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Original Article

Immunogenicity of recombinant F4 (K88) fimbrial adhesin FaeG expressed in tobacco chloroplast

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To test the possibility of producing the novel vaccine in plants against diarrhea normally found in neonatal and newly weaned piglets, the *faeG* gene, encoding a major F4ac fimbrial subunit protein, was introduced into the tobacco chloroplast genome. After two rounds of selection under spectinomycin, we obtained the transgenic plants nearly homoplasmic. RNA gel blot analysis indicated that *faeG* and the antibiotic selective gene aminoglycoside 3′ adenylyltransferase (*aad*A) were highly transcribed as a dicistron, while the translational level of recombinant FaeG in transplastomic tobacco was about 0.15% of total soluble protein. The immunogenicity of recombinant FaeG produced in tobacco chloroplasts was confirmed by the observation that FaeG-specific antibodies were elicited in mice immunized with total soluble protein of transgenic plants, as well as the result that mouse sera stimulated by chloroplast-derived recombinant FaeG could neutralize F4ac enterotoxigenic *Escherichia coli* (ETEC) *in vivo*. This study provides a new alternative for producing the ETEC vaccine using the chloroplast expression system.

Keywords F4ac enterotoxigenic *Escherichia coli*; FaeG; adhesin; transplastomic tobacco; immunogenicity

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Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the main pathogen causing diarrhea in neonatal and newly weaned piglets [1–5]. ETEC has the ability to adhere onto the villi of the intestinal mucosal surface and produce enterotoxin to disrupt the fluid homeostasis in the host's small intestines, causing fluid hyper-secretion and diarrhea [6,7]. Currently, three antigenic variants of F4 fimbriae have been identified in porcine ETEC strains: F4ab, F4ac, and F4ad, which have difference in hemagglutination and porcine enterocyte binding activities. F4ac fimbrial ETEC strains are by far commonly associated with clinical diarrheal disease in young pigs in China [8–10].

F4 fimbriae are encoded by a gene cluster, which contains 10 genes called *faeA–faeJ*, respectively. Among these genes, *faeA* and *faeB* contribute greatly to the regulation of the expression of genes encoding the fimbrial subunits. *faeC* encodes the minor subunit FaeC located at the fimbrial tip with a single copy. FaeD is a transmembrane protein. FaeE is a chaperone protein, and FaeG is the major subunit. *faeF*, *faeH*, and *faeI* are also proposed to be involved in fimbrial biosynthesis [11]. Analysis of the F4 fimbriae showed that they are mainly composed of more than 100 repeats of FaeG subunit, allowing these bacteria to bind F4 specific receptors on the small intestines to induce diarrhea [9,12]. FaeG is thus regarded to play a very important role in the ETEC pathogenesis, and many attempts have been made to express FaeG in various expression systems for the production of a subunit vaccine against diarrhea in piglets. Several investigations demonstrated that the recombinant FaeG expressed in *E. coli* could induce specific immune response in mice [13–15]. Previously we introduced the *faeG* gene into the tobacco nuclear genome and demonstrated that the recombinant FaeG protein has immunogenicity in mice [16,17]. FaeG was also expressed in barley endosperm with an expression level of about 1% of total soluble proteins (TSP) [18]. Moreover, *faeG* has been introduced into the alfalfa nuclear genome, and the recombinant FaeG could induce F4 fimbriae specific antibodies in mice [19].

Compared with the expression systems for recombinant proteins using nuclear transformation systems, the chloroplast expression system was shown to possess more advantages, such as high expression level, no obvious gene silencing and position effect, and less risk of dispersal of the transgene into the environment [20,21]. Therefore, numerous attempts have been done to express vaccine

Table 1 Sequences of primers used in PCR

Name	Primer sequence (5′–3′)	PCR size	Accession number
trnIF	aaaggtaccgactggagtgaagtcgtaacaag	1.18 kb	Z00044.2
trnIR	aaaactagtcttctattcttttccctggcg		104192–105356
trnAF	aaagcggccgcaagcatctgactacttcatgcatg	992 bp	Z00044.2
trnAR	aaaggatcccctcgcccttcacttttaagg		105357–106330
PrrnF	aaatctagatggatttgctcccccgccgtcgttcaatg	137 bp	
PrrnR	aaaggatc**cctcc**ctacaactgtatcca		
aadAF	aaaagatctatgagggaagcggtgatcgccg	827 bp	GQ924777.1
aadAR	aaactgcaggtcgac**cctcc**tagccattatttgccgactaccttggtg		
psbA3′F	aaaaagcttctggcctagtctataggagg	415 bp	Z00044.2
psbA3′R	aaactcgaggcggccgccgaatatagctcttctttcttatttc		144–531
faeGF	gtcgacatgtggatgactggtgatttcaa	804 bp	AJ616238
faeGR	aagcttttagtaataagtaattgctacg		
To1F	aaaacccgtcctcagttcggattgc	1.65 kb	
To1R	ccgcgttgtttcatcaagccttacg		
To2F	ctgtagaagtcaccattgttgtgc	2.5 kb	
To2R	tgactgcccaacctgagagcggaca		

Restriction enzyme recognition sites used in cloning are underlined. The ribosome-binding site is shown in bold.

antigens in chloroplasts, for instance, cholera toxin B subunit [22], tetanus toxin fragment C [23], anthrax protective antigen [24,25], LecA domain of the Gal/GalNAc lectin which is the candidate vaccine antigen of amebiasis [26], human papillomavirus L1 protein [27], etc.

In this study, we constructed a chloroplast expression vector containing *faeG*, which was introduced into the tobacco chloroplast genome. The expression level of FaeG in transplastomic tobacco was about 0.15% of TSP. Moreover, when mice were intraperitoneally immunized with the recombinant FaeG, a specific immune response against F4ac fimbriae was elicited, and the induced antibody could inactivate the F4ac ETEC.

Materials and Methods

Materials

Tobacco (*Nicotiana tabacum* var. Gexin No.1) seeds were kindly provided by Shanghai Academy of Agricultural Sciences, China. BALB/c mice and New Zealand white rabbits were purchased from the Animal Facility of Shanghai Medical College of Fudan University (Shanghai, China). All primers were synthesized by Shanghai Invitrogen Biotechnology Co. (Shanghai, China) and are showed in **Table 1**.

Construction of the chloroplast expression vector

To construct a chloroplast expression vector, we amplified the tobacco chloroplast DNA fragments encoding isoleucine and alanine transfer RNAs (*trn*I and *trn*A) using the chloroplast genome DNA as the template [28,29]. A high salt and low pH method was employed to extract and purify the chloroplast genome DNA [30,31]. These two fragments were then cloned into *Not*I and *Kpn*I sites of pBluescript SK (+) phagemid vector (Stratagene, USA) (pBSK), and the produced plasmid was named as pBSKLR.

The modified 16S rRNA promoter (P*rrn*) was amplified from the plasmid 16-APT containing a Prrn-aadA-TpsbA expression cassette (kindly provided by Prof. Ning Su, the Chinese Academy of Agricultural Sciences) [32], and cloned into the pBSK vector digested by *Xba*I and *Bam*HI, generating pBSK-Prrn. The aminoglycoside 3′ adenylyltransferase (*aad*A) gene was also cloned from 16-APT, integrated into pBSK-Prrn digested by *Bgl*II and *Pst*I, yielding pBSK-Prrn-aadA. The target transcripts were designed to be stabilized by the 3′ untranslated region (3′UTR) of the *psb*A (TpsbA) gene encoding the photosystem II reaction center component. Thus the TpsbA DNA fragment was cloned into the pBSK-Prrn-aadA vector digested by *Hind*III and *Xho*I, generating pBSK-Prrn-aadA-TpsbA.

Eventually, the pBSK-Prrn-aadA-TpsbA vector digested by *Xho*I was blunted by Klenow fragment (TaKaRa, Dalian, China), and then digested by *Xba*I to obtain the fragment Prrn-aadA-TpsbA, which was cloned into the *Spe*I and *Not*I sites of the pBSKLR vector, leading to the chloroplast expression vector pCtLR.

The coding region of *faeG* without the signal peptide was amplified from the plasmid of *E. coli* strain C_{83907} (a standard ETEC strain expressing F4ac fimbriae as determined by the China Institute of Veterinary Drug Control) and cloned into pCtLR digested by *Sal*I and *Hind*III to generate pCtLRG. The schematic diagram of pCtLRG is

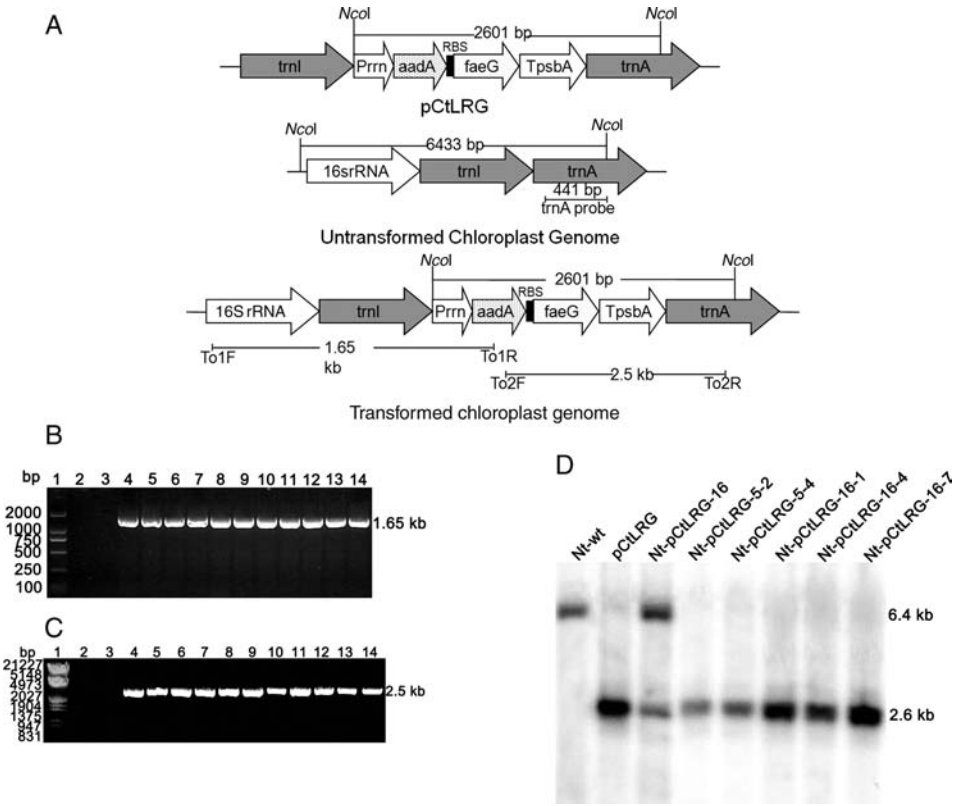

Figure 1 Analysis of *faeG* integration in the chloroplast genome (A) The schematic diagram of the structure of chloroplast transformation vector and targeting region in the plastid genome. *trn*I and *trn*A, the tobacco chloroplast transfer RNA genes encoding isoleucine and alanine, respectively; P*rrn*, the modified 16S rRNA promoter; *aad*A, aminoglycoside 3′ adenylyltransferase gene; *faeG*, a major F4ac fimbrial subunit protein gene; T*psb*A, 3′ untranslated region of *psb*A; 16S rRNA, chloroplast 16S rRNA gene; Primer To1F lands on the native chloroplast genome and To1R lands on the *aad*A gene, generating a 1.65-kb fragment. The primer To2F lands on the *aad*A gene and To2R lands on the *trn*A, generating a 2.5-kb fragment. (B) PCR analysis of pCtLRG transplastomic tobacco with primer pairs To1F/To1R. (C) PCR analysis of pCtLRG transplastomic tobacco with primer pairs To2F/To2R. Lane 1, DNA marker, DL2,000; Lane 2, steriled H_2O used as negative control; Lane 3, 100 ng of DNA from wild-type tobacco used as negative control; Lanes 4–6, 100 ng of DNA from T_0 generation of the transplastomic tobacco; Lanes 7–14, 100 ng of DNA from T_1 generation of the transplastomic tobacco used as template. (D) Southern blot analysis of the integration of expression vector pCtLRG; twenty micrograms of total cellular DNA was digested with *Nco*I and separated on 0.8% agarose gel. The plasmid pCtLRG with same treatment was used as control. A 441-bp DNA fragment amplified by PCR from *trn*A gene was used as the probe. Nt-wt, wild-type tobacco; pCtLRG, chloroplast expression vector; Nt-pCtLRG-16, line of T_0 generation of the transplastomic tobacco; Nt-pCtLRG-5-2, Nt-pCtLRG-5-4, Nt-pCtLRG-16-1, Nt-pCtLRG-16-4, Nt-pCtLRG-16-7: lines of T_1 generation of the transplastomic tobacco.

shown in **Fig. 1(A)**. All the molecular manipulations were performed according to the standard protocols [33].

Generation of transplastomic plants

A particle delivery method was used to transform the tobacco chloroplast genome with the plasmid pCtLRG using the Bio-Rad helium-driven PDS-1000/He System [34,35]. After bombardment, the transformed leaves were incubated in the dark for 48 h at 26°C and were then cut into 1 cm^2 squares and placed on the selection medium (MS basal medium plus 1.5 mg/l 6-BA, 0.15 mg/l IAA, and 500 mg/l spectinomycin) with photoperiods of 16 h light and 8 h dark for one month or longer. Shoots generated from the transformed leaf pieces were cut into small pieces (about 0.2 cm^2) and placed on the same selection medium for the second round of selection. Resistant shoots from the first and second culture cycle were transferred to MS_0 medium supplemented with 500 mg/l spectinomycin. Plants were screened via PCR to verify integration of transgenes in the chloroplast genome. The transplastomic lines were transferred to the greenhouse at 26°C with photoperiods of 16 h light and 8 h dark. Seeds of the transplastomic tobacco were germinated on MS_0 medium containing 500 mg/l spectinomycin [36]. And wild-type tobacco seeds were used as control.

PCR analysis

To determine whether the transgene cassette was site-specifically integrated into the chloroplast genome by homologous recombination, the putative chloroplast transformants were tested by PCR analysis using the primer pairs To1F/To1R and To2F/To2R (their positions are

indicated in **Fig. 1(A)**). The homoplasmy degree of transplastomic tobacco was measured by comparing the copy number of *faeG* and tobacco chloroplast genome reference gene, maturase gene within *trn*K intron (*matK*) using the formula as follows: Homoplasmy degree = copy number of *faeG*/(2 × copy number of *mat*K) [37].

Southern blot analysis

The homoplasmy degree of transplastomic tobacco was also tested by Southern blot analysis [38]. Total DNAs were extracted from the transplastomic tobacco using the cetyltriethylammnonium bromide method [39]. Twenty micrograms of total DNAs were digested with *Nco*I and separated by electrophoresis on 0.8% agarose gel followed by being transferred to the Hybond-N^+ membrane (GE Healthcare, Buckinghamshire, United Kingdom) and hybridized with a probe labeled with α-[^{32}P]-dCTP using a Random Primer DNA labeling Kit (TaKaRa, Dalian, China). A 441-bp DNA fragment amplified from the *trn*A gene of the chloroplast genome was used as the template for probe labeling **[Fig. 1(A)]**. Total DNAs from wild-type tobacco with the same treatment were used as control.

Northern blot analysis

Northern blot was used to test whether the *faeG* gene was transcribed in the transplastomic tobacco [40]. Total RNAs were isolated using TRIzol reagent according to the manufacturer's recommendation (Invitrogen Ltd., Carlsbad, USA). Twenty micrograms of total RNAs were separated on 1.2% formaldehyde agarose gel by electrophoresis, and transferred to a Hybond-N^+ filter membrane (GE Healthcare, Buckinghamshire, United Kingdom). Fragments of *faeG* and *aad*A amplified from pCtLRG were used as probes.

Purification of F4ac fimbriae

F4ac fimbriae were purified from the C_{83907} strain [11]. The protein concentration of purified F4ac fimbriae was determined by Bradford's method with BSA (Bovine serum albumin) as standard [41].

Western blot analysis

Leaves (~0.5 g) of transplastomic and wild-type tobacco plants grown under sterile culture conditions were ground in liquid nitrogen and resuspended in 0.5 ml of ice-cold extraction buffer (20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1% Triton-100). The supernatant of transformed and wild-type plants containing 40 μg of TSP, as determined by Bradford protein assay, was separated by 15% SDS–PAGE, respectively. Samples (40 ng) of purified F4ac fimbriae and rFaeG (recombinant FaeG expressed in *E. coli*) [42] used as positive controls were boiled for 10 min prior to electrophoresis. After electrophoresis, the gel was transferred onto nitrocellulose (NC) membrane (Amersham, Buckinghamshire, United Kingdom). The blot was probed with the polyclonal antibody (1:1000) raised against rFaeG in rabbit [43], followed by goat anti-rabbit alkaline phosphatase-linked secondary antibodies (Proteintech Group, Chicago, USA) with a dilution of 1:2000 to visualize signals.

Quantification of FaeG protein in transplastomic tobacco

Western blot analysis was also used to quantify the expression level of the recombinant FaeG in the transplastomic tobacco. Purified rFaeG was used as standard. The quantity of the chloroplast-expressed FaeG in the TSP was determined by comparing the signal intensity with that of a known quantity of purified rFaeG. The band density was evaluated by BandScan 6.0 software to estimate the concentration of the chloroplast-expressed FaeG in the TSP with three biological replicates.

Moreover, quantitative ELISA [22] was applied to confirm the amount of the chloroplast-expressed FaeG in the TSP, and a standard curve was constructed using rFaeG.

Immunization of mice

To evaluate the immunogenicity of FaeG, TSP from transplastomic tobacco was extracted as described above and dialyzed against PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) overnight. The dialyzed TSP was prepared for animal immunization according to the method previously described [16,18]. A group of eight adult female BALB/c mice (9–12 weeks old) were immunized intraperitoneally four times at 2-week intervals. Each dose for each mouse containing about 7.5 μg of FaeG in the TSP was emulsified with an identical volume of complete Freund's adjuvant at the first immunization, and with incomplete Freund's adjuvant for subsequent immunizations. Mice were bled before each injection. After the last inoculation, the immunized mice were also bled at weekly intervals over a period of 5 weeks. With the same immunization schedule, one group of eight BALB/c mice was immunized with 7.5 μg of purified F4 fimbriae as positive control, and one group of eight BALB/c mice were immunized with the TSP of wild-type tobacco with the same concentration as that of the transplastomic tobacco as negative control. Prior to further analysis, the blood samples were stored at 4°C for about 10 h, and then centrifuged at 8000 rpm for 10 min and the serum stored at −20°C.

Analysis of FaeG antibodies in mice sera

To detect FaeG-specific antibodies, a modified version of indirect ELISA was performed [43]. The ELISA plates

were coated with purified F4ac fimbriae (100 ng/well) and incubated overnight at 4°C. The serum samples from each mouse were serially diluted as much as 10-fold. Alkaline phosphatase-conjugated goat anti-mouse IgG antiserum (Proteintech Group, Chicago, USA) with a dilution of 1:2000 was used as secondary antibody. The antibody titer was defined as the reciprocal of $\log_{10}$ of the highest serum dilution that consistently presented at OD_{405} in at least three independent determinations. The absorbance values at this dilution should be 2.1 times greater than that of the mean absorbance at OD_{405} of the sera from mice immunized with TSP of wild-type tobacco [44].

Rabbit ileal loop analysis

To test whether the antiserum from the mouse immunized with chloroplast-expressed FaeG can neutralize the F4ac ETEC and reduce the symptoms of diarrhea, male New Zealand white rabbits with a weight of ~2.5 kg were used to perform ileal loop assay [16,45]. The rabbits were subjected to fast for 24 h prior to surgery. Laparotomy was performed to externalize the intestine by aseptic technique under anesthesia with chloral through intravenous injection (50 – 75 mg/kg). Each 5–6 cm of jejunum was ligated and each loop was separated with a 0.5–0.6 cm interposing loop. C_{83907} strains were grown from single colonies in Luria–Bertani medium, and the concentration of cells was adjusted to ~10^9 CFU/ml, with each aliquot containing 500 μl of C_{83907}. About 100 μl of antiserum from an intraperitoneally immunized mouse with TSP of transplastomic and wild-type tobaccos were added to ETEC aliquots, respectively, named as Mix1 and Mix2. In addition, physiological saline (PS) and ETEC mixed with the PS of identical volume to those of Mix1 and Mix2 were used as controls. The mixture was incubated for 12 h at room temperature. Then 600 μl of the mixture was injected into each loop, and then the incision was oversewed. After 18 h of incubation, the treated ileal loops were excised, the weights and lengths of the loops were measured, and then the loops were untied to determine the volumes of each share of fluid and to measure the weight of the empty loops. The result was represented by the ratio *V/L* [volume (μl)/loop length (cm)].

Results

Construction of chloroplast expression vector containing *faeG*

To construct the chloroplast-expressing vector, *trn*I and *trn*A were cloned and used as flanking fragments for the integration of foreign genes into the inverted repeat regions of the chloroplast genome through homologous recombination as described previously [27,28]. The coding sequence of mature FaeG protein without the signal peptide encoding sequence was fused to 3′ end of *aad*A gene to form a dicistron *aad*A::*faeG*, which was promoted by P*rrn* and terminated by T*psb*A. And *faeG* had its own ribosome-binding sites (GGAGG). The resultant plasmid pCtLRG is shown in **Fig. 1(A)**.

Determination of *faeG* integration in the chloroplast genome

Through bombardments, 3 independent transformed events in T_0 generation and 25 in T_1 generation were obtained. The primer pairs To1F/To1R and To2F/ To2R were designed to determine whether the *aad*A::*faeG* was integrated into the chloroplast genome by homologous recombination **[Fig. 1(A)]**. We observed the presence of the 1.65-kb PCR products targeting the DNA region spanning 16S rDNA and *aad*A **[Fig. 1(B)]** in 3 lines of T_0 generation **[Fig. 1(B)**, Lanes 4–6**]** and 25 lines of T_1 generation **[Fig. 1(B)**, Lanes 7–14**]**, respectively, showing the PCR result of eight lines of T_1 generation (data not shown). To2F and To2R **[Fig. 1(A)]** were also used to examine whether the dicistron *aad*A::*faeG* was correctly inserted into the prearranged site. And the expected 2.5-kb PCR products targeting the DNA fragment spanning *aad*A and *trn*A in 3 lines of T_0 generation **[Fig. 1(C)**, Lanes 4–6**]** and 25 lines of T_1 generation **[Fig. 1(C)**, Lanes 7–14**]**, respectively, showing the PCR result of 8 lines of T_1 generation (data not shown) were detected, indicating the correct integration of *aad*A::*faeG* dicistron in the transplastomic plants.

Using the effective *TaqMan* quantitative PCR approach, we observed that the homoplasmy degree of 3 lines of T_0 generation was very low (~0.000075) and all the lines of T_1 generation were about 1 by comparing the copy number of *faeG* and tobacco chloroplast genome reference gene, *matK* [37]. To further verify site-specific integration of the *aad*A::*faeG* expression cassette and the homoplasmy degree of transplastomic lines, Southern blot was performed. As shown in **[Fig. 1(D)**, there was only one 6.4-kb DNA fragment probed by a 441-bp *trn*A fragment **[Fig. 1(A)]** in the wild-type tobacco **[Fig. 1(D)**, Lane 1**]**. In the T_0 line, two DNA fragments were detected **[Fig. 1(D)**, Lane 3**]**: one was about 6.4 kb, which was also detected in the wild-type tobacco, and the other was 2.6 kb, which was identical to the positive control **[Fig. 1(D)**, Lane 2**]**. The detection of the 2.6-kb band indicated that the *aad*A::*faeG* expression cassette was specifically integrated into the chloroplast genome, and that of the 6.4-kb DNA fragment represented the existence of the wild-type chloroplast genome in the T_0 generation of the transplastomic tobacco. In T_1 lines **[Fig. 1(D)**, Lanes 4–8**]**, only 2.6-kb DNA products were observed, suggesting that all T_1 lines were nearly homoplasmic. In other words, the homoplasmic tobacco lines were obtained after two rounds of antibiotics selection. The transplastomic tobacco of T_1 generation was

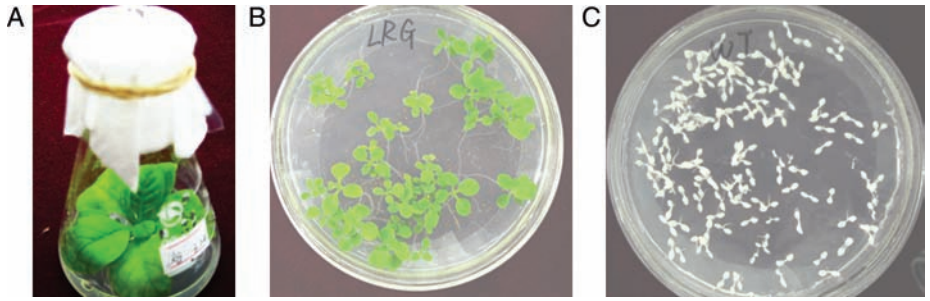

Figure 2 Phenotype of the transplastomic tobacco (A) The antibiotic resistant plant. (B) Seeds of homoplasmic tobacco germinated on medium containing 500 mg/l spectinomycin. (C) Seeds of wild-type tobacco germinated on medium containing 500 mg/l spectinomycin.

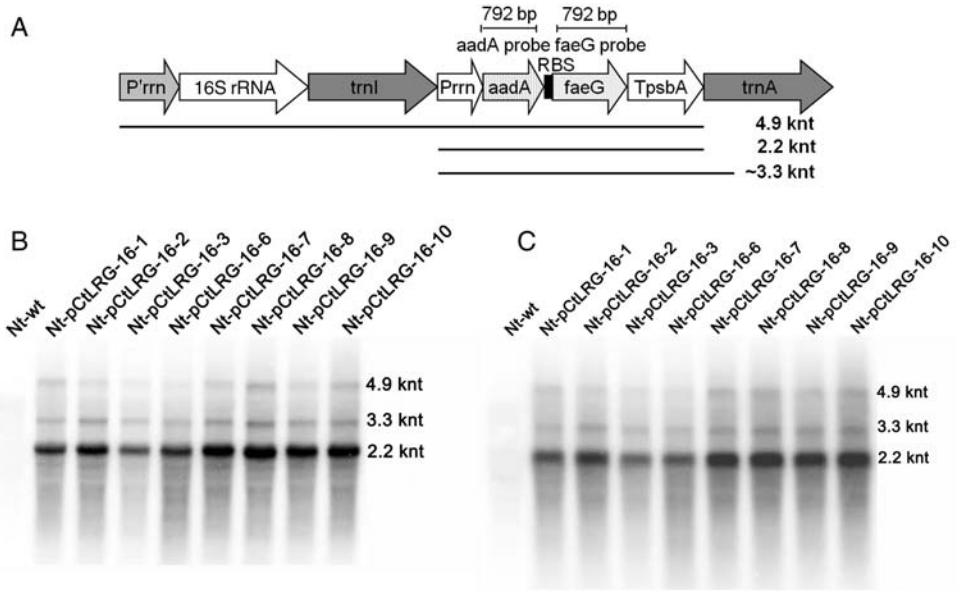

Figure 3 Transcription analysis of *aad*A and *faeG* in transgenic tobacco using Northern blot Twenty micrograms of total RNAs separated on 1.2% formaldehyde agarose gel by electrophoresis and transferred to a Hybond-*N*+filter membrane. The fragments of *faeG* (792 bp) and *aad*A (792 bp) amplified from pCtLRG were used as probes. Nt-wt, wild-type tobacco; Nt-pCtLRG-number, lines of T_1 generation of the transplastomic tobacco. (A) Schematic representation of the *aad*A::*faeG* operon in transgenic tobacco, including the *aad*A gene and the upstream P*rrn* promoter, upstream native chloroplast 16S rRNA gene with its respective promoter (P'rrn) and the *trn*I and *trn*A are shown. Underlines represent expected transcripts and their respective sizes. (B) RNAs hybridized with the *faeG* probe. (C) RNAs hybridized with the *aad*A probe.

also confirmed by the observation that seeds of T_1 lines could be germinated normally on the medium containing 500 mg/l spectinomycin **[Fig. 2(B)]**, but wild-type seedlings were bleached **[Fig. 2(C)]**. The transplastomic tobacco of the T_1 generation was seen to have no adverse pleiotropic effects compared with the wild-type tobacco in the greenhouse **[Fig. 2(A)]**.

Analysis of the transcripts of transgenes

To detect whether *faeG* and *aad*A had been transcribed in transplastomic tobacco, Northern blot analysis was performed using *aad*A and *faeG* probes **[Fig. 3(A)]**, respectively. The result revealed that the most abundant transcript was about 2.2 kilonucleotide (knt) **[Fig. 3(B, C)]** and predicted to be dicistronic, which were putatively transcribed from the P*rrn* promoter and ended downstream of T*psb*A. The transcript with ~4.9 knt was at low intensity, and this transcript might be the read-through of the transcript initiated by the native 16S rRNA promoter (P′*rrn*) and terminated downstream of T*psb*A. One additional transcript with about 3.3 knt was detectable, which might be transcribed from the P*rrn* promoter and ended downstream of the engineered 3′ UTR. No monocistrons were detected in these transplastomic tobaccos. The dicistron containing the two genes of *aad*A and *faeG* accounted for about 90% of the total transcripts detected using the *aad*A or *faeG* probes, respectively.

Immunoblot analysis of chloroplast-expressed FaeG

To observe whether *faeG* is translated in the chloroplast, Western blot analysis was used. The recombinant FaeG from *E. coli* (rFaeG) and the purified F4ac fimbriae were

used as the positive controls. A protein band with the expected size of a molecular weight of 27.6 kDa was detected in the transplastomic tobacco **[Fig. 4(A)]**, indicating that the FaeG protein was expressed properly. The rFaeG was diluted serially and used for quantification of the expression level of the chloroplast-derived FaeG. Our semi-quantitative analysis indicated that the expression level of the FaeG was about $0.148\% \pm 0.0046$ of TSP (w/w) **[Fig. 4(B)]**. Consistently, this expression level was also confirmed by quantitative ELISA approach (data not shown).

Immunogenicity of chloroplast-expressed FaeG in mice

To evaluate whether the chloroplast-derived FaeG protein has immunological activity, mice were immunized subcutaneously with TSP extracted from transplastomic lines, and antiserum titers were examined by indirect ELISA. The results showed in **Fig. 5** indicated that no FaeG-specific antibodies were detected in any animals before primary immunization, but a significant amount of FaeG-specific antibodies were detected in the mice immunized with TSP from the transplastomic tobacco after the second immunizations, reaching the highest titer after the third immunization **(Fig. 5)**. The overall trend of immune response of these mice was nearly similar to that of mice immunized with purified F4ac fimbriae as positive control, except that the antiserum titer was a little lower at the second immunization. Meanwhile, no signal was detected in animals immunized with TSP from the wild-type tobacco **(Fig. 5)**.

Antibodies against chloroplast-expressed FaeG could neutralize ETEC

To test whether the antiserum from mice immunized with the TSP of the transplastomic tobacco could neutralize the adherence of F4ac fimbriae of ETEC and inhibit the expression of enterotoxin in this strain *in vivo*, the ligated ileal loops from treated rabbits were used [16,45]. Results in **Table 2** reveal that the length of the expanded loops treated with Mix1 (mixture of ETEC with the antiserum of mice elicited by TSP from the transplastomic tobacco) was shorter than that in Mix2 (mixture of ETEC with the antiserum of mice elicited by TSP from the wild-type tobacco) or only the ETEC experimental group. The mean values of *V*/*L* [volume (μl)/loop length (cm)] for the ileal loops treated with the Mix1 (135.078) were significantly lower than those in the Mix2 (729.755) and ETEC (699.054) experiment groups ($P < 0.05$), suggesting that antiserum

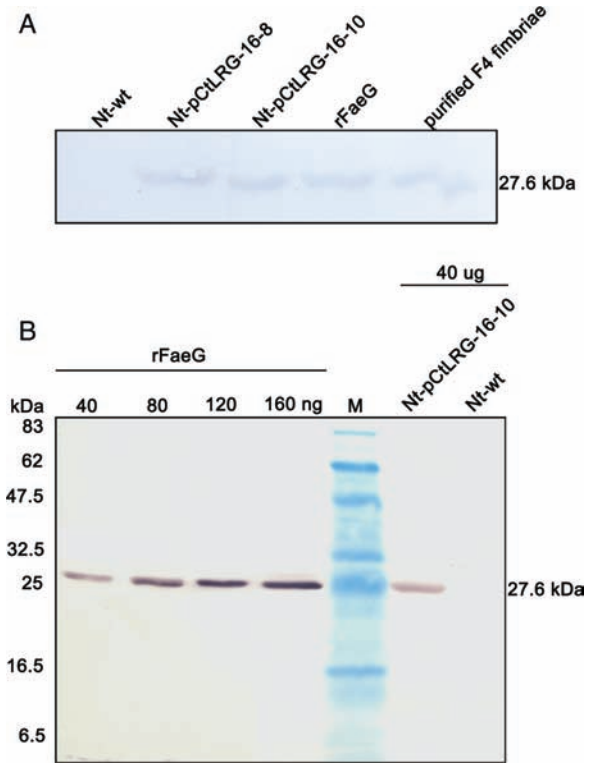

Figure 4 Expression analysis of FaeG in the homoplastomic tobacco (A) Expression analysis of the chloroplast-expressed FaeG by Western blot. Protein extract was separated by 12% SDS-PAGE and transferred onto NC membrane. The blot was probed with the rabbit polyclonal antibody (1:1000) against rFaeG, followed by goat anti-rabbit alkaline phosphatase-linked secondary antibodies (1:2000). Nt-wt, TSP of wild-type tobacco (40 μg); Nt-pCtLRG-16-8, Nt-pCtLRG-16-10, TSP of the T_1 generation of the transplastomic tobacco (40 μg); rFaeG, 40 ng of purified rFaeG; purified F4 fimbriae, 40 ng of purified F4 fimbriae. (B) Quantification of the FaeG from transplastomic tobacco by Western blot analysis.

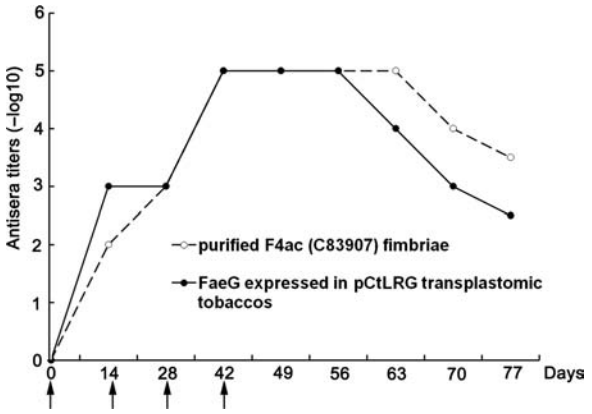

Figure 5 Mean F4-specific serum antibody titers at Day 0, 14, 28, and 42 post-primary immunization of mice Black arrows represent immunization schedule. The serum samples from every mouse were diluted serially as much as 10-fold of the start from 1/10, respectively. Alkaline phosphatase-conjugated goat anti-mouse IgG antiserum with a dilution of 1:2000 was used as a secondary antibody. The antibody titer was defined as reciprocal of the $\log_{10}$ of the highest serum dilution that consistently presented OD_{405} in at least three independent determinations. The absorbance values at this dilution should be 2.1 times greater than that of the mean absorbance at OD_{405} of the sera from mice immunized with TSP of wild-type tobacco.

Table 2 Results of rabbit ileal loop ligation analysis

Samples	Loop length (cm)	Total weight of ileal (g)	Weight of empty ileal (g)	Volume of accumulated fluid (ml)	*V*/*L*
Mix1	5.95 ± 0.08	3.25 ± 0.08	1.34 ± 0.02	0.80 ± 0.00	135.08 ± 0.07*
Mix2	9.93 ± 0.11	9.15 ± 0.01	1.11 ± 0.13	7.24 ± 0.02	729.76 ± 0.43
ETEC	8.49 ± 0.01	8.79 ± 0.02	1.21 ± 0.20	5.94 ± 0.05	699.05 ± 0.32
PS	5.65 ± 0.21	4.20 ± 0.02	1.82 ± 0.05	0.48 ± 0.03	84.01 ± 0.22

Mix1, the mixture of ETEC with the antiserum of mice elicited by TSP from transplastomic tobacco; Mix2, the mixture of ETEC with the antiserum of mice elicited by TSP from wild-type tobacco; ETEC, enterotoxigenic *Escherichia coli* with PS; PS, physiological saline; *, significant difference ($P < 0.05$) compared with Mix2. Data are presented as mean ± SD ($n = 3$).

arose by chloroplast-expressed FaeG could neutralize F4ac ETEC *in vivo*, which might further prevent diarrhea in piglets. Moreover, ileal loops injected with PS solution did not show significant accumulation of fluid **(Table 2)**, suggesting that there was no other infection caused by surgery during ileal loop ligation.

Discussion

In the past decades the chloroplast genetic engineering system has been widely used to produce vaccine antigens and biopharmaceutical proteins owing to its advantages over other expression systems [46]. ETEC is a harmful pathogen of intestinal infections in animals, and postweaning diarrhea is a major problem in piggeries worldwide causing significant economic losses. Induction of a protective immune response against ETEC virulence factors (fimbriae and toxins) would be the first step toward the development of an effective vaccine. In this study, we constructed an *faeG* chloroplast expression vector and introduced this gene into the chloroplast genome with the aim of providing an alternative approach to prevent postweaning diarrhea. The homoplasmic tobacco was achieved in the T_1 generation, which is identical with other studies in the chloroplast expression system [47]. The transcription analysis indicated that the foreign genes were transcribed as a dicistron at a high level, and this was similar to the previous studies [40].

Even several foreign genes can be expressed at high levels in plant chloroplast (>70%) [48], the expression level of recombinant proteins in plant chloroplast varies among target proteins. For example, the expression level of Hepatitis E virus antigen (HEV E2) in tobacco chloroplast was about 0.1% of the TSP [49]; and Type 1 diabetes antigen hGAD65 could only be expressed at 0.25% of TSP in the transplastomic tobacco [46]. In this study, the effective chloroplast expression vector pCtLRG containing the same gene expression cassette as that in previous reports [32,50] was used. Previous studies indicated that the foreign genes could be transcribed at high levels, while the translated protein levels did not fully correlate with transcript abundance. This is explained that in plastids, post-transcriptional gene regulation is of importance and high-level protein accumulation is associated with the choice of 5′ UTR [21,51,52]. The low expression level of FaeG in the pCtLRG transplastomic tobacco may be due to no independent 3′ and 5′ UTR for *faeG* itself, reducing the translation efficiency of the foreign genes. Moreover, the low amount of soluble FaeG protein is possibly caused by self-assembly into fimbriae inside the chloroplasts, or protein instability due to protease digestion.

The biological activity of this recombinant FaeG protein was observed through immunological assay by immunizing mice using plant extract. A significant amount of FaeG-specific antibodies were detected in the mice immunized with TSP from the transplastomic tobacco after the second and third boost immunization, which is similar to that of FaeG produced from transgenic tobacco containing *faeG* in the nuclear genome driven by the CaMV35S promoter [16]. However, we observed that the titers of antisera increased in these mice, reached the highest value after the third immunization, and lasted for four weeks, and then the titer of the elicited antisera began to decline slowly in the following weeks, which was slightly different from our previous observation [16]. The presumptive reason may be that the chloroplast-derived FaeG could be slowly released and protected from degradation by the 'capsule' formed by the plant cell wall and the chloroplast in TSP extracts [46]. The overall trend for immune response of mice immunized with TSP from the transplastomic plant was similar to that of mice immunized with purified F4ac fimbriae, which are the polymers of FaeG subunits with more effective immunogenicity. Moreover, this chloroplast-derived FaeG has the potential to be developed as a vaccine against animal ETEC infections due to the observation that the antibody achieved from these immunized mice is able to neutralize F4ac ETEC *in vivo* through a rabbit ileal loop experiment.

Further researches should be done on this chloroplast-expressed FaeG as an oral vaccine. Also the detailed analysis for the level of IgM, IgG1, and IgG2 in the antisera,

and T cell proliferation to assess the cellular immunity will be performed. Plastid transformation technology has been extended to many other crops, such as oilseed rape, soybean, carrot, cotton, and so on. Especially edible leafy crop lettuce has attracted much attention for its minimized downstream protein-processing costs and high expression level as observed in tobacco, offering an ideal system for oral delivery and easy regeneration [53–55]. Previously we demonstrated that oral immunization of mice with plant-derived fimbrial adhesin FaeG could induce systemic and mucosal K88ad ETEC-specific immune responses [17]. All of these encourage us to further investigate the oral delivery of the chloroplast-expressed FaeG.

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