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ORIGINAL PAPER

Expression of *Helicobacter pylori* urease subunit B gene in transgenic rice

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Abstract *Helicobacter pylori ureB* antigen gene was cloned to the 5′-end of *gus* (*β*-glucuronidase) reporter gene between CaMV35S promoter and the octopine synthase (OCS) terminator in the plasmid, pCAMBIA13011. It was then introduced into rice genome by *Agrobacterium*-mediated transformation. A total of 30 regenerated plants with hygromycin resistance were obtained in the selection media. The putative transgenic individuals were tested for the presence of *ureB* in the nuclear genome of rice plants by PCR analysis. Expression of *ureB* gene in rice plants was verified by RT-PCR and Western blot analysis using polyclonal human antiserum for transcription and translation levels respectively. These results provide a basis for further studies on the accumulation level of UreB recombinant protein in transgenic rice and potential utilization of transgenic rice for delivery of edible vaccines against *Helicobacter pylori*.

Keywords *Helicobacter pylori* · Transgenic rice · *ureB* · Vaccine

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Introduction

The stomach-colonizing bacterium, *Helicobacter pylori*, is the main cause of peptic ulceration and gastric cancer. The standard treatment for *H. pylori* infections has depended on antibiotics in combination with proton pump inhibitors (Bazzoli et al. 2002). Antibiotic-based triple therapies are, however, not practical for global control due to the high cost, patients' non-compliance and low effectiveness due to the development of antibiotic resistance among strains of *H. pylori* (Telford and Ghiara 1996). Vaccination against *H. pylori* is therefore one of the most effective ways to control *H. pylori* infection and, indeed, administration of oral bacterial antigens can protect mice against *H. pylori* infection (Ferrero et al. 1994).

Helicobacter pylori urease is essential for colonization of the stomach as it allows survival under acidic condition. Moreover, urease (including subunit proteins UreA and UreB) is one of the main antigens recognized by the human immune response to *H. pylori*, and UreB

seems to be more protective than UreA (Ferrero et al. 1994). Recently, recombinant urease has been used in vaccination trials to prevent infection by *H. pylori* in mice (Del Giudice et al. 2001). Therapeutic mucosal immunization with recombinant UreB in mice induce a Th2 $CD4^+$ T-cell response (Ermak et al.1998).

The antigen delivery system can influence the immune response qualitatively as well as quantitatively (Sheikh et al. 2000). Although several approaches have been investigated, most have only focused on the bacterial systems. Plant-made vaccines are specially attractive as plant are free of human diseases, reducing screening costs for viruses and bacterial toxins. In addition, expression of vaccines in plants tissues provides a heat-stable environment, and enables oral delivery, thus eliminating injection-related hazards. It has been demonstrated that proteins produced in transgenic plants are capable of invoking protective immune responses against important pathogens (Haq et al. 1995; Mason et al. 1996; Arakawa et al. 1998).

Rice is generally regarded as safe (GRAS) for consumption and is one of the most important food crops in the world, providing the principal source of calories for nearly one third of its population. Expression of recombinant UreB in rice grain for immunization against infection by *H. pylori* is an attractive system. In the present work, we demonstrate that UreB was expressed and accumulated in transgenic rice. The result reveal a potential strategy for utilizing rice grains expressing rUreB as bioreactors for low-cost production and safe delivery of immunization against infection by *H. pylori*.

Materials and methods

Plant material and culture conditions

Mature rice seeds of Zhonghua 11 (*Oryza sativa* L.) cultivar were surface sterilized with 25% (w/v) NaClO for 30 min, thoroughly washed with running tap water followed by sterilization with 70% (v/v) ethanol for 10 min. Seeds were subsequently washed in sterile distilled water 3–5 times. Calli were induced on N6 medium with 2 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/l. For transgenic calli production, the N6 medium was supplemented with 40 μg hygromycin/ml and 500 μg cefotaxime/ml. Explants for inducing shoots were maintained at 24 ± 1°C with 30 mmol m^{-2} s^{-1} illumination from cool white fluorescent lamps under a 16-h photoperiod. Two to three weeks later, regenerated plantlets approximately 1–2 cm in height, were transferred to the rooting medium, which contained half strength MS medium and 40 μg hygromycin/ml.

Construction of plant expression vector pCAMBIA13011-ureB

Helicobacter pylori used in the present study was a clinical strain ZJC02 provided by Professor YL Zhu in second affiliated hospital, college of medicine, Zhejiang University. The *ureB* gene of *H. pylori* was obtained by PCR amplification of a 1710-bp fragment from plasmid pGEM-*ureB*. The oligonucleotides were 5′-CTGGTACCATGAAAAAGATTAGCAGAAAAG-3′ (*Kpn*I *site underlined*) and 5′-ACTCTAGACTAGAAAATGCTAAAGAGTTGC-3′ (*Xba*I *site underlined*). The PCR product was digested by *Kpn*I and *Xba*I and inserted into the corresponding sites of the binary expression vector pCAMBIA13011. The resulting vector, named pCAMBIA13011-*ureB*, includes a constitutive CaMV35S promoter for gene expression and the *gus* (*β*-glucuronidase) reporter gene (Fig. 1). *Agrobacterium tumefaciens* strain EHA105 was used to mobilize the recombinant binary vector pCAMBIA13011-*ureB* by freeze-thaw method (Holsters et al. 1978).

Plant transformation and regeneration

Agrobacterium tumifaciens strain EHA105 harboring the pCAMBIA13011-*ureB* vector was grown overnight at 28°C in YEP medium supplemented with kanamycin (50 μg/ml) and rifampicin (40 μg/ml). The culture was centrifuged and pellets were resuspended in an equal volume of AA medium containing 100 μM acetosyringone. Calli were induced from scutellum of rice seeds on N6 medium, as described by Hiei et al. (1997). The calli were transferred to a co-culture

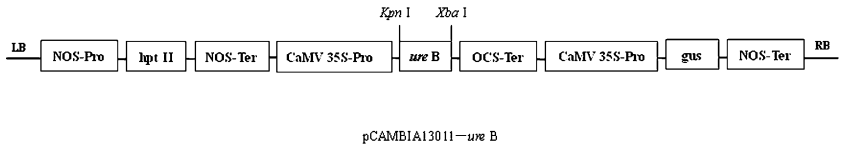

Fig. 1 Schematic structure of the binary plasmid pCAMBIA13011-*ureB* used for *Agrobacterium*-mediated plant transformation

medium and cultured in the darkness at 26 ± 1°C for 3 days and then washed with AA liquid medium containing 500 μg cefotaxime/ml, blotted dry on a sterile filter paper and placed on solid N6 medium with antibiotics (40 μg hygromycin/ml and 500 μg cefotaxime/ml). After two rounds of selection on hygromycin medium, the resistant calli were transferred to regeneration medium for shoot and root development. The rooted plantlets were transferred to soil in a greenhouse, and untransformed plants were also grown under similar conditions as control.

Analysis of β-glucuronidase activity

β-Glucuronidase (GUS) activities were determined in rice calli according to the method of Jefferson et al. (1987). Histochemical staining of rice calli was done in stain containing 2 mM 5-bromo-4chloro-3-indolyl glucuronide (X-Gluc). Samples were infiltrated for 3 min under vacuum and incubated for a further 16 h at 37°C. Samples were washed with 100 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0) buffer and subsequently kept in 70% (v/v) ethanol.

PCR analysis

Genomic DNA was extracted from selected rice plants by CTAB method (Wang et al. 1993). PCR analysis was carried out for the hygromycin resistant plantlets, using a pair of specific primers for amplification of a 1710 bp DNA fragment of the *ureB* gene. PCR reactions were performed in a 25 μl reaction volume, containing 50 ng of rice genomic DNA, 1.25 mM $MgCl_2$, 50 μM of dNTPs and 0.25 μM of each primer and were conducted for 35 cycles by 94°C for 30 s, 58°C for 30 s, 72°C for 120 s.

Total RNA was extracted from wild and transgenic rice plantlet ground in liquid N_2 using Trizol reagent (GibcoBRL) according to a standard protocol. cDNA synthesis was undertaked according to the manufacturer's instructions using a Reverse Transcription System (Promega). Modified half quantitative RT-PCR was used to test the transcription of *ureB*.

Western blot analysis

Total soluble protein was extracted from control and transgenic rice plants (200 mg) by homogenizing in 400 μl of extraction buffer (50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 500 mM Tris HCL, pH 8.0, 1 mM EDTA, 500 mM NaCl, 100 μg PMSF/ml) and centrifuged at $15000 \times g$ for 15 min at 4°C. Protein concentrations were determined using the Bradford method.

For Western blotting, fixed amounts of total protein extracts were boiled for 5 min in 5× SDS sample buffer and separated on 10% SDS-PAGE (1 h, 120 V), and then transferred to PVDF membrane by electroblotting (0.35 A, 1 h). The blocked membrane was incubated with a polyclonal human antiserum raised against *H. pylori* for 1 h at 25°C. After being washed three times with TTBS, the membrane was incubated with goat-anti human IgG alkaline phosphatase conjugate (KPL) for 1 h at 25°C. After being washed three times with TTBS, BCIP/NBT was used to visualize the bound antibody, and the images were recorded on X-ray film.

Results

Vector construct and transformation of rice plants

Helicobacter pylori ureB gene was cloned to the 5′-end of *gus* reporter gene between the CaMV35S promoter and the octopine

synthase (OCS) terminator in the plasmid pCAMBIA13011-*ureB* (Fig. 1). The recombinant pCAMBIA13011-*ureB* plasmid enables selection of transformants on media containing hygromycin and stably integrates into nuclear chromosomal plant DNA.

Callus was induced from the seeds of rice explants within 4 weeks in N6 medium. Transformation of rice with pCAMBIA13011-*ureB* was carried out using *A. tumifaciens* infiltration. The transformed calli were selected on solid N6 medium with antibiotics (40 μg hygromycin/ml and 500 μg cefotaxime/ml). To determine whether the CaMV35S promoter was active in callus tissue, clones were subjected to GUS histochemical assay after 4 weeks of selection. After staining for 16 h, 60% clones showed blue colouration although the intensity of staining varied considerably. The resistant calli were transferred to regeneration medium for shoot and root development. A total of 30 independent transgenic plants were obtained under hygromycin selection and transferred to soil in a greenhouse.

Analysis of the ureB in the transgenic plants

The 30 putative transgenic individuals appeared similar in morphology to wild-type plants. All lines were positive when screened for the presence of the recombinant genes by PCR analysis. The genomic DNA was extracted from leaves and PCR amplified with *ureB* specific primers. The expected amplification product of 1.7 kb *ureB* gene was obtained in transgenic plants, but not in control plants transformed with a non-related gene or plants that had not been transformed (Fig. 2).

Expression of *H. pylori* ureB in transgenic rice plants

Ten independent transgenic lines were selected to test the transcription of *ureB* by reverse transcription PCR. A specific DNA fragment of 1.7 kb was amplified in the transgenic plants, but not in non-transformed plants, indicating that the foreign *ureB* was being transcribed (not shown).

To determine the level of *ureB* gene product in transgenic rice, Western blot analysis was performed using the antibody raised against UreB.

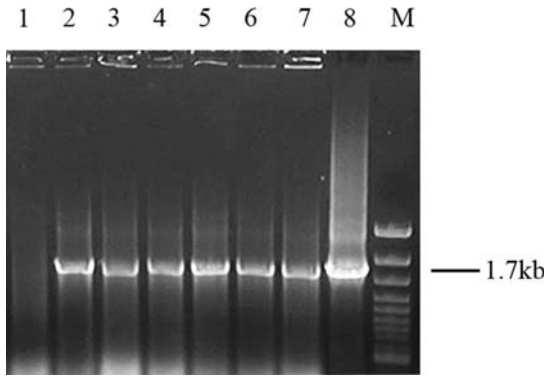

Fig. 2 Representative PCR analysis for the presence of *ureB* gene in transgenic rice plants. Plant DNA was isolated from leaf extracts and PCR was performed with a pair of primers which specifically amplify a 1.7 kb DNA fragment of *ureB*. M: Marker; Lane 1: untransformed plant (negative control); Lane 8: pCAMBIA13011-*ureB* plasmid (positive control); Lanes 2–7: transformed plants

Total protein was extracted from three rice plants that showed different RT-PCR intensities and were analyzed. A specific signal of the 66 kDa protein band was detected in two of the transgenic plants (Fig. 3). However, no specific signal could be detected in the non-transformed plant. These results indicate that the *ureB* gene was successfully transformed into the rice plants some of which could stably accumulate the recombinant protein.

Discussion

Transgenic plants that express therapeutic proteins are ideal vehicles for the production and oral delivery of protective antigens, as the rigid cell walls protect the antigen from the acidic environment of the stomach. The chances of

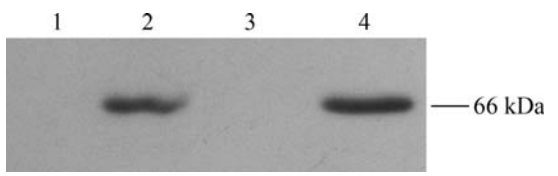

Fig. 3 Detection of the UreB expression in selected transgenic plants by Western blot analysis. Leaf extracts from a non-transgenic plant (lane 1) and three transgenic plant lines no. 2 (lane 2), 3 (lane 3) and 5 (lane 4) were analyzed using a polyclonal antiserum for the detection of UreB

acquiring mucosal immunity against infectious agents that enter the body across a mucosal surface are also increased with oral vaccines. Oral delivery of vaccines through consumption of edible plant tissue has been demonstrated to work effectively and induce both mucosal and systemic immune responses without the need of an adjuvant (Tacket et al. 1998). It has been shown that plants can express, fold, assemble, and process foreign antigens and can provide both a simple vaccine-manufacturing process as well as a matrix suitable for oral immunization (Domansky et al. 1995, Haq et al. 1995, Tacket et al. 1998, Richter et al. 2000). Transgenic plants have been increasingly used for the expression of different antigens, and plant based vaccines are especially attractive as plants offer some distinct advantages over other systems.

We have previously demonstrated the successful expression of UreB in transgenic tobacco plants. However in view of the antigen associated with tobacco plants it may not be the right choice to use as a source of edible vaccine. In the present study, a full-length *ureB* gene from a clinical strain ZJC02 of *H. pylori* successfully expressed a protective antigen in transgenic rice plants. However, the main disadvantage with transgenic-plant based vaccines productions is the low accumulation of the antigen expressed. Several researches are currently focusing on how to increase the expression of recombinant proteins using specific reticulum retention signal sequences (Arakawa et al. 1998, Gomez et al. 2000). Our results represent the first report of *H. pylori ureB* gene expression in transgenic rice plants. This recombinant protein is recognized by human serum antibodies produced in response to a wild-type *H. pylori* infection, thereby opening the possibility of a low-cost alternative production of oral vaccines to *H. pylori*. The immunogenicity and extent of protection offered by oral delivery of the UreB protein expressed in rice remains to be tested. With the demonstration of successful *ureB* gene expression in rice plants, we are poised to move on towards making an edible vaccine against *H. pylori*.

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