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Synthesis and assembly of an adjuvanted *Porphyromonas gingivalis* fimbrial antigen fusion protein in plants

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Abstract

The gram-negative anaerobic oral bacterium *Porphyromonas gingivalis* initiates periodontal disease by binding to saliva-coated oral surfaces. To assess whether edible plants can synthesize biologically active *P. gingivalis* fimbrial antigen, for application as an oral vaccine, a cDNA fragment encoding the C-terminal binding portion of *P. gingivalis* fimbrial protein (FimA), was cloned into a plant expression vector immediately downstream of a cDNA fragment encoding the cholera toxin B subunit (CTB). The chimeric plasmid was transferred into potato (*Solanum tuberosum*) cells and the *ctb–fimA* cDNA fragment detected in transformed leaf genomic DNA by PCR amplification methods. A novel protein band of 21 kDa was detected in transformed potato tuber extracts by immunoblot analysis. Oligomeric CTB–FimA (266–337) fusion protein was identified in the extracts through the binding of anti-CTX and anti-native fimbriae antibodies. The pentameric structure of CTB–FimA fusion protein was confirmed by ELISA measurements of G_{M1} ganglioside receptor binding. Quantification of the CTB–FimA fusion protein by ELISA indicated that the chimeric protein made up about 0.33% of total soluble tuber protein. The biosynthesis of immunologically detectable CTB–FimA fusion proteins and the assembly of fusion protein monomers into biologically active pentamers in transformed potato tuber tissues demonstrate the feasibility of synthesizing adjuvanted fimbrial protein in edible plants for development of adjuvanted mucosal vaccines against *P. gingivalis* generated periodontal disease.

Keywords: Fimbriae; *Porphyromonas gingivalis*; Periodontal disease; Transgenic plants; Subunit mucosal vaccine

Periodontal diseases are caused by a group of oral pathogens that infect the supporting tissue of the teeth, including alveolar bone, gingiva, cementum, and the periodontal ligament. The black-pigmented gram-negative anaerobe *Porphyromonas gingivalis* has been implicated in the pathogenesis of severe periodontal disease in adults [1,2]. The first step in infection of the oral cavity involves bacterial adherence to the oral mucosal surface. Recent studies show that *P. gingivalis* binding to the oral surface is mediated by fimbrial interactions with salivary proteins [3,4], fibronectin [5], and cytokeratins [6]. While the exact role of *P. gingivalis* fimbriae is not yet fully understood, it has been reported that the fimbriae induce expression of inflammatory cytokines in monocytes, dendritic cells, and other types of host immunity providing lymphocytes [7,8,1,2]. These results suggest that fimbriae play a crucial role in bacterial interactions with host tissues and in the pathogenesis of periodontal disease that may be linked to additional inflammation-related diseases such as cardiovascular disease.

Since periodontal disease is found in the majority of adults, even a moderate risk contributed by periodontal

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disease to cardiovascular disease may contribute to significant morbidity and mortality [9]. This relationship is supported by a series of findings that *P. gingivalis* has been identified in atheromatous plaque [10] and that long-term systemic challenge with *P. gingivalis* accelerates atherogenic plaque progression in heterozygous apolipoprotein E deficient mice [11]. Fimbriae facilitate *P. gingivalis* adherence to and the invasion of endothelial cells, stimulating leukocyte-adhesion molecule presentation [12]. Fimbriae are also thought to be important for secretion of monocyte-chemoattractant-1 by endothelial cells [13]. Not surprisingly, the fimbriae-defective mutant of *P. gingivalis* fails to induce periodontal disease and atherosclerosis in apolipoprotein E-deficient mice [14]. Therefore, it is possible that control of *P. gingivalis* may prevent periodontal disease and concomitant atherosclerosis and cardiovascular disease.

Strategies proposed for control of *P. gingivalis*-elicited periodontal disease include the development of oral vaccines. Since fimbriae are one of the critical cell surface virulence factors of *P. gingivalis*, they are of a particular interest to the development of vaccine strategies. Patients with destructive periodontal disease have markedly elevated serum and gingival fluid antibody responses to *P. gingivalis* fimbriae [15,16]. Antibodies raised against the fimbriae provide protection against periodontal disease. Monoclonal antibodies to fimbriae completely inhibit *P. gingivalis* binding [17]. In rabbits, *P. gingivalis* fimbriae induce opsonic antibodies [18]. Moreover, inactivation of the *fimA* gene encoding fimbrillin, a structural subunit of fimbria, results in a decrease in the ability of *P. gingivalis* to interact with salivary components adsorbed to hydroxyapatite beads [19], and the invasion of epithelial cells [20,21]. Fimbriae-defective mutants were found to be significantly less able to cause alveolar bone loss in a rat model of periodontal disease [19,21]. Overall, the above results indicate that fimbriae are important in the progression and control of periodontal disease and that fimbrial antigens may be useful for vaccination against periodontal disease.

Since most pathogens including periodontopathic bacteria cause diseases by colonization or invasion of mucosal surfaces, methods that result in induction of immune responses at these surfaces are under exploration. Oral immunization of mice with fimbriae can generate salivary anti-fimbrial IgA almost exclusively [22,23]. Unfortunately, oral immunization is less effective than parenteral immunization, and requires a higher dose of the immunizing antigen in comparison with parenteral immunization [22]. This condition is due to the elimination of antigens from the digestive tract by hydrolysis of the antigen by stomach HCl and further digestion in the stomach by proteases such as pepsin and digestion in the lumen of the small intestine by chymotrypsin and trypsin. Thus, reduced amounts of intact antigens may reach the mucosal lymphoid tissues to stimulate an immune response [24]. To overcome this problem, novel strategies have been assessed. Fimbriae enclosed in liposomes seem to be effectively directed to gut-associated lymphoid tissue (GALT),[1] elevating salivary IgA antibodies [22]. Co-administration of cholera toxin as an adjuvant with fimbriae was found to augment the production of antigen-specific salivary IgA antibodies [23]. However, both of these strategies require high amounts of the immunogen. Recently, oral immunization of salivary glands using plasmid DNA encoding *P. gingivalis* fimbrial proteins was found to generate significant production of fimbria-specific salivary IgA and IgG antibodies and a cell-mediated immune response in mice [25]. A nonpathogenic strain of the oral bacterium *Streptococcus gordonii* expressing and secreting fimbriae of *P. gingivalis* was generated for use as a live vaccine and alternatively, for use as a direct competitive inhibitor of *P. gingivalis* colonization in the oral cavity [26,27]. These two delivery methods may not prove to be acceptable based on unfavorable routes of administration. Further, these methods demand frequent fimbrial administration to generate desirable levels of immunity.

Alternatively, transfer and expression of genes encoding microbial antigens in plants represent an additional novel and potentially important approach to vaccination against periodontal disease. By combining the ingestion of plants as foods with the production of vaccine subunit components in plant tissues, plant vaccines can be produced at a fraction of the cost of alternative oral vaccine strategies. Furthermore, domestically grown vaccine plants can be readily available when vaccination is required. The advent of plant transformation with foreign genes represents a critical step for production of inexpensive edible immunogens suitable for rapid, palatable, and effective immunization of large populations. Based on these advantages, a variety of bacterial and viral antigens have been expressed in different edible plants and tested for the protection of animals from infectious diseases [24,28,29]. The data obtained from animal immunization experiments were sufficiently encouraging to justify the design of human trials for oral vaccination with transgenic edible plants. The first human study of transgenic plant vaccine containing *Escherichia coli* heat-labile toxin B subunit demonstrated a measurable increase in serum and mucosal immune responses to the antigen [30]. Since preliminary results from human clinical trial studies conducted with plant vaccines have been promising, further development of plant vaccines that target immune stimulation at the mucosal surface of the intestine could be particularly attractive for controlling mucosal diseases.

In previous studies, our laboratory constructed transgenic potatoes expressing bacterial and viral antigens demonstrating that transgenic potatoes can be effective in protection of the host from infection and the progression of enteric diseases [31–34]. In this study, we construct plant expression vectors that express the C-terminal binding domain of *P. gingivalis* fimbriae fused to cholera toxin

[1] *Abbreviations used:* GALT, gut-associated lymphoid tissue; CTB, cholera toxin B subunit; ER, endoplasmic reticulum; IAA, indole-3-acetic acid; GA_3, gibberellic acid; PBS, phosphate-buffered saline; ALP, alkaline phosphate.