientific, Schwerte, Germany).

The tubes were heated at 100°C for 10 minutes, then centrifuged for 10 minutes at 14,000 x g at room temperature. The resulting supernatant was loaded onto Express Plus 4-20% gradient polyacrylamide gels (Genscript Inc., Piscataway, NJ, USA) and electrophoresed at 100 V for 100 minutes. The proteins were subsequently transferred from the gels to polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Turbo transfer system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Following transfer, the membranes were blocked overnight at 4°C in 5% (w/v) skimmed milk dissolved in Tris-buffered saline (TBS; 20 mM Tris, 150 mM NaCl, pH 7.5). Western blotting involved probing the blots with mouse anti-hemagglutinin (HA) primary antibody (Sigma-Aldrich Cat. No. H3663), diluted 1:5,000 in 0.5% blocking solution for 1 hour at room

temperature. After three washes in TBS pH 7.5, the membranes were incubated with goat anti-mouse horseradish peroxidase (HRP)-conjugated antibodies (Bio-Rad #1706516), diluted 1:5,000 in 0.5% blocking solution. Chemiluminescent signals were detected using Enhanced Chemiluminescent detection solution (Biorad Laboratories Inc., Hercules, CA, USA) and imaged with a MicroChemi imaging system using GelCapture acquisition software (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel).

Preparation of chloroplast sub-compartments from transplastomic plants for the analysis of protein localization by immunoblotting

Chloroplast sub-compartments from transplastomic plants were prepared as described by³². Briefly, in a cold room, 400 to 500 g of leaf material from transplastomic plants were harvested and homogenized in a grinding buffer containing 20 mM Tricine-KOH (pH 8.4), 0.4 M sorbitol, 10 mM EDTA (pH 8), 10 mM NaHCO₃, and 0.1% (w/v) bovine serum albumin (BSA) at a 1:4 (w/v) sample-to-buffer ratio. The homogenate was blended, filtered through four layers of Miracloth (Calbiochem), and the liquid extract was collected. The crude cell extract was then centrifuged at 2,070 × g for 2 minutes at 4 °C. The supernatant discarded to obtain concentrated crude chloroplast pellets.

The washing medium (2x), pH 7.6, was prepared with 20 mM Tricine-KOH (pH 7.6), 0.8 M sorbitol, 5 mM MgCl₂, and 2.5 mM EDTA. The pH was adjusted to 7.6 using NaOH pellets. The concentrated chloroplasts were gently resuspended in 1x washing medium. The final chloroplast suspension volume was 36 ml. For chloroplast purification, 200 ml of Percoll gradient was prepared by mixing equal volumes of Percoll and washing medium (2x), resulting in a final concentration of 50% (v/v) Percoll/0.4 M sorbitol. Thirty ml of this preparation were dispensed into 6 tubes and centrifuged at 38,700 × g for 55 min at 4 °C. Six ml of the chloroplast suspension was loaded onto each of the six preformed gradients and centrifuged at 13,300 × g for 10 minutes at 4 °C using a swinging-bucket rotor. The upper phase was discarded, and intact chloroplasts from the lower phase were collected, diluted 3–4-fold with 1x washing buffer, and centrifuged at 2,070 × g for 2 minutes at 4 °C. The pellet of intact chloroplasts was kept on ice.

The intact chloroplasts were lysed using up to 12 ml of hypotonic buffer containing 10 mM MOPS (pH 7.8), 4 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidinium hydrochloride hydrate, and 0.5 mM ε-amino caproic acid. Three milliliters of the lysed chloroplasts were loaded onto step sucrose gradients (0.3 M/0.6 M/0.93 M) and ultracentrifuged at 70,000 × g for 1 hour at 4 °C. Fractions were then collected from the tubes and pooled.

The presence of recombinant protein was determined using western blot analyses. Samples from each step of the experiment were mixed with 2x Laemmli sample buffer, heated at 100 °C for 10 min, and then loaded onto Express Plus 4–20% gradient polyacrylamide gels (Genscript Inc., Piscataway, NJ, USA). The remaining steps of the western blot procedure were carried out following the aforementioned protocol.

Mouse immunization

All mouse immunization procedures were conducted at Cedarlane Labs (4410 Paletta Court, Burlington, ON, Canada) under the approved Animal Use Protocol AUP117AB. This protocol was reviewed and approved by Cedarlane's institutional Animal Care Committee and was developed in accordance with the Canadian Council on Animal Care (CCAC) guidelines for the ethical use of animals in research.

Twenty-four female BALB/c mice, aged six to eight weeks, were assigned to four groups. Pre-immune blood samples of 50 µl were collected from each mouse. Each mouse was subcutaneously immunized with 100 µl of chloroplast extract from transplastomic plants containing 30 µg of recombinant protein. Chloroplasts were isolated from 30 g of transplastomic plant leaves using 120 mL of chloroplast isolation buffer supplemented with 0.1% (w/v) bovine serum albumin (BSA). The homogenate was filtered through six layers

of Miracloth and subjected to differential centrifugation—initially at 200 × g for 3 minutes to remove debris, followed by 1000 × g for 7 minutes at 4 °C to pellet intact chloroplasts. The resulting green pellet was resuspended in 1 mL of PBS and sonicated on ice (amplitude 30%; 30 sec on/30 sec off cycle for 1 minute) to disrupt the chloroplast membrane. Post-sonication, samples were centrifuged at 20,000 × g for 10 minutes at 4 °C. The supernatant was then passed through a 0.22 µm filter to obtain purified chloroplast components. The primary immunization was emulsified with 100 µl of Freund's complete adjuvant, resulting in a total injection volume of 200 µl (100 µl antigen + 100 µl adjuvant). Two booster immunizations were administered on days 21 and 42. For each boost, 100 µl of chloroplast extract from transplastomic plants containing 30 µg of recombinant protein was emulsified with 100 µl of Freund's incomplete adjuvant. In all injections, the resulting 200 µl mixture was administered subcutaneously, with 50 µl injected at four different sites, two injections at the front legs and two at the back legs. The mice were euthanized on day 49 for final bleeds.

Whole-cell *Salmonella* ELISA

Following the methodology outlined by Levinson et al. (2015), the whole-cell *Salmonella* ELISA was performed with some modifications⁷¹. The *S. Enteritidis* strain used in this study was provided by Dr. Ed Topp (London Research and Development Centre, London, Ontario, Canada). In the initial step of coating, 50 µl of a poly-L-lysine solution, diluted at a 1:10 ratio from a 0.01% concentration, was added to each well of ELISA MaxiSorp1 plates (Thermo Fisher Scientific Inc., Waltham, MA, USA). The plates underwent an overnight incubation at 4 °C. For subsequent plate preparation, the wells were emptied without washing. Subsequently, 50 µl of *S. Enteritidis*, washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4) at 4000 × g for 10 minutes at room temperature, was added following a 1:10 dilution. The plate underwent centrifugation at 1500 × g for 3 minutes, followed by a reversal of the plate and a repetition of the process. Following this, 50 µl of a 2% paraformaldehyde solution was added, and the plate underwent a 15 minute incubation at room temperature. After a single wash with PBS-T (0.1% Tween 20), 100 µl of 0.1 M glycine was added, and the plate was incubated at room temperature for 30 minutes. Another wash with PBS-T was conducted, followed by the addition of 100 µl of blocking solution (2% BSA-PBS-T) overnight at 4 °C.

Subsequently, the plate underwent a triple wash with PBS-T. Serial dilutions of Mouse serum (pre-immune and final bleed) were added and incubated for 1 hour at 37 °C. The plate underwent another triple wash with PBS-T, followed by the addition of goat anti-mouse IgG-HRP-conjugated secondary antibody (1:5,000) for 1 hour at room temperature. Subsequently, three washes with PBS-T were performed and antigen-bound antibodies were detected using ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid). The colorimetric reaction was measured at 415 nm using an ELISA plate reader (iMark™ Microplate Absorbance Reader, Bio-Rad, Hercules, California, USA).

Salmonella binding assay

Three millilitres of LB broth (Miller Formulation, Difco, Thermo Fisher Scientific, Ottawa, ON, Canada) were inoculated with 100 µl of a 5 ml overnight culture of *S. Enteritidis* grown at 37 °C. Subsequently, this 3 ml culture was incubated at 37 °C until reaching an OD₆₀₀ of 0.7–0.9. Cells were harvested from 1 ml of the culture by centrifugation at 13,000 × g for 5 min, followed by three 5 minute rinses in PBS. The bacterial pellet was then resuspended in 1 ml of 2.5% paraformaldehyde (PFA) and incubated for 30 minutes at room temperature. After removing excess PFA following centrifugation at 13,000 × g for 5 min, bacterial permeabilization was achieved using 0.1% Triton X-100 in PBS (permeabilization buffer) for 5–10 minutes. The pellet was subsequently resuspended in blocking buffer (2% BSA in PBS) for 1 to 2 hours. Twenty-microliter aliquots of the cell suspension were prepared in separate tubes, centrifuged at 13,000 × g for 5 min, resuspended in serum collected from mice immunized with recombinant proteins (final bleed), diluted in 1% BSA-PBS, and incubated overnight at

4 °C. Serum collected from mice immunized with PBS (final bleed) served as a negative control. Diluted serum was removed by centrifugation at $13,000 \times g$ for 5 min, followed by three washes in PBS for 5 min each.

The cells were then resuspended in Alexa Fluor® 594-conjugated goat anti-mouse IgG (H + L) (Abcam, Cat. No. ab150116) in 1% BSA-PBS for 1–2 hours at room temperature and incubated at 37 °C for 1 h. After washing and rinsing three times in PBS as described previously, and one final rinse in dH₂O, the cells were stained by resuspending them for 2 min in 20 µl aliquots of DAPI (10 mg/ml solution diluted 1:1,000 in dH₂O, Thermo Fisher Scientific Cat. No. D1306), followed by centrifugation at $13,000 \times g$ for 5 min and resuspension in 20 µl of dH₂O. The prepared cells were transferred onto poly-L-lysine coated coverslips (Millipore Sigma Cat. No. S1815) in a 24-well plate and centrifuged at $450 \times g$ for 10 min. Coverslips were then dried and mounted onto glass slides with Aqua-Poly/Mount (Polyscience Inc., Warrington, PA, United States, Cat. No. 18606).

To visualize the binding of antibodies generated in immunized mice to *S. Enteritidis* cells, imaging was performed using an Olympus LSM FV 1200 microscope. Images were captured with a 60× water objective lens. DAPI, employed for staining the DNA, was imaged with excitation at 350 nm and emission at 455–465 nm. The Alexa Fluor® 594-conjugated goat anti-mouse IgG (H + L) was visualized with excitation at 590 nm and emission at 617 nm.

Statistical analysis

Statistical analysis of ELISA results was conducted using R 4.0.3 (R Core Team, 2020). The initial assessment involved checking the data for outliers, zero inflation, balance of categorical covariates, and collinearity. While a linear model was initially employed, it became evident that the assumption of equal variance was violated. Consequently, a generalized least squares (GLS) model was utilized, as it does not require equal variance assumptions. Additionally, a variance of identity residual structure was implemented to allow for distinct variance for each protein. To validate the GLS model, the residual distribution, residuals versus fitted values, and residuals against covariates were thoroughly examined. Subsequently, a two-factor ANOVA was carried out, followed by a Games Howell post hoc test (utilizing the PMCMRplus package), which does not assume equal variance.

Data availability

All data is presented within this manuscript.

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Author contributions

S.S., C.A.C. and C.P.G. designed the constructs. S.S. and A.K. generated the transplastomic plants. S.S. performed protein expression and detection, chloroplast localization, and post-immunization assays, and drafted the

manuscript. T.M. conducted the mouse immunization trials. M.S.S. and R.M. conceived the project, secured funding, and revised the manuscript. R.M.O supervised the work.

Competing interests

The authors declare no competing interests.

Additional information

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