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Brief Communication

Oral booster vaccine antigen—Expression of full-length native SARS-CoV-2 spike protein in lettuce chloroplasts

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Current vaccines continue to save lives during the pandemic but do not prevent virus transmission. Unfortunately, fully vaccinated individuals with repeated boosters also get infected, and breakthrough infections have peak viral loads similar to unvaccinated individuals and transmit SARS-CoV-2 in household settings, with or without symptoms (Singanayagam *et al*., 2022). AI studies of vaccine-resistant mutations in >2.2 million SARS-CoV-2 genomes show that the mutation frequency correlates strongly with the vaccination rates in Europe and America and predicts a complementary transmission pathway, vaccine-breakthrough or antibody-resistant mutations, like those in Omicron (Wang *et al*., 2021). Amidst the emergence of new SARS-CoV-2 variants like the omicron strain resistant to current vaccines, with higher rates of transmissibility, it is prudent to consider additional affordable measures to minimize viral transmission and infection.

Airborne-lifetime-weighed volume of saliva droplets in healthy subjects is 3–5 orders of magnitude higher than breath droplets, and speaking four words transmits more virus than 1 h of maskless breathing (Shen *et al*., 2022). Oral epithelial cells are enriched in ACE2 receptors and GM1 coreceptors, thereby facilitating viral entry (Daniell *et al*., 2022a). Therefore, one approach recently developed involved debulking SARS-CoV-2 or other oral viruses in saliva using virus-trap proteins via chewing gums to minimize self-infection and transmission (Daniell *et al*., 2022a,2022b). Most importantly, proteins bioencapsulated in the chewing gum are stable and fully functional for several years when stored at ambient temperature, thereby making them affordable by the elimination of complex fermenter-based manufacturing processes, expensive cold storage, transportation, and other costs associated with current vaccines. Indeed, this is the first engineered therapeutic protein approved by FDA free of protein purification or cold chain, and clinical trials are in progress to evaluate SARS-CoV-2 infection and transmission (Daniell *et al*., 2022b).

Medicago recently reported transient expression of full-length spike protein in tobacco and the development of an adjuvanted injectable vaccine after purification of VLPs (Hager *et al*., 2022). The native full-length spike protein is challenging to express due to degradation and therefore required modifications of certain amino acids or the addition of tags to enhance stability (Ward *et al*., 2021). Therefore, most of the early publications in this field expressed the receptor-binding domain (RBD) and observed effective immunization and protection in animal models. Interestingly, higher neutralization and affinity to ACE2 have been reported for aglycosylated RBD than glycosylated version (Mamedov *et al*., 2021). While several boosters are needed for continued protection, recent studies show that heterologous boosters (mRNA vaccine boosted with inactivated or nonreplicating adenovirus vaccine—Sputnik, Oxford Astro-Zeneca, or Sinopharm) offered better immunity and protection (Larkin, 2022). Therefore, in this study, we expressed full-length CTB-Spike fusion protein in chloroplasts to facilitate oral delivery, for the eventual development of a cold chain-free heterologous mucosal booster vaccine.

The nucleotide sequence of *S*-gene (Wuhan-Hu-1 (NC_045512.2) strain) encoding full-length native spike protein (Figure 1a) was codon optimized based on the hierarchy of chloroplast *psbA* gene. In this codon optimization process, among 1273 amino acids, 314 codons, including 105 rare codons, were replaced (Figure S1). The full-length synthetic *S*-gene was subcloned downstream of cholera toxin-B (CTB) subunit encoding nucleotide sequence with the hinge (GPGP) and furin cleavage site (RRKR) and was inserted into the marker-free chloroplast vector pLsLF-MF, and the expression cassette is regulated by the *psbA* promoter/5′UTR and 3′UTR (Figure S2).

Lettuce leaf bombarded with pLsLF-MF-CTB-Spike expression cassette containing the spectinomycin-resistant aminoglycoside-3′-adenylyl-transferase (*aadA*) gene regenerated shoots on spectinomycin-containing medium. The *aadA* gene cassette was inserted between two copies of chloroplast-encoded CF1 ATP synthase subunit beta (*atpB*) gene fragment (649 bp) to facilitate marker excision, by utilizing the chloroplast recombinase system (Figure 1b). Among several independent shoots regenerated after the second round of selection, seven were screened by PCR. The 16 s-F/aadA-R primer set anneals to the endogenous chloroplast genome sequence and the *aadA* transgene within the cassette (Figure 1b), which amplified a 3.1 kb DNA fragment (Figure 1c, upper gel). The set of UTR-F/23 s-R primers was used to verify 3′ region of the expression cassette (Figure 1b), which produced a fragment of 2.2 kb (Figure 1c, lower gel). Site-specific integration of CTB-Spike gene into chloroplast genomes of all seven developed transplastomic lines was further evaluated by Southern blots. The detection of two hybridizing fragments (3.09 and 12.99 kbp) in transplastomic lines, when plant DNA was digested with *Hind*III enzyme but not in untransformed WT, confirmed site-specific transgene integration into the lettuce chloroplast genome. Moreover, the absence of 9.5 kb fragment in the transplastomic lines but present in the WT chloroplast genome confirmed the homoplasmy of transplastomic lines. However, the *aadA* gene is not yet excised in these lines but marker-free lines could be generated in T1 generation, as we reported previously. Transplastomic lines are healthy and fertile, and seeds were harvested for the propagation of the next generation (Figure S4).

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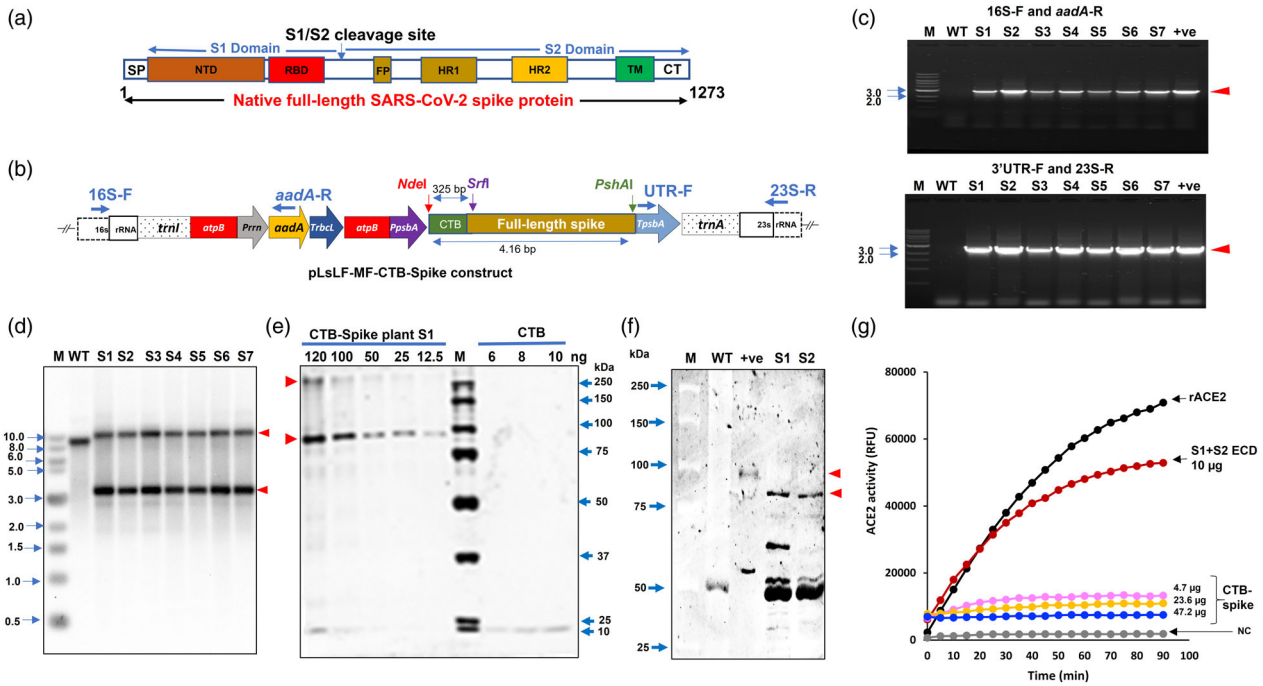

Figure 1 Expression and functional characterization of native full-length SARS-CoV-2 spike protein in lettuce chloroplasts. (a) Schematic diagram of native full-length SARS-CoV-2 spike protein (1273 amino acid) expressed in this study. (b) The codon-optimized spike *s*-gene cloned downstream of the cholera toxin-B (CTB) subunit in marker-free chloroplast transformation pLsLF-MF vector. (c) PCR amplification of 3.1 and 2.2 kb fragment (arrowheads) using primer set 16 S-F and *aadA*-R and 3'UTR-F and 23 S-R, confirms site-specific integration of the *CTB-S*-gene into the lettuce chloroplast genome. (d) Detection of 3.09 and 12.9 kb hybridizing fragments (arrowheads) in Southern blot, after digesting with *Hind*III and probing with the flanking sequence confirms site-specific integration of the CTB-S gene cassette. The absence of 9.1 kb fragment in transplastomic lines confirms homoplasmy. (e) Quantitation of CTB-Spike protein using CTB standard, after loading different concentrations of crude plant extract (concentrations shown on top of each lane). The CTB-Spike quantified by comparing CTB standard using 55.5, 38.6, 23.1, 12.6, and 6.8 ng in total protein loaded (120, 100, 50, 25 and 12.5 ng), respectively resulting in 46.2%, 38.5%, 46.1%, 50.5%, 54.4% of CTB-Spike in total leaf protein. Western blot was done using primary antibody against CTB. (f) Expression of CTB-Spike fusion protein confirmed by Western blotting using antispike primary antibody. Detection of slightly lower than 90 kDa (arrowhead). Purified commercial S1 + S2 ECD used as positive control migrated at ~90 kDa (arrowhead). (g) Functional characterization of CTB-Spike by ACE2 activity inhibition assay in crude plant extract (4.7, 23.6 and 47.2 µg) and purified SARS-CoV-2 S1 + S2 ECD (10 µg). ACE2 activity (RFU—relative fluorescence units) was determined by cleavage of fluorogenic Mca-APK (Dnp) substrate. NC—negative control.

Western blots of crude extracts of transplastomic lines using CTB antibody detected slightly lower than 90 kDa monomer of CTB-Spike protein (Figure 1e and Figure S3). Dimers are also observed, and several transplastomic lines showed the expression of the CTB-Spike fusion protein (Figure 1e and Figure S3). Absence of these proteins in untransformed plants confirms uniquely expressed proteins in transplastomic lines (Figure S3). Expression level of CTB-Spike protein is quite high (38.5–55.4 % TLP, 11.8 mg/g DW), even though data presented are from T0 leaves, and this is expected to significantly increase in subsequent generations. Similar size of CTB-Spike protein (slightly lower than 90 kDa) was also detected in Western blots using the spike antibody in transplastomic lines but not in untransformed (WT) plants (Figure 1f). Commercial source of the spike protein (S1 + S2 ECD) used as a positive control showed a monomeric form of ~90 kDa (Figure 1f); the slightly lower size of the chloroplast spike protein is likely due to a lack of glycosylation in chloroplasts. One protein ~50 kDa detected in both transformed and untransformed WT plants is due to the nonspecific binding of the antispike antibody to a plant protein.

Functionality of the full-length CTB-Spike fusion protein expressed in lettuce chloroplasts was evaluated by analysing the affinity of crude plant extract protein to rACE2 (Recombinant Angiotensin Converting Enzyme 2) and comparing it to that of purified commercial SARS-CoV-2 S1 + S2 ECD. As seen in Figure 1g, the fluorogenic cleaved product of rACE2 was slowly inhibited by 25% after 90 min of this assay, with 30 min of preincubation with 10 µg of purified SARS-CoV-2 S1 + S2 ECD. On the contrary, the ACE2 enzyme activity was inhibited almost immediately after preincubation with 4.7 ug of CTB-Spike protein in crude plant extracts (Figure 1g), and > 80% inhibition was observed after 90 min. Higher concentration of CTB-Spike protein showed further inhibition, demonstrating >10-fold higher affinity to human ACE2 than the glycosylated version of the S1-S2 ECD protein.

In summary, this brief communication reports the first expression of the full-length spike protein in an edible plant. The spike protein is fused with the transmucosal carrier CTB, to facilitate oral delivery of this antigen to the immune system. CTB has been used as a vaccine antigen for several decades in the clinic (Daniell *et al.*, 2019, 2022a,2022b), and more recently, CTB fusion protein has been approved by FDA for evaluation in the clinic (Daniell *et al.*, 2022b). In addition, the spike protein will cross epithelial cells upon oral delivery to the gut lumen by binding to both ACE2 and GM1 receptors because the spike protein enters human cells utilizing ACE2 receptor and GM1 coreceptors (Daniell

et al., 2022a, 2022b). We observed very good levels of expression of the CTB-Spike protein, although the expression was evaluated in T0 plant biomass. In transplastomic lines, subsequent generations show 10- to 20-fold higher levels of expression of foreign proteins. Most importantly, CTB-Spike protein has >10-fold higher affinity to ACE2 and is highly potent in inhibiting ACE2 activity than the glycosylated S1-S2. These observations augur well for advancing the CTB-Spike antigen bioencapsulated in plant cells towards an oral booster vaccine, free of cold chain and expensive purification process. Further studies are in progress for the evaluation of boosting the efficacy of CTB-Spike versus spike antigens to confer mucosal (IgA) and systemic (IgG) immunity.

Several important lessons can be gleaned from prior vaccination campaigns during the continued outbreak of infections. Sabin's live attenuated oral polio vaccine (OPV) is easy to administer, less expensive, and offers better mucosal immunity than Salk's formaldehyde inactivated poliovirus (IPV) injections and is therefore widely adopted around the globe, but OPV2 was recently withdrawn due to recombination with other viruses. Similar to the recent polio virus emergency declared recently in New York counties, Israel detected WPV in their sewer system after prolonged use of IPV and is now administering both bivalent OPV and trivalent IPV (Daniell *et al.*, 2019). Based on these lessons, it is important to develop affordable booster SARS-CoV-2 mucosal vaccines, free of cold chain for ease of global distribution. In this context, several antigens expressed in chloroplasts have been shown to efficiently boost antibody titers (IgG and IgA) and confer protection against viruses (Daniell *et al.*, 2019). Therefore, this report paves the way for developing an oral booster mucosal vaccine against SARS-CoV-2.

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Conflict of interest

The corresponding author (HD) is an inventor of several patents on the expression of foreign proteins in chloroplasts and therefore declares conflict of interest although there is no specific financial COI to disclose. All other authors declare no conflict of interest.

Author contributions

HD conceived this project from designing chloroplast vectors, created transplastomic lines through functional evaluation, interpreted the data, and wrote several sections of this manuscript. YS performed codon optimization (Figure S1), and RS inserted a synthetic gene into the chloroplast vector and confirmed DNA sequence, evaluation of transgene integration into the chloroplast genome by PCR, Southern blots, and expression in Western blots (Figure 1a–f, Figures S2 and S3) and contributed to these sections in the manuscript. SL created transplastomic lines and transferred them to the greenhouse and collected seeds (Figure S4). SKN performed ACE2 inhibition assays (Figure 1g) contributed to this section in the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Codon optimization of S-gene of SARS-CoV-2 isolate Wuhan-Hu-1.

Figure S2 The codon optimized spike gene cloned downstream of CTB in the marker-free chloroplast transformation vector in three steps.

Figure S3 Western blot of CTB-Spike expressing five different transplastomic lines probed with anti-CTB primary antibody.

Figure S4 Transplastomic plants expressing CTB-spike protein grown at the green house (A), and harvested seeds screened on antibiotics plate for germination (B).

Data S2 Supplementary Methods.

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