Accumulation of Recombinant SARS-CoV Spike Protein in Plant Cytosol and Chloroplasts Indicate Potential for Development of Plant-Derived Oral Vaccines

HONG-YE LI, SATHISHKUMAR RAMALINGAM, AND MEE-LEN CHYE[1]

Department of Botany, The University of Hong Kong, Pokfulam Road, Hong Kong, China

Plants are promising candidates as bioreactors for the production of oral recombinant proteins in the biopharmaceutical industry. As an initial step toward provision of an oral vaccine against the severe acute respiratory syndrome coronavirus (SARS-CoV), we have expressed a partial spike (S) protein of SARS-CoV in the cytosol of nuclear-transformed plants and in the chloroplasts of plastid-transformed plants. In the construction of both nuclear and plastid transformation vectors, a 2-kilobase nucleotide sequence encoding amino acids 1–658 of the SARS-CoV spike protein (S1) was modified with nucleotide changes, but not amino acid changes, to optimize codon usage for expression in plants. To investigate the subcellular localization of S1 during transient expression in tobacco leaves, a translational fusion consisting of S1 and the green fluorescent protein (GFP) was generated. Following agroinfiltration of tobacco leaves, analysis by laser confocal scanning microscopy revealed that the S1:GFP fusion protein was localized to the cytosol. In stable transgenic tobacco plants and lettuce plants generated by Agrobacterium-mediated transformation, tobacco and lettuce leaves were observed to express the S1 at high levels from the Cauliflower Mosaic Virus *35S* promoter with Northern blot analysis. When the S1 was expressed in transplastomic tobacco, *S1* messenger RNA and its corresponding protein were detected on Northern and Western blot analyses, respectively. Our results demonstrate the feasibility of producing S1 in nuclear- and chloroplast-transformed plants, indicating its potential in subsequent development of a plant-derived and safe oral recombinant subunit vaccine against the SARS-CoV in edible plants. Exp Biol Med 231:1346–1352, 2006

Key words: severe acute respiratory syndrome coronavirus; plant bioreactor; recombinant subunit vaccines; plastid expression; subcellular localization

[1] To whom correspondence should be addressed at Department of Botany, The University of Hong Kong, Pokfulam Road, Hong Kong, China. E-mail: mlchye@hkucc.hku.hk

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Introduction

The severe acute respiratory syndrome (SARS), which is caused by a novel coronavirus (SARS-CoV) (1, 2), resulted in a worldwide outbreak in 2003 spanning 29 countries, with more than 8000 cases and more than 700 fatalities (http://www.who.int/csr/sars/en/). Given that the virus and its related species are likely endemic in animal populations (3–6), there is a possibility that this infection may recur. Hence, it is pertinent to develop vaccines to prevent another SARS epidemic.

Analysis of the genome sequence of the SARS-CoV revealed that it is distinct from previously characterized members of the family Coronaviridae that cause respiratory and enteric diseases in animals (7, 8). Four of its 11 predicted open reading frames encode structural proteins, including a spike glycoprotein (S) of 1255 deduced amino acids and a membrane glycoprotein (M) of 221 deduced amino acids. The S and M proteins associate to form the viral envelope. The S glycoprotein, a type I membrane protein, functions in the attachment to host receptors and has an N-terminal signal sequence and a C-terminal transmembrane region followed by a cytoplasmic tail (7, 8).

Plants have been demonstrated to be suitable bioreactors for the production of recombinant biopharmaceuticals *via* the expression of foreign genes introduced into the nuclear genome or the chloroplast genome. The generation of transgenic plants by plant nuclear transformation has successfully produced mucosal vaccines against cholera, Norwalk virus, hepatitis B, and foot-and-mouth disease (9). Many viral antigens (10) including S1 (11) from transgenic plants have been demonstrated to be effective in inducing mucosal and serum immune responses in animals. More recently, plastid transformation has been demonstrated to be more effective in enhancing the yield of plant-derived recombinant proteins. In the plant cell, the mere presence of up to 10,000 more copies of the plastid genome compared with a nuclear genome, increases accumulation of the plastid-expressed foreign protein or antigen in the case of

vaccine production (12). The cholera toxin B subunit, the first plant-derived vaccine arising from plastid transformation, accumulates to 4.1% total soluble protein in tobacco leaves (13). Human somatotropin has been observed to accumulate to 7% total soluble protein in plastid transformation, 300-fold greater than in nuclear-transformed tobacco (14), whereas human serum albumin accumulates to 11.1% total soluble protein, 500-fold higher than in nuclear-transformed leaves (15). In comparison, the yield of foreign protein from nuclear-transformed plants rarely exceeds 1% total soluble protein (13). Because plastid transformation appears to be more advantageous than nuclear transformation, we have attempted to express S1 in tobacco plastids as a first step toward the production of plastid-transformed plants that accumulate S1. Tobacco was chosen because it is a model plant for plastid transformation and is closely related to tomato, which produces an edible fruit that is ideal for an oral vaccine, but is a more difficult species in which to manipulate plastid transformation.

Nuclear-transformed tobacco and tomato expressing SARS-CoV viral antigens have been shown to induce immunogenic responses (11). An edible vaccine against the SARS-CoV virus deliverable as fruits (e.g., tomato), leaves (e.g., lettuce), tubers (e.g., potato), seeds (e.g., rice or corn), flowers, stems, or roots, would obliterate purification procedures required of recombinant vaccines from microorganisms. It has further advantages in easy storage, transport, and administration by direct ingestion. Also, edible vaccines in the form of fruits, leaves, tubers, seeds, flowers, stems, or roots can be grown and easily distributed in developing countries, cutting the costs of immunization programs, and omitting the need for refrigeration and delivery by injection, which requires trained health personnel.

Materials and Methods

Cloning of SARS-CoV *S1* cDNA and Site-Directed Mutagenesis to Optimize Codon Usage. Plasmid pCRII-S1, a derivative of vector pCRII (Invitrogen), was provided by Dr. L.L. M. Poon and Prof. J.S.M. Peiris (Department of Microbiology, University of Hong Kong). It contains a polymerase chain reaction (PCR)–amplified fragment of S1 (including amino acids 1–658 of the S protein). The S1-encoding fragment was derived by PCR using forward primer 5′-CTAGTCAGGATCCATGTTTATTTTCTTATTATTTC-3′ (the boldface **ATG** is the start codon for S1) and reverse primer 5′-TTCTA*GTCTAACTGTATGGTAAT*AGCACA-3′ (the corresponding stop codon for S1 on the sense strand appears in boldface; the letters in italics on the sense strand encode additional amino acids [ITIQLD] that precede the stop codon; the underlined codon GCT on the sense strand encodes amino acid 658 [A] on the S peptide). The template used in PCR was cDNA derived from total RNA of Vero cells infected with the SARS-CoV (16). The PCR reaction (50 µl) consisted of 5 µl of cDNA template, 10 pmol of each primer, 1.25 U AmpliTaq Gold (Applied Biosystems), 5 µl of 10× PCR reaction buffer, 6 µl of 25 m*M* $MgCl_2$, and 0.5 µl of each 10 m*M* dNTP. PCR amplification was initiated with denaturation at 95°C for 10 mins, followed by 35 cycles at 95°C for 1 min, 50°C for 50 secs, and 72°C for 1.5 mins. A final extension was carried out at 72°C for 7 mins. The PCR product was analyzed by agarose gel electrophoresis and cloned into TA-cloning vector pCRII (Invitrogen) to yield pCRII-S1.

Subsequently, plasmid pCRII-S1 was used as a mutagenesis template for generating derivatives for optimization of codon usage for heterogeneous gene expression in plants. Mutagenesis was carried out by PCR with *PfuTurbo* DNA polymerase using the QuikChange Multi Site-directed Mutagenesis Kit (Stratagene), according to the manufacturer's specifications. The oligonucleotides used in site-directed mutagenesis are shown in Table 1. A total of 13 nucleotide changes were incorporated for an "optimized" *S1*, which was subsequently used for cloning into plastid and nuclear transformation vectors.

Plasmid Construction for Plastid and Nuclear Transformation. Plasmid pCV1 (Fig. 1), a plastid transformation vector, was constructed for expression of the S1 antigen. It was generated by cloning a 2-kilobase *Spe*I-*Not*I fragment encoding amino acids 12–658 of S1 (minus its start codon and signal peptide) from pCRII-S1 into the *Nhe*I and *Not*I sites of plasmid pMLVHisA.

Plasmid pMLVHisA was derived from chloroplast transformation vector pVSR326 (17). Plasmid pVSR326 utilizes the tobacco plastid genome sequences spanning the *rbcL-accD* region to target the reporter gene encoding β-glucuronidase (*GUS*) into the chloroplast genome by homologous recombination (17). The promoter and terminator for *GUS* are derived from the rice plastid gene *psbA*, which encodes the photosystem II 32-kDa protein. The selectable marker gene *aadA*, which specifies spectinomycin resistance, is expressed from the rice plastid 16S ribosomal RNA operon (*rrn*) promoter; *aadA* is located adjacent to *GUS* (17).

Initially, a 0.04-kb *Nco*I-*Sac*II-*Apa*I-*Nhe*I-*Not*I-*Cla*I-*Sac*I polylinker consisting of annealed oligonucleotides ML328 5′-CATGGCCGCGGGGGCCCGCTAGCAGGCCTGCGGCCGCATCGATGAGCT-3′ (the **ATG** start codon appears in bold and the *Sac*II recognition sequence is underlined) and ML329 (5′-CATCGATGCGGCCGCAGGCCTGCTAGCGGGCCCCCGCGGC-3′) was cloned in the *Nco*I-*Sac*I site of pVSR326 to generate vector pMLV. Subsequently, plasmid pMLV was cleaved with *Sac*II to insert $(His)_5$-encoding annealed oligonucleotides ML366H (5′-GCGGGGTTCT*CATCATCATCATCAT*GGTCCGC-3′; the $(His)_5$-encoding *CAT* codons are in italics) and ML367H (5′-GGACCATGATGATGATGATGAGAACCCCGC GC-3′).

In the resultant derivative pCV1 (Fig. 1), amino acids 12–658 of S1 are fused in-frame to 17 pMLVHisA-derived

Table 1. Oligonucleotides Used for Site-Directed Mutagenesis of S1 for Codon Optimization[a]

Affected residue	Sequence of primer
R18	5′-GTAGTGACCTTGAC***AGA***TGCACCACTTTTGAT-3′
T75	5′-GGGTTTCATACTATTAATCATAC***T***TTTGGCAACCCTGTCATAC-3′
S113	5′-CCATGAACAACAAGTCACAGTC***T***GTGATTATTATTAACAATTCTACT-3′
S169	5′-AGTACATATCTGATGCCTTTTC***T***CTTGATGTTTCAGAAAAGTC-3′
L209	5′-CCTATAGATGTAGTTCGTGATCT***T***CCTTCTGGTTTTAACACTTTG-3′
T247	5′-CAAGACATTTGGGGCAC***T***TCAGCTGCAGCCTAT-3′
A398	5′-GATGATGTAAGACAAATAGC***T***CCAGGACAAACTGG-3′
P507	5′-TCTTTTGAACTTTTAAATGCACC***T***GCCACGGTTTGTGGACC-3′
T509	5′-CTTTTAAATGCACCTGCCAC***T***GTTTGTGGACCAAAATTATC-3′
L597	5′-CTTCATCTGAAGTTGCTGTTCT***T***TATCAAGATGTTAACTGCAC-3′
R620	5′-CAACTCACACCAGCTTGG***AGA***ATATATTCTACTGGAAACAATG-3′

[a] Nucleotides in bold italics are mutated. The affected codons are underlined.

residues, including the "ATG" start codon and a $(His)_5$ tag. The start codon for expression of recombinant $(His)_5$-tagged S1 is derived from the vector pMLVHisA. Plasmid pMLVHisA is designed with a $(His)_5$ tag to enable recognition of the recombinant protein using antisera against the $(His)_5$ tag in Western blot analysis. Also, the $(His)_5$ tag enables easy purification of the recombinant protein using Ni-NTA Agarose (Qiagen) affinity columns. The rice plastid *psbA* promoter (P_{psbA}) drives expression of the recombinant protein and the terminator (T_{psbA}) is also *psbA*-derived. The presence of flanking *rbcL* and *accD* sequences from the tobacco plastid genome enables homologous recombination, resulting in the incorporation of *S1* (and *aadA*) into the plastid genome in the target plant. P_{rrn} regulates expression of the spectinomycin-resistance marker *aadA*, and the *rbcL* terminator (T_{rbcL}) functions as terminator.

A nuclear transformation plasmid pCV2 (Fig. 1) for expression of the S1 antigen was constructed by cloning a 2-kb *Bam*HI-*Xho*I fragment of *S1* encoding amino acids 1–658 from pCRII-S1 into the *Bam*HI and *Sal*I sites of binary vector pSa7. Plasmid pSa7, a derivative of pBI121 (18), is a plant nuclear transformation vector. In contrast to plasmid pCV1, the signal peptide of S1 (amino acids 1–13) is retained in plasmid pCV2 because previous reports have suggested that the presence of endoplasmic reticulum–targeting signals in plant nuclear-expressed foreign proteins results in improved protein stability (19, 20). In plasmid pCV2, *S1* is placed between the strong and constitutive Cauliflower Mosaic Virus (CaMV) *35S* promoter and the nopaline synthase (*NOS*) terminator.

Plasmid pCV12 (Fig. 1), the nuclear transformation vector for expression of a protein fusion consisting of the SARS-CoV S1 protein fused with green fluorescent protein (GFP), was generated by cloning a 0.8-kb *Xho*I-*Spe*I *GFP* fragment from pGFP2 (a gift from Prof. Nam-Hai Chua, Rockefeller University, New York, NY) in the *Sal*I-*Xba*I sites of pCRII-S1 to create an *S1-GFP* fusion. Subsequently, a 2.8-kb *Bam*HI-*Apa*I fragment containing the *S1* cDNA (encoding amino acids 1–643) and *GFP* cDNA was cloned into the *Bgl*II-*Apa*I sites of pGD (21) resulting in the loss of *Bam*HI and *Bgl*II sites.

Agroinfiltration of Tobacco for Expression of the S1:GFP Antigen. Production of transgenic tobacco expressing a protein fusion consisting of the SARS-CoV S1 protein fused with the GFP was carried out using Agrobacterium-mediated transformation (22) with plasmid pCV12. Two days after agroinfiltration, representative tobacco leaf epidermal cells were selected and observed by confocal microscopy (23) of *Agrobacterium tumefaciens* LBA4404 harboring plasmid pCV12 expressing the S1:GFP-fusion protein or LBA4404 harboring pGDG expressing GFP alone.

Plastid Transformation and Analyses on Transplastomic Lines. Plastid transformation for S1 antigen production was carried out according to the method described by Svab and Maliga (24) by bombarding tobacco leaves with tungsten particles coated with pCV1 DNA. Aseptical seeds were germinated on Murashige and Skoog (MS) medium (25), supplemented with agar (5 g/liter) and sucrose (30 g/liter).

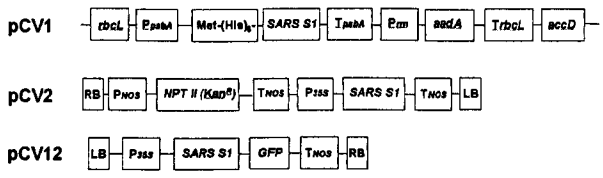

Figure 1. Diagram showing *S1* constructs in plasmids pCV1, pCV2, and pCV12. pCV1: The flanking regions *rbcL* and *aacD* derived from the tobacco plastid genome function in homologous recombination during plastid transformation; P_{psbA}, *psbA* promoter for expression of inserted gene; T_{psbA}, *psbA* terminator; P_{rrn}, *rrn* promoter driving expression of the spectinomycin-resistance marker *aadA*; T_{rbcL}, *rbcL* terminator; Met, start codon for recombinant $(His)_5$-tagged protein. pCV2: RB and LB represent right and left borders of T (transfer)-DNA for random insertion into the plant nuclear genome; P_{NOS}, nopaline synthase (*NOS*) promoter; T_{NOS}, *NOS* terminator; NPTII (KanR), neomycin phosphotransferase specifying kanamycin-resistance; P_{35S}, Cauliflower Mosaic Virus *35S* promoter. pCV12: Nuclear transformation vector for expression of a protein fusion consisting of the SARS-CoV S1 protein fused with GFP. RB and LB represent the right and left borders of T-DNA.

For bombardment, leaves were placed abaxial side up on RMOP medium consisting of MS salts, 6-benzylaminopurine (1 mg/liter), α-naphthalene acetic acid (0.1 mg/liter), thiamine (1 mg/liter), myo-inositol (100 mg/liter), agar (5 g/liter) at pH 5.7, and sucrose (30 g/liter). Bombardment was carried out using the particle delivery system PDS 1000-He (Bio-Rad) and its accessories. Tungsten particles (M 17) were coated with plasmid DNA and leaves were bombarded. After bombardment, leaves were cut into small pieces and placed on RMOP selection medium containing spectinomycin dihydrochloride (500 mg/liter). The regenerated plantlets obtained following plastid transformation of tobacco were passaged for five more cycles on selection medium to obtain homoplastomic plastid–containing plants.

Plantlets were tested using PCR analysis with *S1* primers ML560 (5′-CAGAGAGAGTTTCCCGGATTC-3′) and ML567 (5′-CAACCTATAGATGTAGTTCG-3′) followed by Northern blot analysis using a ^{32}P-radiolabeled *S1* probe. The PCR reaction (25 μl) consisted of 250 ng of template, 5 pmol of each primer, 0.75 U AmpliTaq Gold (Applied Biosystems), 2.5 μl of 10× PCR reaction buffer, 1.5 μl of 25 m*M* $MgCl_2$, and 0.25 μl of each 10 m*M* dNTP. PCR amplification was initiated with denaturation at 95°C for 3 mins, followed by 35 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min.

Nuclear Transformation and Analyses on Transgenic Plant Lines. Nuclear transformation of tobacco and lettuce for expression of S1 antigen was carried out with plasmid pCV2 using Agrobacterium-mediated transformation (18, 22). Following Agrobacterium-mediated transformation, shoots of tobacco and lettuce were rooted and the regenerated shoots were used in PCR analysis with primers *35S* and *NOS*-ter, which are located within the CaMV *35S* promoter and the *NOS*-terminator, respectively. The PCR reaction (25 μl) consisted of 250 ng of template, 5 pmol of each primer, 0.75 U AmpliTaq Gold (Applied Biosystems), 2.5 μl of 10× PCR reaction buffer, 1.5 μl of 25 m*M* $MgCl_2$, and 0.25 μl of each 10 m*M* dNTP. PCR amplification was initiated with denaturation at 95°C for 3 mins, followed by 35 cycles at 95°C for 1 min, 56°C for 2.5 mins, and 72°C for 1 min. Southern blot analyses were performed according to standard procedures (26) to confirm the presence of PCR-amplified *S1* DNA using a ^{32}P-labeled *S1* probe.

Western Blot Analysis. To indicate transient expression of S1:GFP in tobacco leaves following agroinfiltration, Western blot analysis was carried out using antibodies against GFP. Total protein (20 μg) extracted from tobacco leaves, infiltrated with plasmid pGDG expressing GFP alone or plasmid pCV12 expressing S1:GFP fusion, were separated on an 8% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis gel, blotted onto Hybond-C filters according to the method described by Sambrook *et al.* (26), and cross-reacted with antibodies against GFP (Clontech). To demonstrate $(His)_5$-S1 expression in plastid-transformed tobacco, total soluble protein was extracted in buffer (50 m*M* sodium phosphate buffer pH 7.0, 5 m*M* dithiothreitol, 1 m*M* Na_2EDTA, 0.1% SDS, and 0.1% Triton X-100) and purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) (27). Western blot analysis was performed as described by Sambrook *et al.* (26). The QIAexpress Ni-NTA AP Conjugate (Qiagen) was used according to the manufacturer's instructions to detect the presence of $(His)_5$-tagged recombinant proteins.

Laser-Scanning Confocal Microscopy. A Zeiss LSM 510 inverted confocal laser-scanning microscope equipped with helium/neon lasers was used for the analysis of GFP localization following the settings described by Goodin *et al.* (21) with minor modifications. GFP fluorescence was excited at 488 nm, filtered through a primary dichroic (UV/488/543), a secondary dichroic of 545 nm, and subsequently through BP505–530 nm emission filters to the photomultiplier tube detector. The images were processed using the LSM 510 software (Zeiss).

Results

Transient Expression of S1:GFP Antigen in the Cytosol of Agroinfiltrated Tobacco Leaves. Figure 2 shows representative tobacco leaf epidermal cells examined by confocal microscopy 2 days following agroinfiltration of *Agrobacterium tumefaciens* LBA4404 harboring plasmid pCV12 expressing the S1:GFP fusion protein (Fig. 2A, C) or LBA4404 harboring pGDG expressing GFP alone (Fig. 2B, D). GFP alone was localized to the cytosol and the nucleus as expected; accumulation of GFP to the nucleus has been previously reported to occur by passive diffusion (28). The S1:GFP fusion was localized to the cytosol, and also to the periphery of the nucleus as well, implicating that S1:GFP was secreted through the endoplasmic reticulum network.

In Western blot analysis using antibodies against GFP, tobacco leaves infiltrated with plasmid pCV12 expressing the S1:GFP fusion (Fig. 2E, lane 2) showed an expected band (calculated size 99.1 kDa), demonstrating that the S1:GFP fusion protein was successfully expressed.

Production and Analyses of Nuclear-Transformed Tobacco and Lettuce Expressing S1. Regenerated plantlets obtained by Agrobacterium-mediated nuclear transformation of tobacco and lettuce using vector pCV2 were analyzed by PCR using primers *35S* and *NOS*-ter followed by Southern blot analysis with a ^{32}P-radiolabeled *S1* probe (Fig. 3A–E). Figure 3A shows plants regenerated from tobacco leaves after Agrobacterium-mediated transformation. Figure 3B indicates a representative regenerated tobacco shoot used in PCR analysis. Figure 3C shows shoot regeneration from lettuce cotyledons after Agrobacterium-mediated transformation. Figure 3D displays representative regenerated lettuce shoots analyzed by PCR. Northern blot analysis on these plantlets (Fig. 3E) suggests the presence of a 2.1-kb *S1* hybridizing band in two independent tobacco lines (lanes 2 and 3), and two

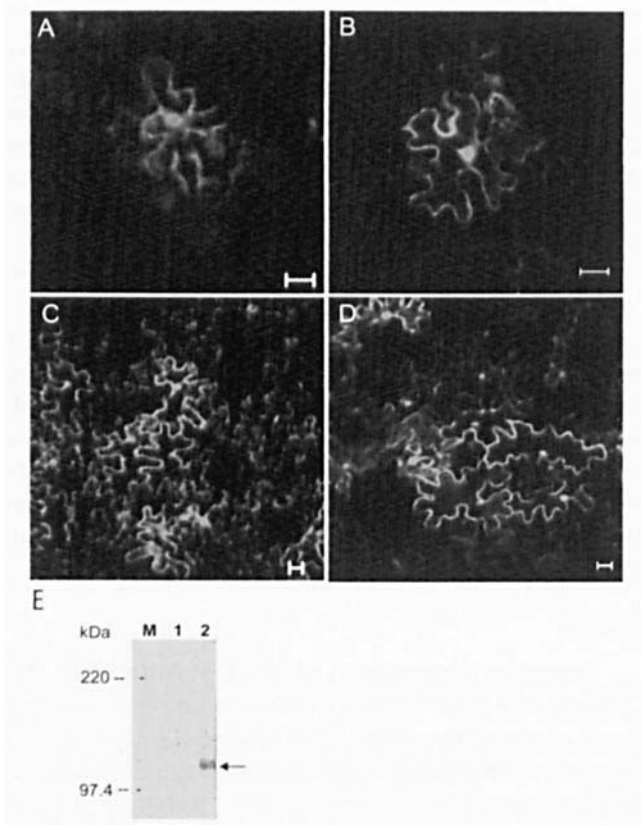

Figure 2. Transient expression of S1:GFP in agroinfiltrated tobacco leaves. Representative tobacco leaf epidermal cells are shown by confocal microscopy 2 days following agroinfiltration of *Agrobacterium tumefaciens* LBA4404 harboring plasmid pCV12 expressing S1:GFP fusion protein (A, C) or LBA4404 harboring pGDG expressing GFP alone (B, D). Bar represents 20 μm. Western blot analysis using antibodies against GFP show transient expression of S1:GFP in tobacco leaves following agroinfiltration. GFP alone (lane 1) or plasmid pCV12 expressing S1:GFP fusion (lane 2). Arrow indicates cross-reacting S1:GFP band (calculated size 99.1 kDa). M, molecular mass markers.

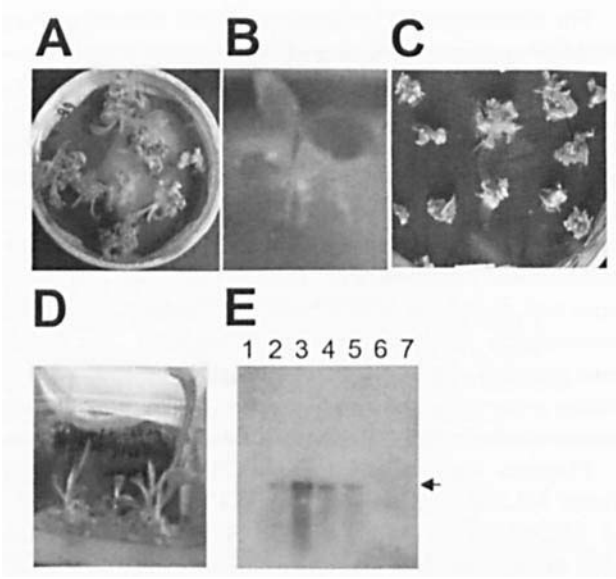

Figure 3. Plants obtained following nuclear transformation and their analysis. (A–E) Regenerated plantlets obtained following Agrobacterium-mediated transformation of tobacco and lettuce using nuclear transformation vector pCV2 and analysis of these plantlets by PCR using primers *35S* and *NOS*-ter followed by Southern blot analysis with a ^{32}P-radiolabeled S1 probe. (A) The plants regenerated from tobacco leaves after Agrobacterium-mediated transformation. (B) The regenerated tobacco shoot used in the PCR analysis. (C) The plants regenerated from lettuce cotyledons after Agrobacterium-mediated transformation. (D) The regenerated lettuce shoot used in the PCR analysis. (E) The presence of a 2.1-kb *S1* hybridizing mRNA band (arrowed) in Northern blot analysis of two independent tobacco lines (lanes 2 and 3), and two independent lettuce lines (lanes 4 and 5), and the absence of which in wild-type tobacco (lane 6) and wild-type lettuce (lane 7). One other tobacco line tested negative (lane 1).

independent lettuce lines (lanes 4 and 5), and the absence of this band in wild-type tobacco (lane 6) and wild-type lettuce (lane 7). One other tobacco line tested negative (lane 1).

Production and Analyses of Plastid-Transformed Tobacco. Plantlets regenerated after plastid transformation of tobacco using vector pCV1 were analyzed by PCR using *S1* primers followed by Northern blot analysis with a ^{32}P-radiolabeled *S1* probe (Fig. 4A–D). Figure 4A and B show the plantlets regenerated from tobacco leaves following bombardment in plastid transformation. Figure 4C indicates the specific 0.7-kb PCR-amplified *S1* band in regenerated tobacco (lane 2), which is absent in the wild type (lane 1). Figure 4D shows the presence of a 2.1-kb hybridizing *S1* mRNA band (arrowed) in a tobacco plastid-transformed line (lane 2), which was absent in the wild type (lane 1). Figure 4E displays results of Western blot analysis of transplastomic tobacco using Ni-NTA conjugate in the detection of His-tagged protein. The detection of an expected 73-kDa $(His)_5$-S1 band demonstrates that the $(His)_5$-tagged S1 protein was successfully expressed in tobacco chloroplasts. The yield of the $(His)_5$-tagged S1 protein in T_0 plastid-transformed tobacco is estimated to be approximately 0.2% total soluble protein. It is expected to increase in subsequent generations upon culture on selective media as homozygosity of S1-expressing plastids is attained.

Discussion

This study reports on both transient and stable expression of S1:GFP and S1, respectively, in the cytosol of transgenic plants and the chloroplasts of transplastomic plants as proof-of-principle for the eventual production of an oral vaccine against the SARS-CoV. Viral antigens expressed in transformed plants are effective in inducing mucosal and serum immune responses in animals, irrespective of parenteral or oral delivery. Viral antigens expressed in transformed plants could also be applied as reagents for antibody detection in serological tests.

Although the SARS epidemic was eventually controlled by case isolation, the high infectivity and mortality rate, the lack of specific treatment, and the potential for reemergence make it imperative to develop safe and effective methods of vaccination against SARS. A number of candidate vaccines using several approaches are under

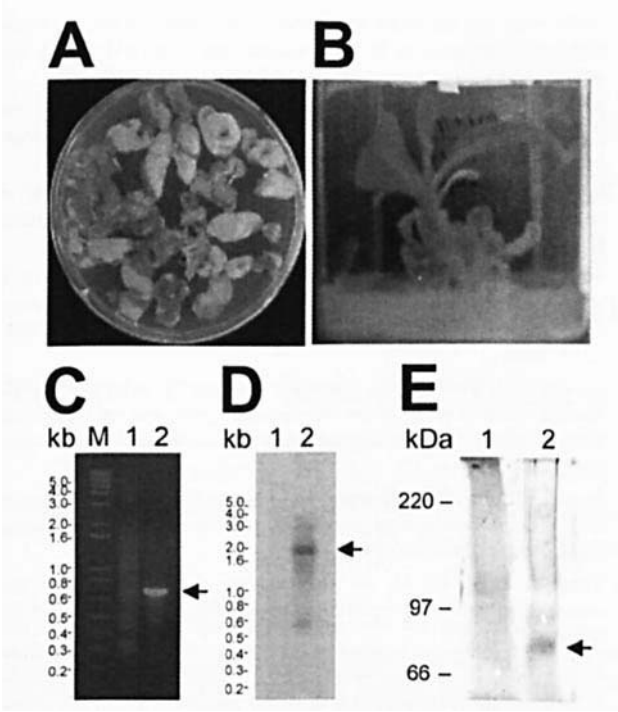

Figure 4. Plants obtained following plastid transformation and their analysis. (A–E) Regenerated plantlets obtained following plastid transformation of tobacco using plastid transformation vector pCV1; PCR analysis of these plantlets using *S1* primers and Northern blot analysis with a ^{32}P-radiolabeled *S1* probe. (A, B) Plantlets regenerated from tobacco after bombardment transformation. (C) Specific 0.7-kb PCR band in regenerated tobacco (lane 2), which is absent in the wild type (lane 1). (D) Presence of a 2.1-kb hybridizing *S1* mRNA band (arrowed) in a tobacco line (lane 2), which is absent in the wild type (lane 1). (E) Western blot analysis of transplastomic tobacco using Ni-NTA conjugate for detection of $(His)_5$-tagged protein. The arrow indicates the expected 73-kDa $(His)_5$-S1 band.

development. The first vaccine made from the inactivated form of SARS-CoV has already been tested in clinical trial (29). Several live attenuated, genetically engineered or vector vaccines encoding the SARS-CoV spike protein are in preclinical tests. These vaccine candidates are effective in terms of eliciting protective immunity in the vaccinated animals. However, caution must be advised in the use of whole virus or full-length S protein-based immunogens in humans due to the potential induction of harmful immune or inflammatory responses. Several studies have demonstrated that the S protein of SARS-CoV is a major virion structural protein. It is not only a functional domain that mediates virus-receptor binding but also a major inducer of neutralizing antibodies. It has been demonstrated that the receptor binding domain (RBD) of S is a linear epitope-dominant region capable of inducing highly potent neutralizing antibody responses and protective immunity (30). The screening and identification of linear B-cell epitopes of SARS-CoV with a synthesized 10-mer overlapping peptide library that spanned the major structural proteins of SARS-CoV also demonstrated that S (471–503), a peptide located at the RBD domain of SARS-CoV S1 subunit, could specifically block the binding between the RBD and angiotensin-converting enzyme 2, resulting in the inhibition of SARS-CoV entrance into host cells *in vitro* (31). That study suggested that the S (471–503) peptide was a potential immunoantigen for the development of safe and effective SARS vaccines (31). These findings may provide the basis for the development of immunity-based prophylactic, therapeutic, and diagnostic clinical techniques for SARS.

Therefore, in this study, the S1 subunit of SARS-CoV S protein was used for developing a safe and effective subunit SARS vaccine. The S protein of the SARS-CoV was chosen for expression in two plant species (tobacco and lettuce) to produce a potential SARS-CoV recombinant antigen. Lettuce is an easy-to-transform crop plant with abundant edible leaf tissue available for direct utilization and or downstream processing. Tobacco is an extremely efficient plant transformation system and is frequently used in plant biotechnology as the system of choice. After preliminary molecular characterization, transgenic plants were tested for the presence of recombinant S protein by Western blot analysis, which revealed the expression and accumulation of recombinant S protein in transient and stable transgenic or transplastomic plants. Our study provides a possibility of establishing a safe and inexpensive vaccination strategy against SARS. Because the plant chloroplast genome sequences are highly conserved, the same construct used here in tobacco plastid-transformation could be applied to tomato, which would constitute an oral vaccine in its edible fruit.

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