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Plant-made subunit vaccine against pneumonic and bubonic plague is orally immunogenic in mice

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Abstract

Yersinia pestis, the causative agent of plague, is an extremely virulent bacterium but there are no approved vaccines for protection against it. Our goal was to produce a vaccine that would address: ease of delivery, mucosal efficacy, safety, rapid scalability, and cost. We developed a novel production and delivery system for a plague vaccine of a *Y. pestis* F1–V antigen fusion protein expressed in tomato. Immunogenicity of the F1–V transgenic tomatoes was confirmed in mice that were primed subcutaneously with bacterially-produced F1–V and boosted orally with transgenic tomato fruit. Expression of the plague antigens in fruit allowed producing an oral vaccine candidate without protein purification and with minimal processing technology.
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1. Introduction

Plague is a disease caused by the bacterium *Yersinia pestis*. It is transmitted between animals and humans by the bite of infected fleas or by direct contact or inhalation of infective materials. The most common form of the disease is bubonic plague, which arises following a bite from a flea that has previously fed on an infected animal. Infection by *Y. pestis* in the bubonic form results in swollen and tender lymph nodes called “bubos”, hence the name of the disease. Pneumonic plague arises when there is a colonization of the alveolar spaces leading to pneumonia. This is the most feared form of plague because of the rapidity with which the disease develops (1–3 days); the high mortality rate in infected individuals (almost 100%); and the rapid spread of the disease from person to person as a consequence of inhalation of aerosolized, infected droplets [1].

Y. pestis is generally recognized to have caused three major pandemics of disease in the 1st, 14th–17th and 19th centuries, with 200 million deaths worldwide. In one of the epidemics during the second plague pandemic, known as the Black Death, it is estimated that over 30% of the population of Europe died as a direct result of infection [1]. Today, plague can be found predominantly in Southeast Asia, Southwest USA, Madagascar and other parts of Africa. Clearly, *Y. pestis* still has the potential to cause large-scale outbreaks of plague. Examples include an occurrence in India in 1994 and, most recently, in Congo in February 2005 [2]. These outbreaks reminded the world that plague is still a potential problem. Of greater concern is the possibility that *Y. pestis* might be used as a bio-terrorism or biological warfare agent because it has an alarming potential for causing massive morbidity and mortality in an exposed population [3]. Pneumonic

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plague is the most likely outcome in the illegitimate use of *Y. pestis* as a weapon. Unfortunately, the last commercial plague-vaccine, available in the US until 1999, offered poor protection against pneumonic plague. It was a whole cell vaccine containing formaldehyde-killed *Y. pestis* strain 195/P cells. The predominant protective immunogen present in this vaccine was the F1-antigen and therefore, could not provide protection against F1-negative strains of *Y. pestis*. In addition, it required a course of vaccination over six months and carried a significant risk of transient, and severe, side effects [4].

Antibiotic treatment of bubonic plague is usually effective but pneumonic plague is difficult to treat and death often results even with antibiotic therapy. Therefore, to circumvent a potential catastrophe after a natural or intentional outbreak of *Y. pestis*, it is crucial to obtain an easy to deliver and economic vaccine for the different forms of plague, particularly, pneumonic plague.

Of all the *Y. pestis* antigens tested, only F1 and V induce a good protective immune response against a challenge with the bacterium [5]. The F1 (Fraction 1) antigen is the major capsular protein. It forms a polymer composed of a protein subunit and plays an important role in inhibiting phagocytosis by macrophages [6]. The V antigen is a secreted protein that regulates the translocation of the cytotoxic effector proteins from the bacterium into the cytosol of mammalian cells [7]. The effector proteins (termed “Yops”) have a range of functions like promoting the death of phagocytic host cells and inhibiting the normal inflammatory response [8].

Protection afforded by candidate plague vaccines have been significantly enhanced by combining the F1 and V proteins [9,10]. Previous investigations have co-expressed the F1 and V proteins or an F1–V fusion protein [10–12]. Producing the F1–V antigen fusion protein may be advantageous to combining individual F1 and V antigens as purifying and characterizing one protein, rather than two, should lead to lower manufacturing costs [10]. Also, an injected subunit vaccine based on F1 and V antigens, using Alhydrogel as an adjuvant, provides good protection against an airborne challenge with *Y. pestis* in mice [12–15]. Promising results were recently reported with a two-dose intramuscular F1 and V subunit vaccine in humans [16]. However, there is still a clear need to provide alternative, economical vaccines more suited to the large-scale immunization of populations. Such a vaccine would be ideally administered non-invasively and promote a much better mucosal immunity against the infection. This is particularly desirable since aerosolization of *Y. pestis* as a bio-weapon would deliver the pathogen to a mucosal surface. The concept of a common mucosal immune system predicts that induction of immunity at one mucosal surface, such as the gut, can provide immunity at another mucosal surface, such as the lung [17]. Thus, the potential exists for oral delivery of *Y. pestis*’ antigens to elicit a protective immune response to plague.

We report production of an economical alternative plague vaccine candidate and investigate its elicited immune response in mice. We intend to address key issues such as: mucosal efficacy, ease of delivery, rapid scalability, safety, and cost. The F1–V-antigen fusion protein was expressed in transgenic tomato plants that were then molecularly characterized. The F1–V transgenic tomato fruit were pooled and freeze-dried to concentrate and standardize the dose of the antigen. The immunogenicity of the oral plant-made vaccine was tested in BALB/c mice which were primed subcutaneously (s.c.) using bacterially produced F1–V and boosted orally with freeze-dried, powdered, F1–V transgenic tomato fruit. The vaccine elicited IgG1 in serum and mucosal IgA in fecal pellets.

2. Materials and methods

In the ELISAs and Western-blots described in this section, the proteins used as standards for F1–V, F1, and V detection were recombinant bacterial forms (named F1–V, rF1 or rV) obtained as described by Heath et al. [10]. The primary antibodies (anti-F1–V, anti-V, and anti-F1–V) and the antigens used as standards were provided by A. Friedlander, USAMRIID, Ft. Detrick, MD.

2.1. F1–V design and insertion into different plant expression cassettes

We designed a synthetic gene encoding a fusion of the *Y. pestis* F1 and V proteins [10] which was optimized for expression in dicotyledonous plants. The gene encoded the same amino acid sequence as found in the bacterial-derived protein but plant preferred codons were used and spurious mRNA processing signals were removed (Genebank accession # DQ229852). The gene was assembled by a commercial supplier (Retrogen, San Diego, CA) and cloned into a commercial PCR cloning vector, PCR Blunt 3.5 kb (Invitrogen, Carlsbad, CA), to give pTopoF1–V. The 1456 bp *Nco*I–*Sac*I fragment from pTopoF1–V was inserted into the plant expression vector pIBT210.1 [18] to make pF1–V-IBT210.1. The expression cassette contained the strong constitutive CaMV 35S promoter, tobacco etch virus 5′-UTR and the soybean *vspB* 3′ region. The expression cassette was purified from pF1–V-IBT210.1 after digestion with *Hind*III and *Eco*RI and ligated into pGPTV–BAR [19] to yield p35SF1–V (Fig. 1A). pGPTV–BAR, a binary vector for plant transformation, carries the bar gene that confers resistance to the herbicide bialaphos [20].

We obtained the Cassava Vein Mosaic Virus (CsVMV) promoter (without a leader sequence) [21] on a proprietary plasmid from Dow AgroSciences (Indianapolis, IN) and incorporated it into an expression cassette with the soybean *vspB* 3′ region [18]. The F1–V fusion gene was ligated into this cassette using *Nco*I and *Sac*I restriction sites and the cassette was transferred into the binary vector pGPTV–Kan [19] to make pCFV110 (Fig. 1A). The N-terminal signal peptide from the soybean *vspA* coding sequence [22] was inserted

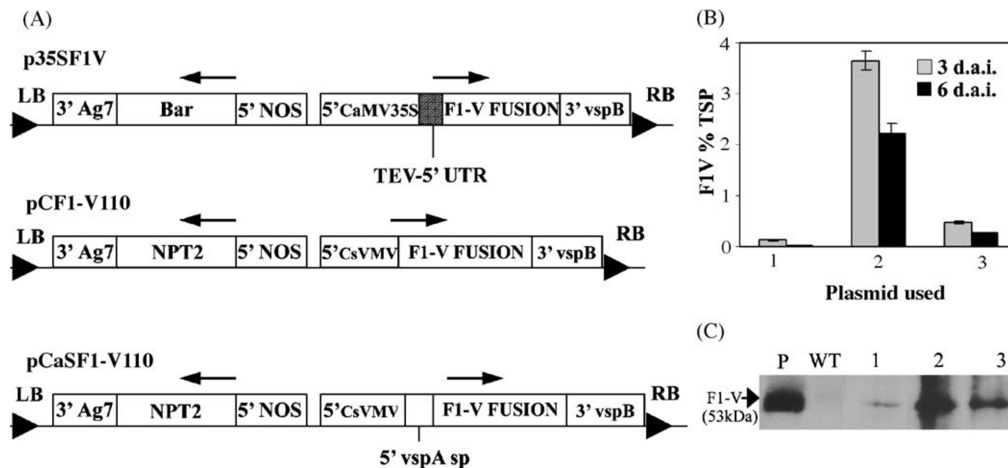

Fig. 1. (A) Structure of the T-DNA region of binary vectors used for transient expression of F1–V in *N. benthamiana* leaves. pCaSFV110 has the vspA signal peptide (ER-targeted) and pCFV110 does not (cytoplasm targeted). In both binary vectors pCaSFV110 and pCFV110, F1–V is under the control of the Cassava Vein Mosaic Virus (CsVMV) promoter. In 35S-F1V, transcription of F1–V is driven by the cauliflower mosaic virus 35S promoter (5′ CaMV 35S), and the tobacco etch virus 5′ untranslated region (TEV-5′ UTR) mediates enhancement of translation initiation. The 3′ UTR and polyadenylation signal of soybean vegetative storage protein gene (3′ *vspB*) lie 3′ of F1–V and mediates 3′ end processing of the transcript. Ag7: *Agrobacterium* gene 7 polyA signal. Analysis of the F1–V fusion protein transient expression in *N. benthamiana* leaves by: (B) F1–V ELISA; and (C) anti-F1–V Western-blot. Lanes 1, 2 and 3: F1–V transient expression driven by plasmids pCaSF1–V110, p35SF1–V and pCF1–V110, respectively. The total soluble protein (TSP) was extracted three and six days after the agro-infiltration. Bars are the means ± S.E.M. values obtained for three different leaves. d.a.i: Days after infiltration; F1–V% TSP: F1–V percentage of total soluble protein; P: 70 ng of purified bacterially produced F1–V.

into the *Nco*I site at the 5′ end of the F1–V gene to make pCaSFV110 (Fig. 1A).

2.2. Transient expression of F1–V in Nicotiana benthamiana leaves

The binary vectors p35SF1–V, pCF1–V110 and pCaSF1–V110 were introduced separately into *A. tumefaciens* strain LBA4404 [23] by electroporation. The transformed bacterial strains were then used to infiltrate *N. benthamiana* leaves. Bacteria were grown for 24 h at 28 °C, centrifuged at 6000 × *g* (AC 50.10 angle rotor, Jouan CR3i), and the pellet resuspended in infiltration buffer [10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.5, 300 μM acetosyringone] to an optical density at 600 nm of 0.5 using the SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA). The *Agrobacterium* solution was injected into fully expanded *N. benthamiana* leaves through a small puncture using a 3 ml needle-less syringe. The leaf tissue of the transfected area was collected on the third and sixth day after the agroinfiltration. The tissues were assayed immediately or frozen at −80 °C. This protocol was modified from Huang and Mason [24].

2.3. Stable tomato transformation

Agrobacterium-mediated transformation of tomato cotyledons (*Lycopersicon esculentum* variety Tanksley TA234TM2R) was performed according to Walmsley et al. [25] except that seeds were sterilized in 20% bleach for 20 min and rinsed three times in sterile distilled water. Briefly after, sterilization seeds were planted on half-strength Murashige and Skoog (MS) medium (50 mg l^{-1} myo-inosytol, 2 mg l^{-1} thiamine HCl, 0.5 mg l^{-1} pyridoxine HCl, 0.5 mg l^{-1} nicotinic acid, 10 g l^{-1} sucrose and 8 g l^{-1}Difco bacto-agar, pH 5.8). Cotyledons were excised before the first true leaves emerged and incubated in the *Agrobacterium* suspension (optical density at 600 nm of 0.5–0.6) for 10 min. Two strains of *A. tumefaciens*, EHA105 [26] and LBA4404 [23], each containing the binary plasmid 35SF1–V (Fig. 1A), were used for stable transformation. After co-cultivation, cotyledon explants were cultured with the adaxial sides up on selective 2Z medium (4.3 g l^{-1} MS salts, 20 g l^{-1} sucrose, 100 mg l^{-1} myo-inosytol, 2 mg l^{-1} glycine, 10 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine HCl, 0.5 mg l^{-1} Thiamine HCl, 0.5 mg l^{-1} folic acid, 0.5 mg l^{-1} d-biotin, 5.2 g l^{-1} agar, 300 mg l^{-1} timentin, 3 mg l^{-1} bialaphos, pH was adjusted to 6.0). When shoots were approximately 2 cm tall, they were transferred to selective rooting medium (4.3 g l^{-1} MS salts, 30 g l^{-1} sucrose, 2 mg l^{-1} glycine, 10 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine HCl, 0.5 mg l^{-1} Thiamine HCl, 0.5 mg l^{-1} folic acid, 0.5 mg l^{-1} d-biotin, 8 g l^{-1} bacto-agar, 300 mg l^{-1} timentin, 2 mg l^{-1} bialaphos, pH was adjusted to 6.0). Plants that rooted in selective rooting medium containing bialaphos were selected for PCR and F1–V ELISA analysis. Individual lines possessing the best F1–V fruit expression were self-pollinated and the resulting seeds germinated on MS medium supplemented with 2 g l^{-1} of bialaphos.

Subscript text used to describe a transgenic plant line, for example, T_0 indicates the number of sexual cycles that have

occurred after the transformation event. Hence, T_0 is used to identify the primary transgenic events (first generation) and T_1 are plants from the primary events (second generation).

2.4. Nucleic acids analysis

Fresh leaves from the tomato seedlings that regenerated on medium containing the herbicide bialaphos were analyzed using REDExtract-N-Amp Plant PCR kit (Sigma–Aldrich, Saint Louis, MO). Using primers specific for TEV (5′-GCATTCTACTTCTATTGCAGC-3′) and VSP (5′-GATACAGTCTCAGAAGACC-3′) sequences, an amplicon of 1.6 kb was expected in transgenic plants containing the F1–V gene. The icycler thermo-cycler (Bio-Rad, Hercules, CA) was programmed to run the PCR using an annealing temperature of 55 °C and 35 cycles. A negative control of wild type tomato genomic DNA and a positive control of p35SF1–V plasmid were included in each experiment. PCR samples were run on a 1% (w/v) agarose gel against a 1 kb ladder (New England Biolabs, Ipswich, MA).

Genomic DNA was prepared from 1 g of young leaf tissue using a CTAB extraction protocol [27]. Fifteen micrograms of DNA was analyzed by Southern-blot hybridization according to Sambrook et al. [28]. The DNA probe was made and labeled by PCR using the 35SF1–V plasmid as a template with the same primer set described above. Digoxigenin (DIG) labeled dUTP was incorporated into the 1.6 kb amplicon according to the manufacturer's instructions (PCR DIG probe synthesis kit, Roche Applied Science, Indianapolis, IN).

The reconstruction of the gene copy number was done using 39.5 pg, 197.5 pg and 395 pg of the 35S-F1V plasmid digested with *Hind*III and *Eco*RI (amounts equivalent to 1, 5 and 10 copies of F1–V gene per tomato haploid genome, respectively). Those amounts were calculated taking into account the size of the tomato haploid genome (655,000,000 bp) and the amount of plant genomic DNA used in this assay (9 μg). The F1–V gene copy number was determined for each T_1 tomato plant using densitometric analysis and comparing the intensities of each band in the Southern-blot with those corresponding to 1, 5 and 10 copies.

Hybridization was done for 18 h at 45 °C using the Dig East Hyb solution (Roche Applied Science, Indianapolis, IN) and 5 $\mu l\,ml^{-1}$ Dig PCR-labeled F1–V probe. The membrane was first washed three times, 10 min each, with low stringency buffer (0.30 M sodium chloride, 0.030 M sodium citrate, 0.1% (w/v) SDS) at room temperature. Afterwards, the membrane was washed twice, 30 min each, with high stringency buffer (0.03 M sodium chloride, 0.003 M sodium citrate, 0.1% (w/v) SDS). The detection was done as per the Manufacturer's instructions (DIG block and wash buffer set and DIG Luminescent Detection Kit, Roche Applied Science, Indianapolis, IN). Southern analysis of the plant lines was visualized after exposing the membrane to KODAK BioMax MS film.

Total RNA samples were isolated from 500 mg of young leaf tissue using RNAqueous (Ambion, Austin, TX) and Plant RNA isolation Aid (Ambion, Austin, TX). The RNA samples were quantified with the NanoDrop 1000 A Spectrophotometer (Ambion, Austin, TX). Five micrograms of total RNA was denatured with formaldehyde/formamide, separated on a 1% (w/v) agarose MOPS-acetate-EDTA gel, and stained with SYBR safe (Invitrogen, Carlsbad, CA). Afterwards, the RNA was transferred to a Zeta Probe membrane (Bio-Rad, Hercules, CA), by capillary action, as described by Sambrook et al. [28], and fixed by UV cross-linkage. The membrane hybridization, washes, and detection were done as with Southern analysis.

2.5. Protein extraction from leaves and freeze-dried fruit

Between 50 and 100 mg of young leaf or freeze-dried tomato powder was collected in 2 ml micro-centrifuge tubes for fast-prep (Fisher Scientific, Pittsburgh, PA). The tissue was treated with liquid nitrogen and pulverized using a micro-spatula. The powder was resuspended in 3 ml of PBS extraction buffer (phosphate-buffered saline, pH 7.2 and 10 $\mu g\,ml^{-1}$ leupeptin) per gram of leaf, or in 8 ml fruit extraction buffer (50 mM sodium phosphate, pH 6.6; 100 mM NaCl; 1 mM EDTA; 10 $\mu g\,ml^{-1}$ leupeptin) per gram of fruit powder. One bead (1/4 in. ceramic spheres beads; Qbiogene, Carlasbad, CA) was added to each tube before homogenization in a fast-prep machine (Fast-prep FP120 Bio, QBiogene, Carlasbad, CA) for 15 s at speed 4. The tubes were then centrifuged at 20,800 × *g* (Eppendorf microcentrifuge 5417R) for 5 min and the supernatant transferred to a new 1.5 ml tube. The total soluble protein concentration of the extracts was determined by Bradford Assay [29] (Bio-Rad, Hercules, CA), using known concentrations of bovine serum albumin (BSA) as the protein standard.

2.6. Western-blot analysis to detect F1–V fusion protein in transgenic plants

Five micrograms of total soluble protein (TSP) per sample was added to 6 μl 6 × SDS gel loading buffer (300 mM Tris–HCl, pH 6.8, 600 mM dithiothreitol, 12% SDS, 0.6% Bromophenol Blue, 60% glycerol), boiled for 10 min and placed on ice. Bacterially produced F1–V fusion protein was used as a positive control. The samples were centrifuged at 20,800 × *g* (Eppendorf 5417R microcentrifuge) for 5 min at 4 °C and then loaded on a SDS-polyacrylamide gel (10.5–14% Tris–HCl, 4% stacking, Bio-Rad, Hercules, CA). The gel was run at 30 mA for 3 h using Tris–glycine running buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS). The separated proteins were transferred from the gel to a PVDF membrane (Bio-Rad, Hercules, CA) using a Mini-Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA) and run left overnight at 17 V (Bio-Rad, Hercules, CA). The membrane was then blocked with 2.5% dry milk in PBST for Western (PBS buffer plus 0.1% Tween 20) for 2 h at room temperature. After a brief wash in PBST, the membrane was incubated with rabbit polyclonal antibody against F1–V, at

dilution 1:2000 in 1% dry milk in PBST, for 1 h at 37 °C. After a 15 min wash followed by three 5 min washes, the membrane was incubated with horseradish peroxidase conjugated goat polyclonal IgG antibody against rabbit (Sigma–Aldrich, Saint Louis, MO), diluted at 1:10000 in 1% dry milk in PBST. The membrane was then washed in PBST for 15 min followed by three washes of 5 min. Protein detection was performed using ECL Plus (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

2.7. Enzyme-linked immunosorbant assay analysis (ELISA)

The total soluble protein (TSP) was extracted from freeze-dried fruit and fresh leaves as previously described. The extracts were incubated in the ELISA plate (high bind polystyrene EIA/RIA 96 well microplate, Corning, NY) for 2 h at 37 °C, followed by three washes with PBST for ELISA (PBS plus 0.05% Tween 20), and blocking with 5% (w/v) dry milk in PBST for 1 h at 37 °C. After another three washes, rabbit anti-F1–V polyclonal antibody (or anti-V or anti-F1 polyclonal antibody for V- or F1-ELISA, respectively) at a dilution of 1:4000, was added and incubated for 2 h at 37 °C. After another three washes with PBST, the plate was incubated with peroxidase labeled goat anti-rabbit (Sigma–Aldrich, Saint Louis, MO) at a dilution of 1:3000 in PBST and final detection performed using TMB peroxidase substrate (Bio-Rad, Hercules, CA). The reaction was stopped after 5 min with 1 N H_2SO_4 and the optical density was read at 450 nm using the Microplate Reader Thermo Max (Molecular Devices Inc., Sunnyvale, CA).

2.8. Tomato fruit processing

A fresh fruit sample from each T_0 and T_1 tomato plant were collected, kept overnight at −20 °C and then freeze-dried (100 SRC Virtis freeze-drier) for at least 72 h. The dried fruit coming from the same plant was then pulverized to a powder, pooled and stored in vacuum-sealed plastic bags at room temperature.

2.9. Animal trials: prime-boost strategy

Female BALB/c mice, six–eight weeks old, raised under specific-pathogen-free conditions (Charles River Laboratories), were used in this study. The mice were divided into groups of five or six for immunization using a prime-boost strategy. On day 0, 11 mice received a sub-cutaneous (s.c.) prime of 10 μg purified, bacterially produced F1–V fusion protein adsorbed to the aluminum hydroxide adjuvant, Alhydrogel (AL), at 0.19 mg per dose (Heath et al., 1998 [10]), suspended in a volume of 0.2 ml MilliQ ultrapure (Millipore, Billerica, MA) sterile water. The test diet in the feed treatments consisted of 2 g of freeze-dried tomato fruit powder blended with 7 g of food mixed with a blender comprised of: two whole apples (small Washington Delicious apples), one-half cup of mixed nuts (peanuts, cashews, almonds, halberts, brazils), two tablespoons of honey, and one-fourth cup of water. The pH of the food mix was adjusted to 7. Five of the 11 mice were fed with control tomato powder and the other six with transgenic tomato powder containing 300 μg of F1–V on days 21, 28 and 35 and a final higher dose of 1200 μg on day 42. In the first three boostings only tomatoes from T_0 plants were used. On the fourth and last boost, green tomatoes from the T_1 plants were used increasing the dosage by four-fold. On the day of the boosting, mice were removed from their cages to individual holding cages with water and fasted for 18 h. Before delivery of the orally administered transgenic or non-transgenic tomato material, 10 μg of purified bacterial cholera toxin (Sigma–Aldrich, Saint Louis, MO) was given to the mice as an adjuvant in 0.25 ml sodium carbonate buffer (350 mM), pH 8.5 by gastric intubation (gavage) [30]. Residual test diet, if any, was collected and weighed to determine the amount ingested by the animals. All the animals were housed separately so that fecal pellets could be collected. Samples of fecal pellets and serum (100 μl by way of tail bleed using a 22 G needle) were collected on days −1, 20, 27, 34 and 41 to determine anti-F1 and anti-V IgG or IgA antibody by ELISA. Animals were provided with food and fresh water ad libitum during the experiment except for the days of boosting when they were only provided with water and the test diet. The experimental protocol involving mice was approved by the Arizona State University Animal Care and Use Committee.

2.10. Determination of IgG_1 and IgG_2 titers in serum and IgA titers in fecal samples

ELISA plates (high bind polystyrene EIA/RIA 96-well microplate, Corning, NY) were coated with 2.5 $\mu g\,ml^{-1}$ rF1 or rV antigen in PBS and incubated overnight at 4 °C. After three washes with PBST for ELISA (PBS, pH 7.4, plus 0.05% Tween 20), plates were blocked with 1:10 dilution of fetal calf serum (FCS) in PBS and incubated for 1 h at 37 °C. After three washes with PBST, the serum was serially diluted two-fold in the plates using FCS and starting at 1:100. The plates were then incubated for 1 h at 37 °C before being washed three times with PBST and incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse-IgG1 (Southern Biotech), at a dilution of 1:4000 in blocking buffer (1:10 dilution FCS in PBS), for 1 h at 37 °C. For IgG2a, the plates were incubated with HRP conjugated goat anti-mouse-IgG2 antibody (Southern Biotech), at a dilution of 1:4000 in blocking buffer, for 1 h at 37 °C. After washing four times with PBST, the detection was done using TMB peroxidase substrate (Bio-Rad, Hercules, CA) for 5 min. The reaction was stopped with 1 N H_2SO_4 and then the absorbance read at 450 nm using the Microplate Reader Thermo Max (Molecular Devices Inc., Sunnyvale, CA). Titers were estimated as the reciprocal of the maximum dilution of serum giving an absorbance reading of 0.1 units after subtraction of non-specific binding in serum from non-treated animals (negative control). Concentrations

of serum anti-F1 or anti-V IgG1 or IgG2a were determined by linear regression from a standard curve of mouse myeloma IgG1 or IgG2a (Sigma–Aldrich, Saint Louis, MO).

Fecal samples were prepared by adding 5 ml of extraction buffer (PBS, 0.1% Tween 20 and 10 μg ml^{-1} leupeptin) per gram of material. Suspensions were kept for 30 min at 4 °C and thoroughly homogenized with a QBiogene Fast Prep machine for 1 min. After incubating for another 30 min at 4 °C, suspensions were centrifuged for 10 min at 20,800 × g (Eppendorf microcentrifuge 5417R), at 4 °C. The supernatant was collected and clarified again by centrifuging at 20,800 × g at 4 °C for 1 min. The twice-clarified supernatant was collected and stored at −20 °C until it was assayed or immediately analyzed by ELISA. The total soluble protein (TSP) in each sample was tested using the Bradford Assay (Bio-Rad, Hercules, CA). Two-fold serially diluted samples, starting at 50 μg of TSP, were applied onto the plates (previously coated with rF1 or rV and blocked as was already described for serum samples) and incubated for 1 h at 37 °C. After washing three times with PBST, plates were incubated for 1 h at 37 °C with anti-mouse-IgA antibody (Sigma–Aldrich, Saint Louis, MO) diluted 1:1000 in blocking buffer. Detection was performed as was previously described for serum samples.

2.11. Statistical analysis

All statistical analyses were performed using the software SPSS 13.0 for Windows. Regardless of the test applied, results were considered statistically significant if $P < 0.05$. A repeated measure ANOVA with a Turkey-Kramer multiple comparisons test was used to determine significant difference between detected F1–V, F1 and V in the same protein extract. The same test was applied to determine significant difference between F1–V percentage of total soluble protein (F1–V% TSP) of fresh leaves, green and red powdered tomato. The one-way ANOVA test, with a post hoc Dunnett T3 test, was used to determine significant differences in F1–V percentages of total soluble protein (F1–V% TSP) between different tomato ripening fruit stages. The same test was used to compare F1–V transient expression in *N. benthamiana* leaves using three different plasmid constructs (pCF1–V110, pCaSF1–V110 or 35SF1–V). A two-tailed paired Student's t-test was applied to determine significant differences in serum F1- and V-specific IgG1 and IgG2a before and after boosting with control or F1–V transgenic tomatoes (intra-groups comparison). The Levene's test was applied to determine equality of variances in the antibody concentrations between the mice group boosted with control tomatoes or the group boosted with F1–V transgenic tomatoes (inter-groups comparisons). When the variances were equal ($p > 0.05$), the unpaired Student's t-test for equality of the means was applied to determine significant differences in pre-boost or post-boost antibody concentrations. When the variances were unequal ($p < 0.05$ in Levene's test), an unpaired Student's t-test with Welch corrections for different variances was used. Fisher's exact test was applied to determine significant differences in the number of transgenic plants obtained using *A. tumefaciens* strain LBA4404 or EHA105.

3. Results

3.1. Transient expression of F1–V in N. benthamiana leaves

We studied and compared the F1–V transient expression in *N. benthamiana* leaves using three different plasmid constructions: p35SF1–V, pCF1V110 and pCaSF1V110 (Fig. 1A). *N. benthamiana* leaves were infiltrated with three different cultures of *A. tumefaciens* strain LBA4404 bearing one of the three different plasmid constructs. The soluble proteins extracted three and six days after agroinfiltration were analyzed by ELISA (Fig. 1B) and Western-blot (Fig. 1C). The transient F1–V expression using plasmid p35SF1–V is significantly higher ($P = 0.006$) than that obtained using plasmids pCaSF1–V110 or pCF1–V110. Both constructs use the CsVMV promoter to drive expression of F1–V. However, pCaSF1–V110 contains sequences coding for the soybean vegetative storage protein signal peptide vspA (VSP alpha S) fused to F1–V, which is expected to target F1–V to the endoplasmic reticulum (ER). pCF1–V110 does not contain this sequence, hence expression should be targeted to the cytoplasm. Transient F1–V expression using pCF1–V110 is significantly higher ($P = 0.007$) than that found with pCaSF1–V110. Transient, F1–V expression is significantly higher ($P < 0.05$) at 3 days after infiltration (d.a.i.) than at 6 d.a.i. (Fig. 1B) with all three constructs. ELISA analysis of *Agrobacterium* cultures containing the F1–V constructs verified that there was no expression of the fusion protein by the bacteria (data not shown) and, therefore, the expression observed in leaves was derived only from leaf cells.

A specific band of 53 kDa corresponding to the F1–V fusion protein was detected by Western-blot in the positive control (lane "P", bacterially produced F1–V) and in all the sample lanes, except for the W.T. (wild type) control lane (Fig. 1C).

The plasmid p35SF1–V was used for stable transformation of tomato on the basis of the higher levels of transient expression.

3.2. Screening of transgenic plants

We used two different *A. tumefaciens* strains, EHA105 and LBA4404, in stable tomato transformation experiments. Out of 175 explants (cotyledons) transformed with the p35SF1–V vector carried by *Agrobacterium* strain LBA4404, 58 tomato plants resistant to the herbicide bialaphos were regenerated and 29 (50%) of them were positive for the F1–V gene by PCR. The same number of explants were transformed with *Agrobacterium* strain EHA105 resulting in 61 tomato plants

being selected using bialaphos. Of these, 37 (61%) were positive for the F1–V gene by PCR. Regardless of the strain used for transformation, there is no significant difference ($P = 0.27$) in the number of F1–V or bialaphos transgenic tomato plants obtained. However, use of EHA105 resulted in more rapid plant regeneration.

A total of 66 out of 119 plants regenerated on Bialaphos selective medium were positive by PCR for F1–V. In addition to the specific F1–V amplification product of 1.6 kb, there was a spurious band of about 600 bp that was always present even in the W.T. negative control (Fig. 2). The intensity of this non-specific extra band was proportional to the amount of DNA template in all cases. Therefore, we were able to use it as a positive internal control for the PCR reaction.

3.3. F1–V fusion protein expression in T_0 and T_1 tomato plants

Fresh leaves of the 66 T_0 tomato plants found positive by PCR were analyzed by Indirect F1–V ELISA. Five T_0 plants (transformants 4, 8, 21, 22 and 23) were considered and were carried forward because of their higher F1–V expression. Tomato fruit from the same plants expressing the fusion protein F1–V were pooled and freeze-dried to increase their shelf-life. Fig. 3 shows the comparison of the F1–V expression in fresh leaves with red and green freeze-dried tomatoes in different T_0 and T_1 plants.

We chose a total of nine T_1 plants (4.6, 4.12, 4.14, 4.48, 4.51, 4.52, 22.1, 23.7, 23.13) because of their high F1–V expression levels in leaves and fruit (Fig. 3B). Expression of F1–V varied from 0.9 to 4.6% TSP in fresh leaves. There is no significant difference between the percent of TSP in both green and red fruit for plant 4.6 or between leaves and green fruit for plant 4.14. However, all the other T_1 lines had significantly higher percentages of TSP in green freeze-dried fruit than in fresh leaves ($P < 0.05$) and red freeze-dried fruit ($P < 0.01$). In all the cases, the T_1 tomato plants showing high antigen expression in fruit or leaves had indistinguishable phenotypes from non-transgenic control plants or plants with low antigen expression levels.

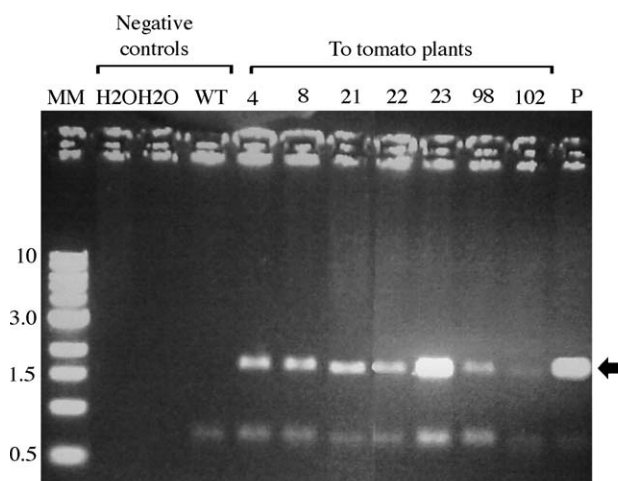

Fig. 2. PCR analysis of genomic DNA extracts from T_0 tomato plants. MM: molecular marker 1 kb ladder. Band sizes are in kb. W.T.: wild type (non-transformed tomato). H_2O: amplification without plant DNA using water as the template. P: positive control (amplification product from p35SF1V). The arrow points out the specific F1–V amplification product (1.6 kb).

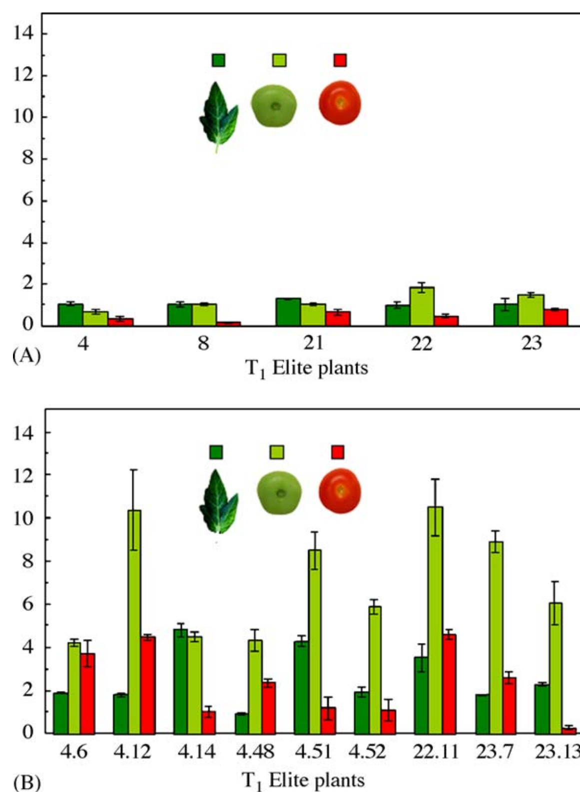

Fig. 3. F1–V fusion protein expression in pooled, fresh leaves, green and red freeze-dried tomato fruit from: (A) T_0 lines and (B) T_1 lines. F1–V% TSP: F1–V percentage of total soluble protein. The name of each T_1 plant begins with the parent T_0 plant before the dot and the specific T_1 plant number after the dot. Bars are means of three repetitions ± S.E.M.

There are six tomato fruit ripening stages according to the California Tomato Commission: green, breakers, turning, pink, light red, and red (http://www.tomato.org/reflex/food/color.html). We analyzed pooled, freeze-dried tomato fruit at each stage, from the same T_1 plant, T23.13, by F1–V ELISA (Fig. 4A). We found that the F1–V percent of TSP in pooled fruit at ripening stage 1 (fruit fully developed but still completely green) is significantly higher than in all the other stages ($P < 0.01$ in all cases). Similar results were found with all other T_1 lines (data not shown), therefore, we used only green fruit for vaccine powder production from T_1 generation plants for the final boosting. Red tomato fruit were collected only for seed harvest.

Western-blot analysis was used to investigate the integrity of the F1–V fusion protein in green, pooled, freeze-dried tomato fruit from six of the T_1 lines. A strong band of 53 kDa corresponding to the F1–V fusion protein was present in all the sample lanes except in the W.T. (wild type or negative

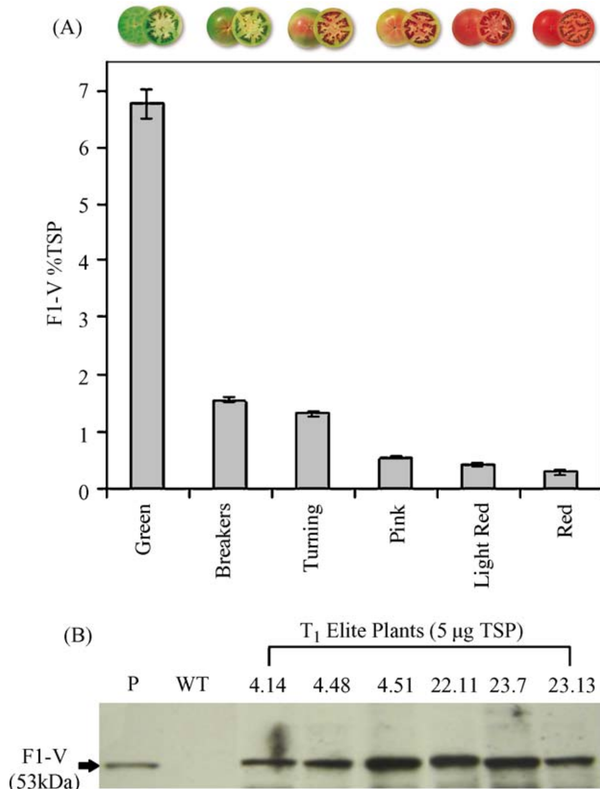

Fig. 4. Expression of F1–V fusion protein in pooled, freeze-dried fruit. (A) F1–V% TSP in six different fruit ripening stages, from the T_1 line 23.13. (B) Western-blot for F1–V in pooled, green, freeze-dried tomato fruit from six different T_1 lines. All samples were standardized to contain 5 μg of total soluble protein (TSP). F1V% TSP: F1–V percentage of total soluble protein. W.T: wild type (non-transformed plant). Bars are the means of three repetitions ± S.E.M. P: positive control (10 ng of purified bacterial recombinant F1–V).

control) (Fig. 4B). The lower molecular weight bands may correspond to minimal partial degradation products of the mature protein.

The antigenicity of the plant-made F1–V fusion protein and each of its individual components (F1 and V), was studied using F1, V and F1–V ELISA of the same protein extracts from three T_1 lines (Fig. 5). The amount of F1–V per gram of dried fruit determined by F1–V ELISA is significantly higher than F1 or V antigen alone in the three T_1 plants analyzed ($P < 0.05$ in all the cases). However, there is no significant difference between F1 or V antigen levels in the three plants studied. The F1-antibody recognizes the plant-derived F1–V (54 kDa. band, lane 4.51, Fig. 6A); the recombinant bacterially expressed F1–V and F1, but not V (F1-Western-blot, Fig. 6A). The V-antibody recognizes the plant-derived F1–V (54 kDa band); the recombinant bacterially expressed F1–V and V, but not F1 (V-Western-blot, Fig. 6B).

3.4. Nucleic acid analysis of T_1 tomato plants

It is well known that transgene copy number can affect the level and stability of transgene expression, determining

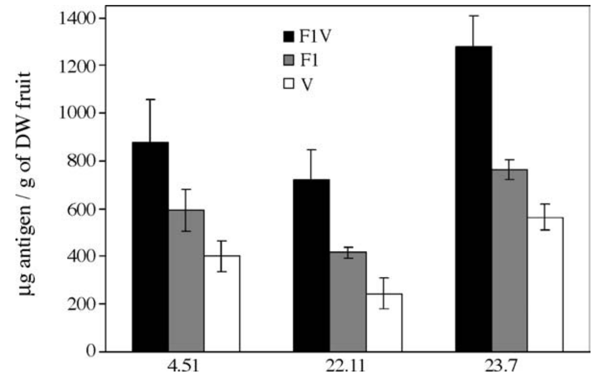

Fig. 5. Comparison of F1, V and F1–V antigen detection by F1, V and F1–V ELISAs in the same protein extracts from green freeze-dried fruits of T_1 plants 4.51, 22.11 and 23.7. In each case the standard curve was constructed using bacterial F1, V and F1–V, respectively. DW: dry-weight. Bars are means of three repetitions ± S.E.M.

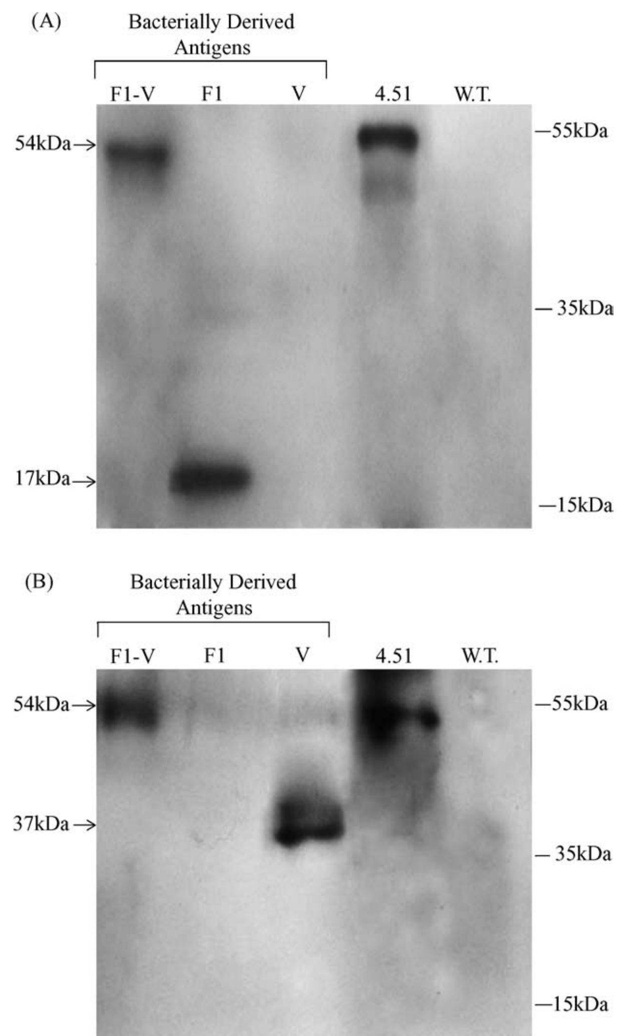

Fig. 6. Antigenicity study of the F1–V fusion protein in green, freeze-dried fruit. (A) F1-Western-blot using F1-specific antibody. (B) V-Western-blot using V-specific antibody. Lanes 4.51 and W.T.: protein extracts from green freeze-dried fruit from T_1 plant 4.51 and wild type, respectively.

in most of the cases the susceptibility of the gene to silencing [31,32]. Hence, the F1–V transgene copy number of the T_1 tomato plants was estimated by Southern-blot analysis. An expected specific band of about 2800 bp, corresponding to the 35S-F1V cassette digested with *Eco*RI and *Hind*III, was present in all the lines except in the W.T. (wild type or non-transformed tomato plant) (Fig. 7A). An accurate calculation of gene copy number in sibling plants 23.7 and 23.13 was confounded because of bands with higher molecular weights in addition to the expected size of 2.8 kb. These bands likely correspond to different F1–V insertion sites where at least one of the restriction sites at the border of the 35SF1–V cassette, for *Hind*III or *Eco*RI, has disappeared by mutation or deletion. The same could have happened with the 4.8 kb extra band in lanes 1, 2 and 3 (plants 4.12, 4.14 and 4.51, all siblings coming from plant T_4). Table 1 summarizes the estimated F1–V gene copy number in each T_1 plant. There is a weak positive correlation between the F1–V gene copy number and the F1–V protein levels in green, freeze-dried tomatoes in the T_1 plants (Pearson's correlation $r = 0.2848$). Only 8% of the variance in the F1–V protein levels in green fruit can be explained by variation in F1–V gene copy number (coefficient of determination $r^2 = 0.08$).

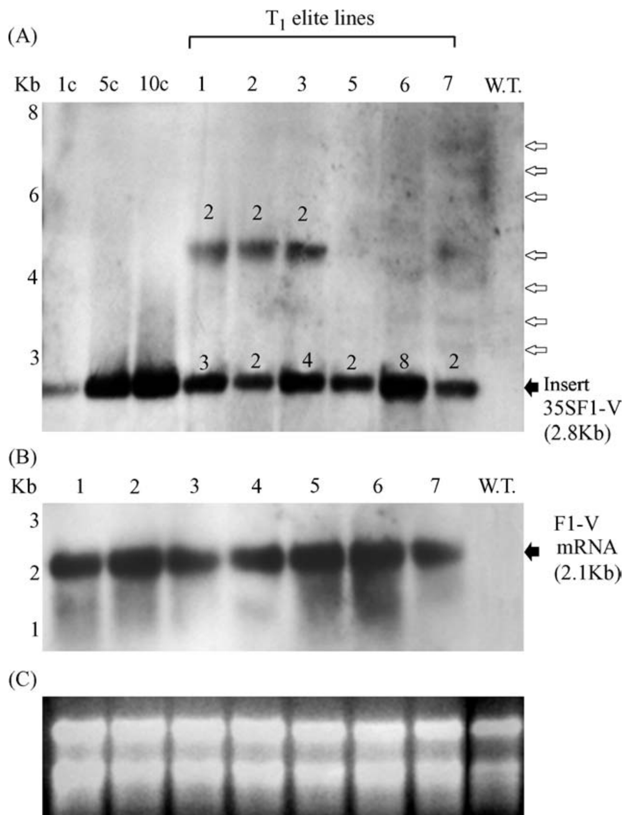

Fig. 7. Nucleic acid analyses of T_1 tomato plants. (A) Southern-blot of genomic DNA digested with *Eco*RI and *Hind*III that cut at both extremes of the 35S-F1V cassette. A DIG-labeled F1–V probe was used to detect the gene. 1c; 5c; and 10 c: 1, 5 and 10 copies of 35S-F1V, respectively. W.T: wild type. Numbers above each band represent the estimated gene copy number determined by densitometric analysis of the intensity of each band compared to 1, 5 and 10 copies of the 35S-F1V (for total F1–V gene copy number per plant see Table 1). White arrows point out extra high molecular weight bands (additional to 2.8 kb) corresponding to more gene insertion sites. (B) Total RNA (5 μg) from wild type (W.T.) and transformed T_1 tomato plants was separated on a 1% (w/v) formaldehyde agarose gel followed by capillary transfer and hybridization with F1–V probe labeled with digoxigenin. (C) rRNA quality in SYBR stained 1% (w/v) agarose gel. Lanes 1–7 correspond to the following T_1 plants: 4.12; 4.14; 4.51; 4.52; 22.11; 23.7 and 23.13, respectively.

Table 1
F1–V gene copy number, and fusion protein expression in green freeze-dried pooled tomato fruit

T_1 plant	μg F1–V g^{-1} DW[a] (green fruit)[b]	F1–V gene copy number[c]
4.12	1669 ± 184	5
4.14	860 ± 23	4
4.51	880 ± 50	6
22.11	1660 ± 134	2
23.7	1694 ± 60	About 15 copies
23.13	1392 ± 134	About 9 copies

[a] DW: dry-weight.
[b] Values are means ± S.E.M. (three repetitions per plant).
[c] Estimated by Southern-blot.

Northern-blot analysis of total RNA extracted from leaves of T_1 plants revealed a band of approximately the predicted size (2.1 kb) in all the experimental plants but not in the W.T. lane (Fig. 7B). In spite of the fact that the same amount of total RNA was loaded per sample (Fig. 7C) and the plants have different F1–V gene copy numbers (Fig. 7A), the F1–V mRNA band intensity was similar for all the transgenic plants (Fig. 7B).

3.5. Oral immunogenicity of dried F1–V tomato in mice

The ability of F1–V in freeze-dried tomato fruit to induce serum and mucosal antibodies when fed to BALB/c mice was tested in prime-boost experiments. F1- and V-specific serum immunoglobulin G1 (IgG1) was detected in 100% of the mice that were primed subcutaneously with bacterial F1–V and boosted with control or F1–V transgenic tomato. The peak of the serum F1- and V-specific IgG1 occurred at 21 days after the last boost (a.l.b.) at day 63 post-primary immunization. Fig. 8 shows the analysis by ELISA of serum and fecal pellets at 21 days after the last boost. The average of serum F1- and V-specific IgG1 ($n = 6$) is significantly higher after the mice were boosted with F1–V tomatoes ($P = 0.030$ and $P = 0.023$, respectively) (Fig. 8A and B, respectively). In the control group of mice ($n = 5$), there is no significant increase in serum F1-specific ($P = 0.79$) or V-specific ($P = 0.08$) IgG1 after boosting with control tomato powder. However, the F1- and V-specific IgG1 are significantly higher ($P = 0.04$ and 0.03, respectively) in mice boosted with transgenic F1–V tomatoes than in the mice boosted with control tomato fruit (Fig. 8A and B, respectively).

F1- and V-specific serum immunoglobulin G2a (IgG2a) was detected in 100% of the mice immunized with subcutaneous bacterial F1–V in sera collected on day 21 a.l.b. There is a significant difference in the average serum F1-specific

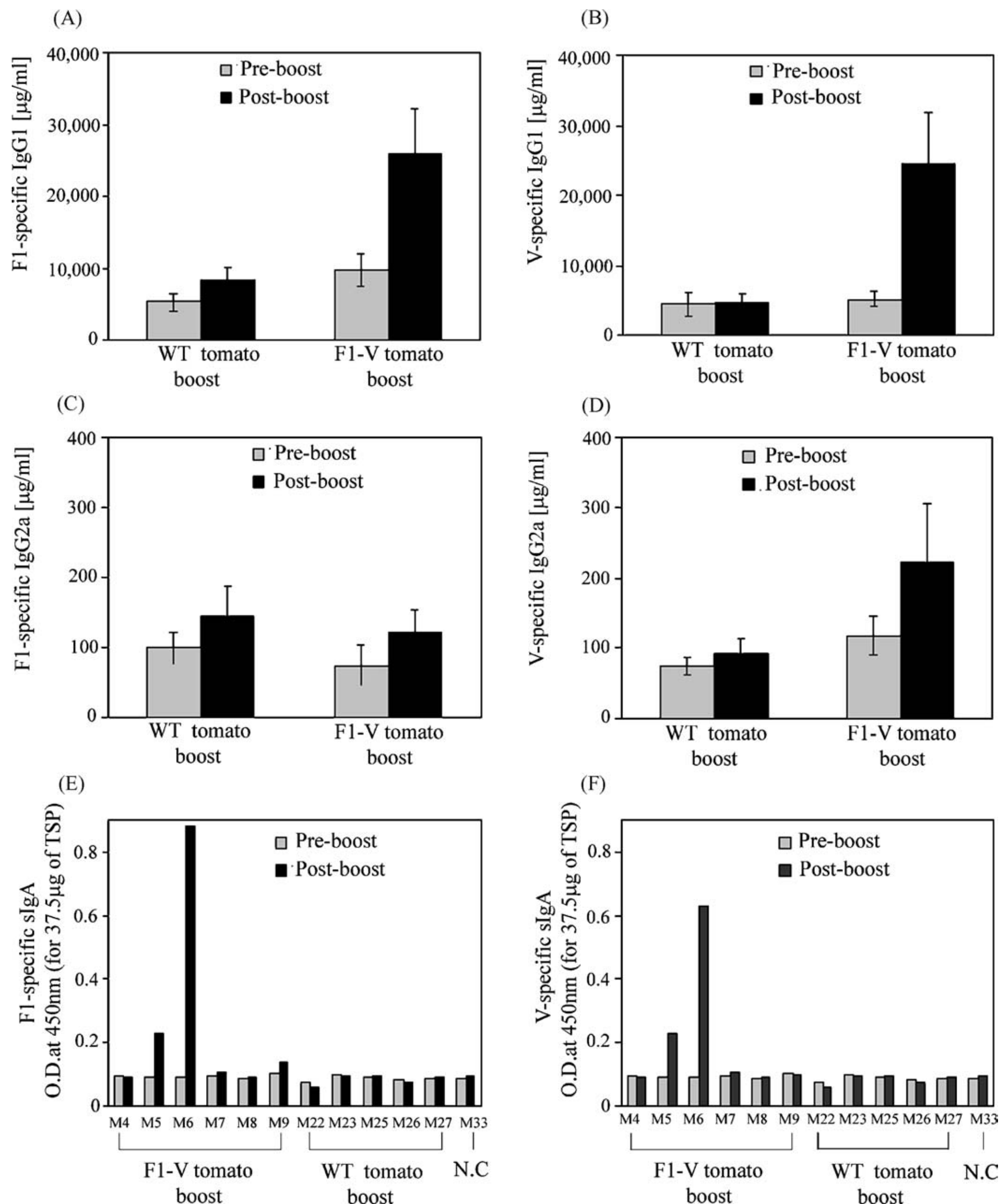

Fig. 8. Prime-boost experiments in BALB/c mice. Prime: subcutaneous F1–V; Boost: W.T. (wild type) or F1–V tomato. (A) Average serum F1-specific IgG1 ($n = 6$); (B) average serum V-specific IgG1 ($n = 6$); (C) average serum F1-specific IgG2a; (D) average serum V-specific IgG2a. Bars represent the group mean endpoint concentrations in ($\mu g\,ml^{-1}$). Error bars are ±S.E.M. Mucosal IgA in fecal pellets per mouse: (E) F1-specific mucosal IgA; (F) V-specific mucosal IgA.

IgG2a ($P = 0.012$) after mice ($n = 6$) were boosted with transgenic tomato (Fig. 8C). However, the difference in the average serum V-specific IgG2a after boosting with F1–V transgenic tomatoes is not significant ($P = 0.18$) (Fig. 8D), and neither is the difference in F1- and V-specific IgG2a in mice boosted with control tomatoes ($n = 5$).

F1-specific and V-specific mucosal IgA was detected in fecal pellets in two out of six mice 21 days after the last boosting with transgenic F1–V tomato (Fig. 8E and F). Particularly, mouse number 6 (M6) had an eight-fold increase in the F1-specific sIgA and a six-fold increase in the V-specific sIgA in fecal pellets after the last boost with the transgenic tomato fruit. In mice immunized only subcutaneously with bacterially produced F1–V or in those boosted with control tomato, F1- or V-specific sIgA was not detected at any time during the assay (an OD at 450 nm lower than 0.1 was considered background).

4. Discussion

There have been previous initiatives to devise an alternative plague vaccine that could be administered needle-free and they are currently being tested in animal trials by dif-

ferent research groups. One of these is a micro-encapsulated preparation of F1 and V antigens, delivered intranasally to mice, that protects against parenteral and inhalation challenges with *Y. pestis* [33]. Another utilizes oral immunization with a recombinant *Salmonella enterica* expressing *Y. pestis*' antigens that has also been reported to provide protection against a subsequent challenge with the bacteria [34–36].

The production of therapeutic proteins in plants represents an economical alternative to fermentation-based expression systems, especially for the manufacturing of high-volume reserves of subunit vaccines (for a review see [37]). Plants have been shown to provide both an encapsulated antigen and an oral delivery system. Plant-made vaccine antigens can be delivered to a mucosal surface (for example, when provided orally or intranasally). Additionally, plants can be grown locally and inexpensively using the standard growing methods of a given region and can also be produced virtually indefinitely from seeds [38]. Oral delivery is made possible because it is believed that the plant cell wall provides enough protection against degradation to allow much of the vaccine antigen expressed in the cells to reach the gut-associated lymphoid tissue (GALT) in an intact and immunogenic state. Since plant-made vaccines were first described by Curtis and Cardineau [39], different groups have experimented with transgenic plants for expression and oral delivery of recombinant vaccine antigens. The six human clinical trials accomplished with plant-made vaccines have shown the potential of using the plant-made vaccine technology [40–45].

In this paper, we describe the development and evaluation of an alternative oral subunit vaccine candidate against plague, produced by expressing the F1–V fusion protein in tomato. To our knowledge, this is the first report of a plant made, orally delivered plague-vaccine. Tomato has many advantages over other host plants for the production of oral vaccines. Tomato yields large masses of palatable fruit that are edible raw (avoiding heat denaturation of the antigens) and has well established industrial greenhouse culture and fruit processing. Unfortunately, a vaccine expressed in fresh tomato fruit has a short shelf-life. For this reason, fresh tomato fruits expressing the fusion protein F1–V were pooled and freeze-dried. Freeze-drying is a well-established technology that is inexpensive and provides antigen stability at room temperatures, batch consistency and concentrated antigen. The integrity and antigenicity of the F1–V fusion protein in the freeze-dried, tomato fruit powder was confirmed by ELISA and Western-blot analyses.

We constructed three different plasmid binary vectors bearing the F1–V fusion protein: p35SF1–V, pCaSF1–V110 and pCF1–V110 (Fig. 1A). Transient expression of these vectors found: (a) the plant-made F1–V fusion protein retained its native antigenicity since it was recognized by polyclonal antibody targeted against the bacterially produced F1–V; (b) CaMV35S promoter/TEV-5′ UTR is significantly better than CsVMV promoter at driving high expression of F1–V in the transient assay; (c) the F1–V expression is significantly higher when it is cytoplasm rather than endoplasmic reticulum targeted in the transient assay; and (d) the F1–V transient expression using any of the three plasmid constructs is significantly higher at 3 d.a.i. than at 6 d.a.i.

Of 66 primary transformants positive for F1–V by PCR, only five plants were selected as lines, based on F1–V expression analyses, and advanced to the T_1 second generation. We selected nine second generation tomato plants expressing high concentrations of the fusion protein F1–V in fruit (4–10% of TSP, 600–1700 $\mu g\,g^{-1}$ of green freeze-dried tomato fruit). We analyzed the antigenicity of the F1–V fusion protein in extracts from pooled, freeze-dried tomato fruit. There is a significant difference ($P < 0.05$) in the F1–V amount detected by F1–V ELISA compared to the F1 and V amounts detected by F1 and V ELISA, respectively, in the same protein extract (Fig. 5). This fact could be a consequence of a lower antigenicity of the F1 and V proteins when they are part of the plant-made fusion protein as opposed to separated bacteria-made proteins. However, the antigenicity of F1 and V in the plant-derived F1–V fusion protein was sufficient to be recognized by the antibodies specific for the bacterial F1 and V (Fig. 6A and B, respectively), as well as to induce an immune response in mice (Fig. 8).

It is generally accepted that the murine model of plague is able to provide a meaningful indication of the efficacy of plague vaccines. This model has previously been approved by the US Public Health Service for the testing of plague vaccines [46]. Glynn et al. [47] demonstrated that alternating routes for delivery of the priming and booster doses (known as prime-boost strategy) in immunizations with F1–V fusion protein, can be as or more effective than homologous boosting for induction of serum anti-F1–V IgG1 responses. We used BALB/c mice that were previously primed with parenteral F1–V to test the immunogenicity of the orally administered F1–V transgenic tomatoes. In the first three boosts, we used 2 g of freeze-dried transgenic tomatoes (300 ug of F1–V) derived from the first generation (T_0) of transgenic plants. The fourth and final boost also consisted of 2 g of freeze-dried transgenic tomatoes but was derived from the second generation plants (T_1) that have at least a four-fold increase in F1–V expression in the fruits as compared to the first generation. For this reason, the final dose was increased from 300 ug to 1200 μg.

The F1- and V-specific IgG1 concentrations were significantly higher in mice boosted with the transgenic tomato fruit than in mice boosted with W.T. (non-transgenic tomato fruit). Williamson et al. [48] found that the combined titers of the IgG1 subclass, developed to F1 plus V, correlates significantly ($P < 0.05$) with protection against a challenge with *Y. pestis* s.c. at 10^7 CFU in BALB/c mice. Based on this study, we estimated the possible degree of protection in the mice under the prime-boost experiments with control or F1–V transgenic tomato fruit (Table 2). The average predicted percentage of protection for those values of $\log_{10}$ IgG1 titers to (F1 + V) is 50–90% for the mice boosted with transgenic tomato fruit but only 10% for the five mice boosted with control tomato. When the $\log_{10}$ F1 + V IgG1 was calculated per

Table 2
Predicted protection against challenge with *Y. pestis* s.c. at 10^7 CFU in BALB/c mice primed with s.c. bacterially produced F1–V and boosted with control or F1–V transgenic tomato fruit

IgG1 specificity	Avg. $\log_{10}$ F1 + V IgG1 titer after boosting with TG F1–V tom. ($n = 6$)[a]	Predicted % of protection[b]	Avg. $\log_{10}$ F1 + V IgG1 titer after boosting with W.T. tom. ($n = 5$)[a]	Predicted % of protection[b]
F1	5.21 (4.89−5.53)	90	4.53 (4.30−4.76)	10–50
V	5.06 (4.75−5.37)	10	4.71 (4.45−4.73)	<10
F1 + V	10.27 (9.65−10.89)	50–90	9.24 (8.75 + 9.49)	10

Mouse	Oral boost	$\log_{10}$ F1 + V IgG1	Predicted protection[b]
M4	TG F1–V tom.	9.42	Low
M5	TG F1–V tom.	10.62	High
M6	TG F1–V tom.	10.92	High
M7	TG F1–V tom.	10.62	High
M8	TG F1–V tom.	10.92	High
M9	TG F1–V tom.	9.12	Low
M22	W.T. tomato	9.42	Low
M23	W.T. tomato	9.42	Low
M25	W.T. tomato	8.83	Low
M26	W.T. tomato	9.12	Low
M27	W.T. tomato	9.42	Low

[a] Numbers in parenthesis are the confidence limits at 95% of the mean. TG: transgenic; W.T: wild type or non-transformed tomato; CFU: colony-formation units.

[b] Predictions were based in the study of Williamson et al. [48] that correlated total IgG1 titer to (F1 + V) with protection.

mouse, the predicted protection in four out of six of the mice boosted with F1–V tomato was high (at least 90%) in clear contrast with the low protection in the five mice boosted with control tomato.

In mice boosted with F1–V transgenic tomato, the ratio of average F1- and V-specific IgG1/IgG2a concentrations was 200 and 117, respectively. This indicates a type 2 T-helper cell immune response (Th2), which is associated particularly with a humoral response, the appropriate response to generate protection against a predominantly extracellular bacterium like *Y. pestis* [49].

F1- and V-specific mucosal IgA was elicited only in mice boosted with oral transgenic F1–V tomato. It was not detected in mice boosted with control tomato at any time during the assay or in those mice treated only with subcutaneous bacterial F1–V (positive controls). This confirms previous findings that mucosal surfaces are usually poorly protected with IgA following parenteral administration of vaccines. Mucosal vaccination offers the added advantage that systemic immunity can be induced in concert with local responses because of translocation of antigenic material from sub-epithelial compartments to systemic immunoresponsive tissues such as spleen [33]. For plague, although mucosal IgA may be important for protection of the upper respiratory tract, it is IgG to F1 and V that is protective against an aerosol challenge [13]. Thus, effective mucosal vaccines for pneumonic plague must have the capacity to stimulate not only mucosal, but good systemic responses as well.

Using transgenic tomato plants to produce an oral vaccine in fruit without any protein purification and with minimal processing may provide a cost-effective alternative to current vaccine production strategies. In this paper we show that an antigen from a non-enteric human pathogen (*Y. pestis*) can be orally immunogenic when produced and delivered in plant tissues. Plant-expressed F1–V has the potential to be useful as a booster vaccine against plague since it is able to elicit specific mucosal sIgA and serum IgG1 responses. A prime-boost vaccine for plague also has practical implications. In an imminent or post-release bioterrorism event, the ability to dispense a parenteral priming dose with the distribution of tomato powder pills that could be self administered would greatly improve national preparedness.

In future experiments we will test higher doses of plant-made F1–V using only green fruit from second or later generation plants, and will also test the addition of various potential oral adjuvants. Increased protein levels might induce a stronger and more prolonged immune response without the need for previous priming with parenteral F1–V. The vaccinated mice will be challenged with *Y. pestis* to determine the degree of protection achieved.

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