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ORIGINAL ARTICLE

Production of monoclonal antibodies against 53-kDa protein of *Porphyromonas gingivalis* in transgenic rice cell suspension culture

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Abstract Periodontitis is a bacterial infection-induced inflammatory disease that affects the tissues surrounding the teeth and is the main cause of tooth loss in adults. *Porphyromonas gingivalis*, a black-pigmented gram-negative anaerobe, is a major causative agent of periodontitis. A 53-kDa protein of the bacterium has been demonstrated to be highly immunogenic and recognized by sera from most periodontitis patients, and it is now considered as a potential target of protective immunity. In this study, complementary DNA encoding monoclonal antibodies to 53-kDa protein of *P. gingivalis* A7A1-28 was cloned from hybridoma cell lines expressing the antibodies. The heavy and light chain genes were constructed into plant expression vectors under the control of the rice α-amylase 3D (RAmy3D) promoter. The recombinant plant expression vectors were introduced into rice cells (*Oryza sativa* L. cv. Dongjin) using particle bombardment transformation method. Transgenic rice calli were selected by genomic DNA PCR and used to establish cell suspension cultures. The expression of the heavy and light chain genes of the monoclonal antibodies in the rice cell suspension cultures was detected by western blot analysis. The plant-produced monoclonal antibodies showed specific reactivity with 53-kDa proteins of *P. gingivalis*. These results suggest that 53-kDa protein-specific monoclonal antibodies produced in the rice cell suspension cultures are biologically active against the bacterial protein and can be used for passive immunization to prevent *P. gingivalis*-induced periodontal disease.

Keywords Plant-produced monoclonal antibody · *Porphyromonas gingivalis* · 53-kDa protein · Minor fimbriae · Periodontitis · Rice amylase 3D promoter system

Introduction

Periodontitis is an inflammatory disease that affects the tissues surrounding the teeth and is the main cause of tooth loss in adults (Loesche and Grossman 2001). Periodontitis is initiated by specific invasive oral pathogens that colonize dental biofilms, and dysregulation of host immune response to the infection and inflammation plays a central role in the disease pathogenesis (Saini et al. 2009). Chronic periodontitis is a constant potential source of infection and inflammation, and thus is linked to a number of systemic diseases such as cardiovascular disease, cerebrovascular diseases, type 2 diabetes mellitus, adverse pregnancy outcomes, rheumatoid arthritis, osteoporosis, respiratory diseases, and cancers (Kim and Amar 2006; Hajishengallis 2015).

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Recent data from metagenomic, metatranscriptomic, and mechanistic studies have proposed a new model of the pathogenesis of periodontitis, in which bacterial synergy and active bacterial subversion of the host immune response enable the persistence of pathogens in the local inflammatory environment of periodontitis and the induction of pathology or complications at distant sites (Hajishengallis 2015). *Porphyromonas gingivalis,* a black-pigmented gram-negative anaerobe, has been considered as one of the most important pathogens implicated in periodontitis (Hamada et al. 1994; Lamont and Jenkinson 1998). In the new model, *P. gingivalis* is yet a keystone pathogen owing to its ability to induce dysbiotic microbial communities (Hajishengallis 2015). Therefore, control of *P. gingivalis* infection is crucial in the treatment and prevention of periodontitis and related systemic diseases.

Strategies proposed for control of *P. gingivalis*-induced periodontitis include the development of vaccines. To make an excellent vaccine, it is important to identify protective antigens which are often surface-exposed and immunogenic. Key pathogen virulence determinants are usually included in modern subunit vaccines (Strugnell et al. 2011). Sera from patients with chronic and aggressive periodontitis were found to react strongly with a 53-kDa major outer membrane protein of *P. gingivalis* (Kokeguchi et al. 1990; Kurihara et al. 1991), and furthermore, sera from some of the patients reacted mainly with the protein (Guo et al. 2000). *P. gingivalis* expresses two distinct types of fimbriae: major/long fimbriae (FimA) and minor/short fimbriae (Mfa1) (Enersen et al. 2013). This highly immunogenic 53-kDa outer membrane protein was reported to be another type of minor fimbriae (Arai et al. 2000). Recently, it was shown that the 53-kDa protein is another major subunit protein of Mfa1, of which 75-kDa (or 67-kDa due to aberrant migration in SDS-PAGE) protein had been known to be the only major subunit protein (Nagano et al. 2015). These observations suggest that the 53-kDa protein represents a potential target of protective immunity.

The humoral immune response to infectious disease by nature usually provides the most comprehensive approach to combating an infection, and generates the greatest potential for mounting effective protective immunity (Vigil et al. 2010). However, it was observed that some patients with periodontitis fail to elicit antibodies to periodontal pathogens (Clifano et al. 1997). To gain immunity, artificial active immunization can be introduced. Active immunization with subunit proteins is considered as safe, but limitation of the subunit approach is that a recombinant or cell culture-synthesized protein may not correctly form the three-dimensional structure and thus may not induce protective antibodies (Strugnell et al. 2011). In these cases, passive immunization may be an alternative.

The monoclonal antibodies with high affinity for respective antigen molecules can be used in clinical diagnosis, commercial analysis, and passive immunotherapy. Passive immunization with antibodies against specific pathogens has the advantage of an immediate immune response, low toxicity, and high specific activity against the infection (Casadevall 2002). However, protection offered by passive antibody therapy is short-lived and thus requires repeated treatment or high doses, increasing cost of the therapy. Transgenic plants represent an economical alternative solution for these disadvantages of the therapy. Transgenic plants have been utilized to produce full-size monoclonal antibodies of the IgG (Zeitlin et al. 1998; Kim et al. 2014), secretory IgA (Ma et al. 1995; Larrick et al. 2001), single-chain variable fragments (Tavladoraki et al. 1993; Schouten et al. 1997), and Fab (antigen-binding fragments) (De Neve et al. 1993). They have potential for large-scale, low-cost biomass production using agriculture and low risk of mammalian pathogen contamination (Orzáez et al. 2009; Kolotilin et al. 2014).

Transgenic plants and plant cell suspension cultures have been widely used for production of industrial or pharmaceutical recombinant proteins. Transgenic plant cell suspension cultures can be used to secrete and accumulate these recombinant proteins into the culture medium. The recombinant proteins have been highly expressed in transgenic rice cell suspension culture using the rice amylase 3D promoter system, which shows strong expression of recombinant proteins under sugar starvation conditions (Shin et al. 2003). To overcome the accumulation of amylase and secreted proteinase in suspension culture medium when rice α-amylase promoter was activated under the condition of sugar starvation, secreted amylase and cysteine proteinase into the culture medium were reduced by RNA interference, and the accumulation of recombinant proteins in the rice cell suspension culture was increased (Kim et al. 2013). In our previous study, the fully-assembled monoclonal antibodies of IgG against the major subunit protein of major fimbriae (FimA) of *P. gingivalis* were produced in transgenic rice cell suspension culture, and the plant-produced monoclonal antibodies purified from the suspension culture were demonstrated to be equally effective as the parental monoclonal antibodies in inhibiting *P. gingivalis* binding to saliva-coated hydroxyapatite beads and invasion of KB oral epithelial cells (originally derived from an epidermal carcinoma of the human mouth) by the bacterium, and in enhancing intracellular killing of *P. gingivalis* by polymorphonuclear neutrophils (Kim et al. 2014).

Minor fimbriae are abundant surface appendages of *P. gingivalis* and are homopolymers of either 75- or 53-kDa protein (Arai et al. 2000; Nagano et al. 2015). Since 53-kDa protein is immunogenic and distinct from FimA in

the antigenicity (Hamada et al. 1996), it should be considered as one of the most important target antigens along with FimA for a better control of *P. gingivalis*-induced periodontitis. In this study, 53-kDa protein-specific monoclonal antibodies were produced and accumulated in rice cell suspension culture for easy purification, and the plant-produced monoclonal antibodies were observed to be biologically active against 53-kDa protein of *P. gingivalis*.

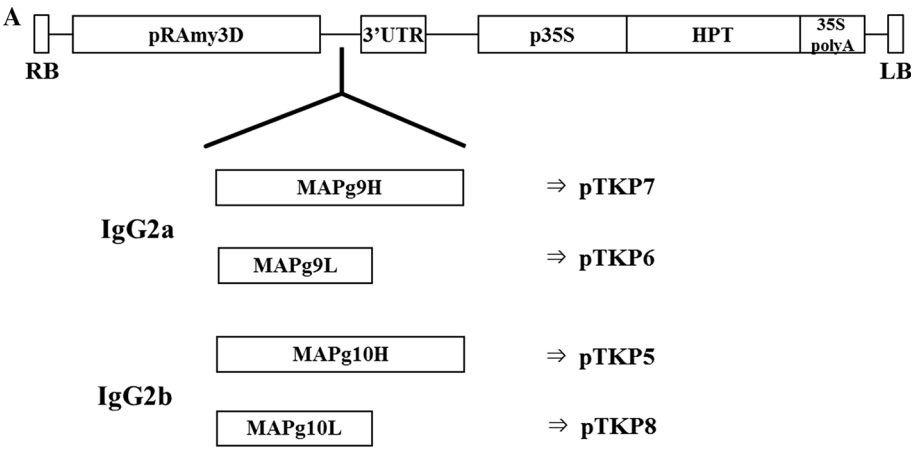
Materials and methods

Construction of plant expression vectors

Monoclonal antibodies were generated from mice immunized with whole cells of *P. gingivalis* strain A7A1-28 according to the method described in our previous study (Koh et al. 2011). Hybridoma cell lines, MAPg4, MAPg9, and MAPg10, expressing monoclonal antibodies against 53-kDa protein of *P. gingivalis* were selected to obtain the genes encoding the monoclonal antibodies. Total RNAs were extracted from the hybridoma cell lines, and single-stranded DNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Complementary DNA for heavy and light chain genes of the monoclonal antibodies were amplified by PCR with primer sets specific for the heavy and light chain genes, in which the appropriate restriction enzyme sites (bold) were included for convenient subcloning (Fig. 1). PCR products were cloned into a pGEM®T-Easy vector (Promega, Madison, WI), and the sequence of each gene was confirmed by DNA sequence analysis (Genotech, Seoul, Korea). The sequencing result of MAPg4 showed one nucleotide deletion when compared with the previously reported sequences of IgG2 heavy chain gene. For construction of recombinant plant expression vectors, the digested fragments of the cloned genes with *Xba*I and *BamH*I or *Bgl*II were introduced into the *Xba*I and *BamH*I sites of the plant expression vector, pMYV657 (Kim et al. 2012a). The resulting recombinant plant expression vectors containing the heavy or light chain gene from MAPg9 and MAPg10 were designated pTKP7, pTKP6, pTKP5, and pTKP8, respectively (Fig. 1). The heavy and light chain genes are under the control of the promoter and the 3′ untranslated region of rice amylase 3D (*RAmy3D*) gene. These plant expression vectors harbored the hygromycin phosphotransferase gene as a selection marker for plant transformation.

Rice transformation

Scutellum-derived callus tissues from mature rice seeds (*Oryza sativa* L. cv. Dongjin) were transformed via particle bombardment-mediated gene transfer (Chen et al. 2002). Briefly, dehusked rice seeds were sterilized and cultured on N6 medium supplemented with 2,4-dichlorophenoxyalic acid (2,4-D) (2 mg L^{-1}), sucrose (30 g L^{-1}), and kinetin (0.2 mg L^{-1}) for 7 days to ensure callus induction. Gold particles (0.6 μm) were coated with 10 μg of each recombinant plant expression vector (pTKP6 and pTKP7 for



B

Primers	Sequence (5′ → 3′)
MAPg9HCFX	GC**TCTAGA**GCCACCATGATGGTGTTAAGT
MAPg9HCRBG	GC**AGATCT**CATTTACCCGGAGA
MAPg9LCFX	GC**TCTAGA**GCCACCATGAAGTTGCCTGT
MAPg9LCRB	**GGATCC**CTAACACTCATTCCT
MAPg10HCFX	GC**TCTAGA**GCCACCATGGGATGGAGCTGT
MAPg10LCFX	GC**TCTAGA**GCCACCATGGTGTCCTCAGCTT

Fig. 1 Construction of plant expression vectors. Complementary DNAs of heavy (MAPg9H and MAPg10H) and light chains (MAPg9L and MAPg10L) of MAPg9 and MAPg10 monoclonal antibodies specific for 53-kDa protein of *P. gingivalis* strain A7A1-28 were constructed into plant expression vectors under the control of a promoter (pRAmy3D) and 3′ untranslated region (3′UTR) of the rice amylase 3D gene. The hygromycin phosphotransferase gene (HPT) was under the control of a promoter (p35S) and terminator (35S polyA) of the Cauliflower mosaic virus 35S (**a**). The PCR primer sets specific for the target genes were used for DNA amplification (**b**)

MAPg9, pTKP5 and pTKP8 for MAPg10). After bombardment with the plasmid-coated gold particles, the calli were cultured on N6 co-culture medium supplemented with 2,4-D (2 mg L^{-1}), sucrose (30 g L^{-1}), kinetin (0.2 mg L^{-1}), and glucose (10 g L^{-1}) without antibiotics for 3 days in dark conditions, and then transferred to N6 selection medium supplemented with the antibiotic hygromycin B (50 mg L^{-1}) for the selection of transgenic callus.

Genomic DNA PCR

Genomic DNA in putative transgenic and wild-type rice calli was purified using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) and used as template for genomic DNA PCR analysis. The presence of the light and heavy chain genes of the monoclonal antibodies were determined using gene specific forward and reverse primers, which were used for the construction of the recombinant plant expression vectors. The PCR mixture contained 100 ng of genomic DNA, 10 pmol primers, 200 μM dNTPs, 1× *Taq* polymerase buffer (1 mM Tris–Cl, pH 8.8, 5 mM KCl, and 0.01 % Triton X-100), 1.5 mM $MgCl_2$, and 2 U i-*Taq* Polymerase (iNtRON Biotechnology, Seoul, Korea) in a total reaction volume of 20 μL. The amplified PCR products were separated on a 1 % agarose gel and visualized with ethidium bromide.

Establishment of rice cell suspension culture

Transgenic rice calli showing positive signals in the genomic DNA PCR analysis were cultured at 28 °C in a shaking incubator at a rotation speed of 110 rpm in the dark. The rice cell suspension culture was maintained in 50 mL of N6 medium containing 2,4-D (2 mg L^{-1}), kinetin (0.02 mg L^{-1}), and 3 % sucrose (Thompson et al. 1986) in a 300-mL flask, and a 10-mL inoculum was transferred to fresh medium every 9 days for subculturing. For induction of the monoclonal antibody gene expression under the control of the *RAmy3D* promoter, the rice cell suspension medium was removed via aspiration, and the rice calli were transferred to fresh N6 medium without sucrose, adjusting the callus volume to 10 % (volume of wet callus/volume of medium). After 7 days of the transgenic rice cell suspension culture under sugar starvation conditions, the cell suspension culture medium containing secreted proteins was harvested by aspiration for the following analysis.

Western blot analysis

Proteins in the harvested suspension culture medium were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or non-reducing conditions and transferred to Hybond™ C nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ) using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA) for western blot analysis. The protein-blotted membranes were blocked with 5 % skim milk in TBST (Tris-buffered saline with 0.05 % Tween-20) and incubated with the following antibodies: goat anti-mouse IgG (whole molecule) conjugated with alkaline phosphatase (Sigma A3562, St. Louis, MO), goat anti-mouse IgG (γ-chain specific) conjugated with alkaline phosphatase (Sigma A3438), and rat monoclonal anti-mouse kappa light chain (Sigma K2132). Goat anti-rat IgG (whole molecule) conjugated with alkaline phosphatase (Sigma A8438) was the secondary antibody for the membranes treated with the rat monoclonal anti-mouse kappa light chain. The immunoreactive complexes were detected by adding BCIP/NBT color development solution (Sigma B6404).

Quantification of plant-produced monoclonal antibodies

Expression of MAPg9 monoclonal antibody in the suspension culture medium was observed to be higher than that of MAPg10. Using the rice cell suspension culture of MAPg9, the time-dependent expression level of the monoclonal antibody was measured by quantitative ELISA after the induction under sugar starvation conditions. Briefly, wells of microtiter plates were coated with goat anti-mouse IgG2a (Sigma ISO2) to detect MAPg9 monoclonal antibody. The wells were washed three times with TBST and blocked with 1 % bovine serum albumin in PBS at 37 °C for 2 h. Samples collected at 2-day intervals for 14 days from the suspension culture medium containing the monoclonal antibody were added to the wells and incubated at 37 °C for 2 h. Known amounts of mouse IgG2a isotype from murine myeloma (M5409 Sigma) as control were used to make a standard curve for MAPg9 monoclonal antibody. After washing the wells of the microplates with TBST, anti-mouse IgG (H + L) conjugated with horseradish peroxidase (Promega W4021) was added. The microplates were incubated at 37 °C for 2 h and washed three times with PBST. The plates were incubated with 100 μL per well of TMB substrates L (PharMingen, Fallbrook, CA) for 30 min at room temperature, and the optical density of the end-product was measured at 405 nm in an ELISA reader (Packard Instrument Co, Downers Grove, IL).

Results

Cloning of monoclonal antibody genes against 53-kDa protein

Mice were injected with whole cells of *P. gingivalis* to obtain B hybridoma cells expressing monoclonal antibodies against 53-kDa protein of *P. gingivalis*. Three (MAPg4, MAPg9 and MAPg10) hybridoma cells were shown to

react strongly with the protein in the western blot analysis. Total RNAs were prepared from the hybridoma cells, and the first single-stranded DNA was synthesized to amplify heavy and light chain genes of the monoclonal antibodies. The PCR products for cDNA of MAPg9 and MAPg10 monoclonal antibodies were detected and cloned. PCR products of MAPg4 monoclonal antibody were obtained, but deletion of one particular base pair was invariably observed when their nucleotide sequences were compared with the previously reported sequences of IgG2 heavy chain genes. Therefore, the PCR products of MAPg4 monoclonal antibody were excluded from this study.

The nucleotide sequences of the heavy and light chain genes of the monoclonal antibodies were determined (Supplementary Figs. 1 and 2). The constant region sequences of the heavy chains of MAPg9 and MAPg10 monoclonal antibodies were matched with those of the reported mouse line gamma 2a and gamma 2b genes, respectively. The light chain gene sequences of MAPg9 and MAPg10 monoclonal antibodies were matched with those of the reported mouse line kappa.

Construction of plant expression vector and genomic DNA PCR analysis

The cDNAs encoding the heavy and light chain genes of the monoclonal antibodies against 53-kDa protein of *P. gingivalis* were introduced into a plant expression vector under the control of the rice amylase 3D promoter (Fig. 1), and then transformed into rice embryogenic calli using bombardment-mediated gene transfer. The integration of the light and heavy chain genes of the monoclonal antibodies into putative transgenic rice calli was confirmed by genomic DNA PCR analysis with gene-specific primers. Sixteen out of 24 MAPg9-transgenic rice calli generated PCR products of the heavy chain gene, and all of the 24 MAPg9-transgenic rice calli generated PCR products of the light chain gene, as positive PCR signals with the gene-specific primers represented the expected sizes of the heavy and light chain genes: 1.4 and 0.7 kb, respectively. Nineteen out of 24 MAPg10-transgenic rice calli yielded PCR products of the heavy chain gene, and 23 transgenic rice calli generated PCR products of the light chain gene. No PCR product of the heavy or light chain gene was detected in wild type rice calli (Fig. 2).

Expression of monoclonal antibodies in transgenic rice calli

The transgenic rice calli showing the positive PCR signals of the monoclonal antibody genes were selected, and the expression of the genes was induced for 7 days under sugar starvation condition because RAmy3D promoter is activated in the absence of sugar in the medium. The expected molecular weights of the light and heavy chains of MAPg9 and MAPg10 monoclonal antibodies are 26.2, 52.4, 25.8, and 51.3 kDa, respectively. The bands of MAPg9 (approximately 157 kDa) and MAPg10 monoclonal antibodies (approximately 154 kDa) in the callus extracts were detected by western blot using anti-mouse IgG (H&L) (data not shown).

Transgenic rice calli expressing the monoclonal antibodies were propagated in rice cell suspension culture. Expression of the monoclonal antibodies was induced for 7 days under sugar starvation conditions. Secretion and accumulation of the light chains, heavy chains, and heavy-light chain assembled antibodies were detected in the transgenic rice cell suspension culture by western blot analysis using heavy chain-specific or light chain-specific antibodies under reducing and non-reducing conditions. In non-reducing condition, bands of MAPg9 and MAPg10 monoclonal antibodies detected by the heavy-chain specific antibody were located at the same position as those detected by the light chain-specific antibody, indicating that the heavy and light chains were assembled into whole antibody molecules (Figs. 3, 4). In reducing condition, monomers of the heavy chain and light chain were detected as bands of 55 and 25 kDa, respectively (Figs. 3, 4). Additional several bands detected in the western blot analysis might indicate complex forms and truncated forms of the antibodies (Figs. 3, 4).

Biological activity of monoclonal antibodies produced in transgenic rice calli

After the expression of the monoclonal antibodies in the transgenic rice cell suspension culture, the plant-produced monoclonal antibodies were to confirm their reactivity with 53-kDa protein of *P. ginvalis*. In the western blot analysis, MAPg9 and MAPg10 monoclonal antibodies produced in the rice suspension cultures reacted with the recombinant 53-kDa protein (with an apparent molecular weight of 56 kDa: 3-kDa vector fusion peptide connected to 53-kDa protein) purified from *E. coli* (Fig. 5) and 53-kDa protein in the sonic extract of *P. gingivalis* strain A7A1-28 (Fig. 6). No immunoreactive band was detected in the sonic extract of *P. gingivalis* strain ATCC33277, which is known not to have 53-kDa protein (Nagano et al. 2015).

The expression levels of the monoclonal antibodies in the transgenic rice callus suspension were measured by ELISA at 2-day intervals for 14 days after the induction under conditions of sugar depletion. The highest expression level of MAPg9 monoclonal antibody was 14.9 μg mL^{-1} on day 8 after the induction under sugar starvation conditions (Fig. 7).

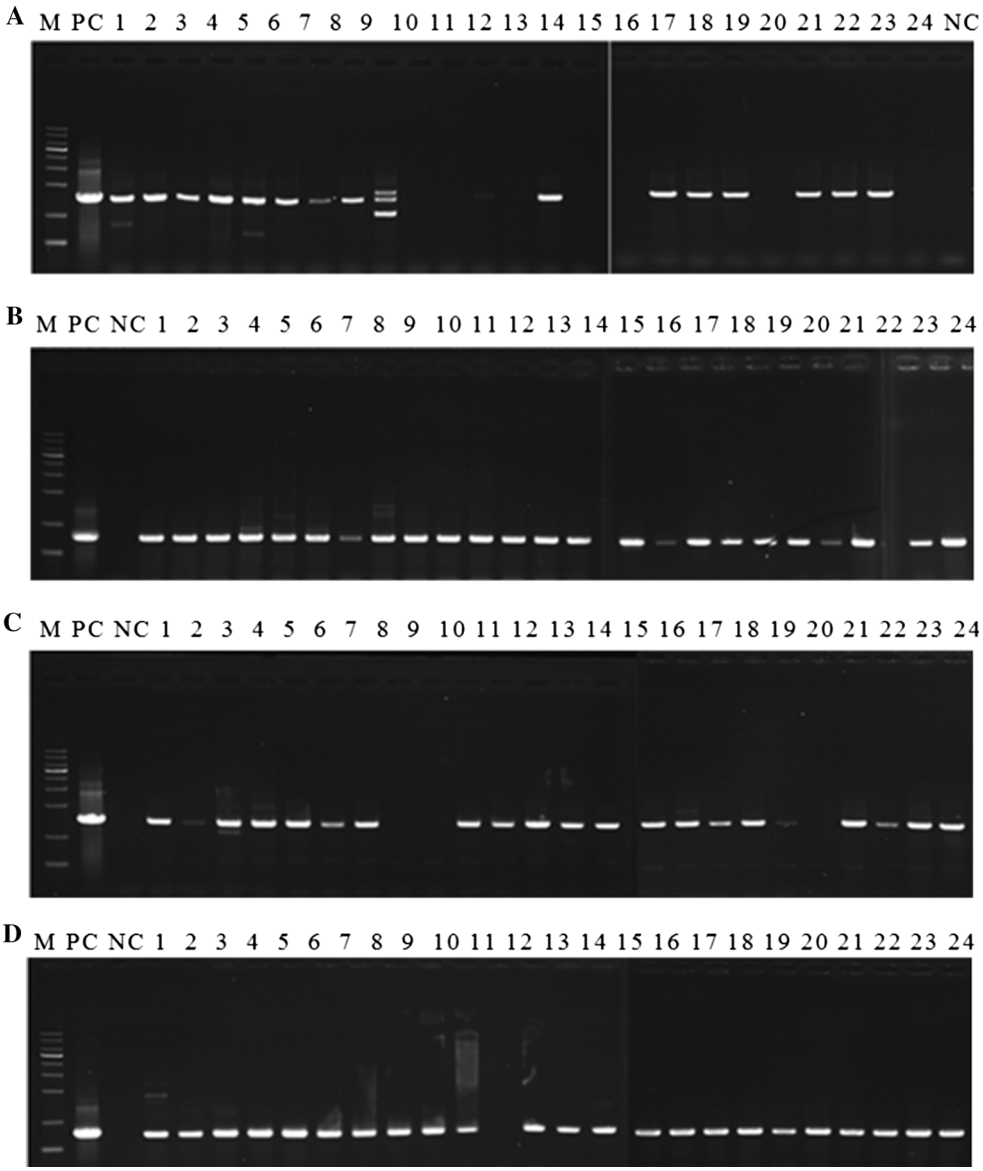


Fig. 2 Genomic DNA PCR analysis of MAPg9 and MAPg10 monoclonal antibody genes in putative transgenic rice calli. The heavy chain (**a**, **c**) and light chain (**b**, **d**) genes of MAPg9 (**a**, **b**) and MAPg10 (**c**, **d**) monoclonal antibodies in putative transgenic calli were amplified by genomic DNA PCR amplification with the gene-specific primers. *Lane M* is 1 kb DNA ladder (ELIPIS Biotech, Seoul, Korea). *Lane PC* is the plant expression vectors containing the cloned genes as positive control. *Lane NC* is the genomic DNA of wild-type rice callus as negative control. *Lanes 1–24* are the putative transgenic rice lines with MAPg9 or MAPg10 monoclonal antibody genes

Discussion

Periodontitis defined as infection-induced inflammatory disease of periodontal tissue surrounding and supporting the tooth, if untreated, can eventually lead to the loss of teeth, affecting not only oral health but also whole being wellness. Even if treated, since once-damaged periodontal tissue is unable to be restored to its original condition and may remain continuously vulnerable to bacterial infection, periodontitis often recurs at a later time, continuously damaging the tissue (Iwata et al. 2014; Machtei et al. 1997). Moreover, ample evidence strongly indicates that periodontitis is associated with a number of systemic diseases (Kim and Amar 2006; Hajishengallis 2015). Therefore, periodontitis should be now regarded as a potential overall health threat, and strategies for effective control and prevention of the disease should be considered from not only an individual, but also a public oral health perspective. Strategy of passive immunization using monoclonal antibodies with biological activities against pathogens involved in periodontitis can be one of ways to control the disease. However, production of monoclonal antibodies in conventional animal-based expression systems has risk of animal pathogen contamination and results in high production costs. It has been shown that the production of recombinant therapeutic proteins in plant expression systems allows large-volume manufacturing for vaccines, antibodies, and other biopharmaceuticals. Besides, plant-based platforms for production of monoclonal antibodies offer significant advantages over the

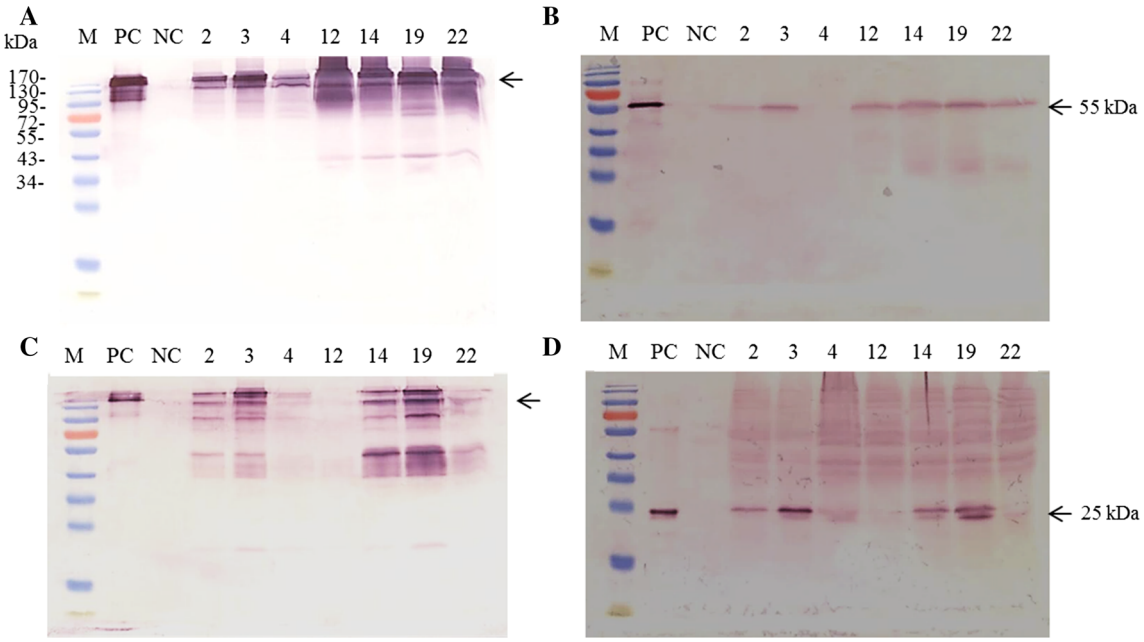


Fig. 3 Western blot analysis of MAPg9 monoclonal antibody in transgenic rice cell suspension cultures. MAPg9 monoclonal antibody was detected by heavy (**a**, **b**) or light (**c**, **d**) chain-specific antibody under non-reducing (**a**, **c**) and reducing conditions (**b**, **d**). The accumulated MAPg9 monoclonal antibody secreted into the suspension culture medium was detected 7 days after sugar starvation conditions. *Lane M* is pre-stained protein molecular weight standards (Fermentas, Glen Burnie, MD) for SDS-PAGE. *Lane PC* is the plant-produced anti-FimA monoclonal antibody #123 in the previous study (Kim et al. 2014) as positive control. *Lane NC* is the wild-type rice cell culture medium as negative control. *Lanes 2*, *3*, *4*, *12*, *14*, *19*, and *22* are the culture media from the transgenic rice cell cultures of MAPg9

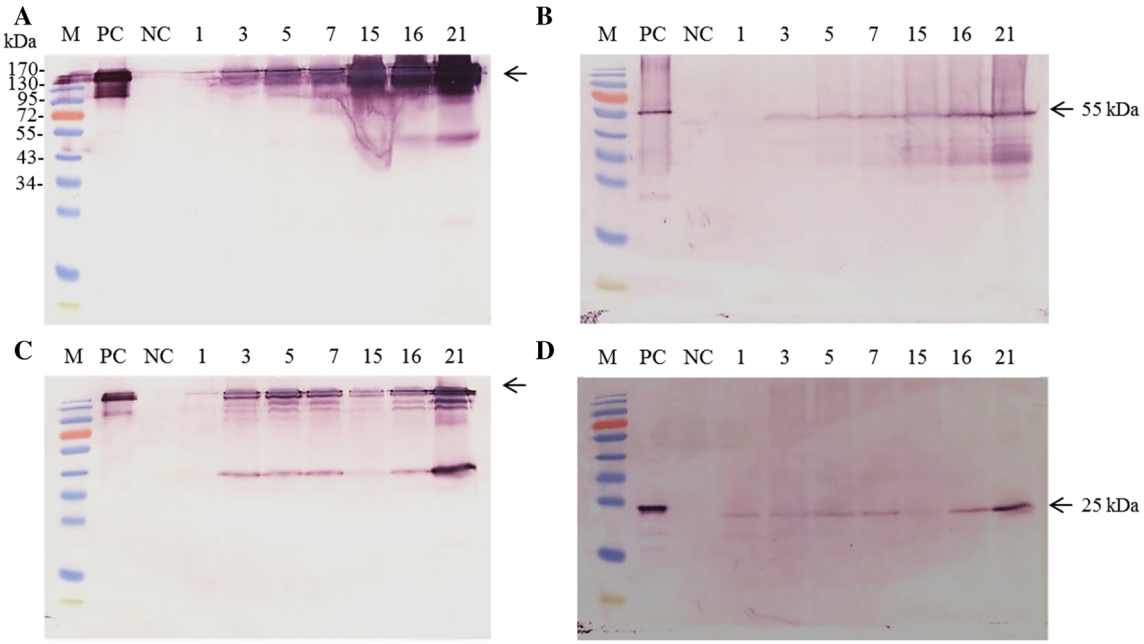


Fig. 4 Western blot analysis of MAPg10 monoclonal antibody in transgenic rice cell suspension cultures. MAPg10 monoclonal antibody was detected by heavy (**a**, **b**) or light (**c**, **d**) chain-specific antibody under non-reducing (**a**, **c**) and reducing conditions (**b**, **d**). The accumulated MAPg10 monoclonal antibody secreted into the suspension culture medium was detected 7 days after sugar starvation conditions. *Lane M* is pre-stained protein molecular weight standards. *Lane PC* is the plant-produced anti-FimA monoclonal antibody #123 as positive control. *Lane NC* is the wild-type rice cell culture medium as negative control. *Lanes 1*, *3*, *5*, *7*, *15*, *16*, and *21* are the culture media from the transgenic rice cell cultures of MAPg10

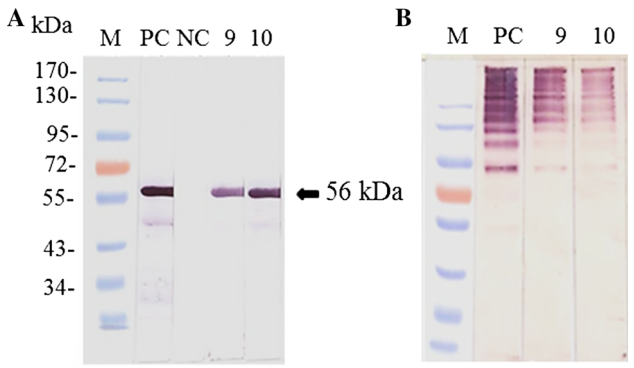


Fig. 5 Immunoreactivity of the monoclonal antibodies produced in transgenic rice callus suspension cultures with recombinant 53-kDa protein. Recombinant 53-kDa protein purified from *E. coli* was used to detect the immunoreactivity of the monoclonal antibodies produced in the transgenic rice callus suspension cultures under the reducing (**a**) or non-reducing (**b**) conditions. *Lane M* is pre-stained protein molecular weight standards. *Lane PC* is the parental mouse monoclonal antibodies against 53-kDa protein as positive control. *Lane NC* is the wild-type rice cell culture medium as negative control. *Lanes 9* and *10* are MAPg9 and MAPg10 monoclonal antibodies produced in the transgenic rice cell suspension cultures. Note that the actual molecular weight of the recombinant 53-kDa protein is ~56 kDa since ~3-kDa pRSET/B vector (Invitrogen, Carlsbad, CA) fusion peptide was connected to the N-terminus of 53-kDa protein

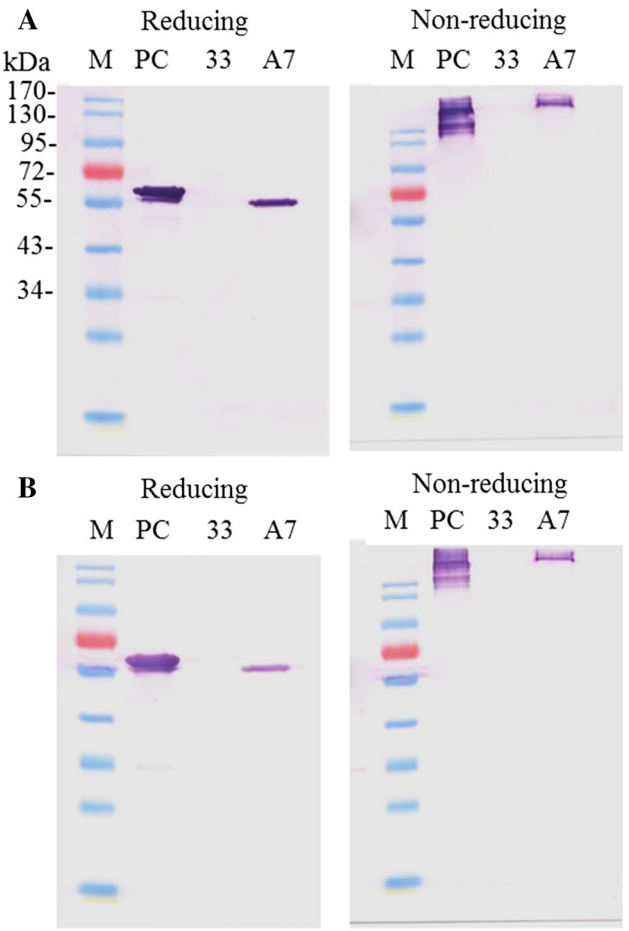


Fig. 6 Immunoreactivity of the monoclonal antibodies produced in transgenic rice callus suspension cultures with 53-kDa protein of *P. gingivalis*. Sonic extract of *P. gingivalis* A7A1-28 containing native 53-kDa protein was used to detect the immunoreactivity of the monoclonal antibodies produced in the transgenic rice cell suspension cultures of MAPg9 (**a**) and 10 (**b**) under the reducing and non-reducing conditions. *Lane M* is pre-stained protein molecular weight standards. *Lane PC* is the recombinant 53-kDa protein purified from *E. coli* as positive control. *Lane 33* is the sonic extract of *P. gingivalis* ATCC33277, which does not have 53-kDa protein. *Lane A7* is the sonic extract of *P. gingivalis* A7A1-28

conventional expression systems, including lack of animal pathogens and low production costs (Orzáez et al. 2009; Kolotilin et al. 2014). Therefore, passive immunization using plant-produced monoclonal antibodies can be considered as a cost-effective public health intervention. The monoclonal antibody against *Streptococcus mutans*, the main causative agent of dental caries, was expressed in transgenic plants, and passive immunization using this antibody prevented the bacterial recolonization for at least 4 months (Ma et al. 1998). In our previous studies, monoclonal antibodies against native FimA proteins of *P. gingivalis* were produced in *N. benthamiana* by agroinfiltration (Kim et al. 2012b) and rice cell suspension culture systems (Kim et al. 2014). The plant-produced monoclonal antibodies in the transgenic rice cell suspension culture were easily purified by protein G-affinity chromatography. FimA-specific monoclonal antibodies produced in the rice suspension culture showed biological activity against *P. gingivalis*, inhibiting the bacterial binding to saliva-coated hydroxyapatite beads and invasion of KB cell by the bacterium, and enhancing the intracellular killing of *P. gingivalis* by polymorphonuclear neutrophils. These results suggest that passive immunization with the plant-produced monoclonal antibodies may also be feasible to achieve protection against periodontitis.

It has been shown that 53-kDa protein of *P. gingivalis*, which is one of the most important periodontal pathogens, is the major antigen that is recognized by sera from periodontitis patients (Kokeguchi et al. 1990; Kurihara et al. 1991). In the present study, the whole cells of *P. gingivalis* A7A1-28 were used to elicit monoclonal antibodies. Three monoclonal antibodies that induced a strong agglutination of *P. gingivalis* A7A1-28 were selected, and all these antibodies were observed to react with 53-kDa protein of the bacterium in western blot analysis (data not shown). These observations suggest that 53-kDa protein has an important role in eliciting protective immune responses and can be used as a vaccine candidate for periodontitis.

In western blot analysis under non-reducing conditions, monoclonal antibodies secreted into the suspension culture medium were detected, and the bands of the monoclonal antibodies detected by heavy chain- or light chain-specific antibodies were located at the same positions

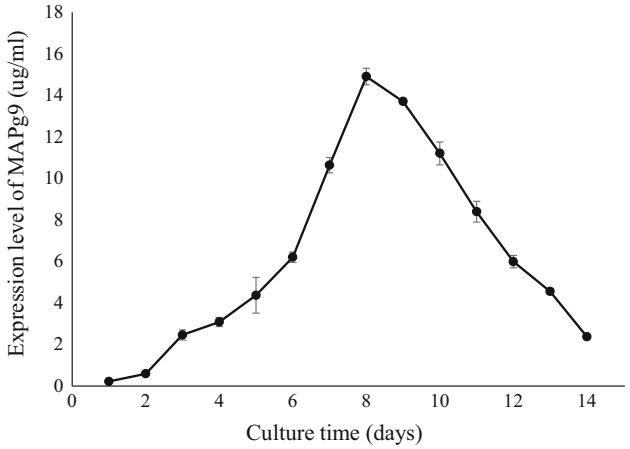


Fig. 7 Time-dependent expression level of MAPg9 monoclonal antibody in transgenic rice cell suspension culture. The amount of MAPg9 monoclonal antibody in the rice cell suspension culture medium accumulated was measured by ELISA after the induction of the monoclonal antibody gene in sugar depletion medium. Data are expressed as mean ± SD ($n = 3$)

corresponding to the size of a whole antibody, indicating that the heavy and light chains of the monoclonal antibodies were assembled into a biologically full-length antibody after being secreted into the rice suspension culture medium. The several additional bands detected via western blot analysis might indicate complex forms and truncated forms of antibodies (Figs. 3, 4).

The monoclonal antibodies produced in the rice cell suspension cultures were immunoreactive with the recombinant 53-kDa protein purified from *E. coli* as observed by western blot analysis (Figs. 5, 6), in which the resulting bands were the same as those with the mouse monoclonal antibodies against 53-kDa protein. The result suggests that the plant-produced monoclonal antibodies have the same immunoreactivity with 53-kDa protein as the parental mouse monoclonal antibodies. The plant-produced monoclonal antibodies also reacted with the sonic extract of *P. gingivalis* A7A1-28 but did not with the extract of *P. gingivalis* ATCC33277, which does not express 53-kDa protein. The immunoreactive bands in the western blot analysis using the recombinant 53-kDa protein under non-reducing conditions showed a ladder-like pattern, indicating that the recombinant protein exists as oligomeric and polymeric forms. When the sonic extract of *P. gingivalis* A7A1-28, which contains native 53-kDa protein, was used under the same conditions, sizes of the immunoreactive bands appeared to be higher than those generated by the antibodies with the recombinant 53-kDa protein (Fig. 6). The bands were stacked nearly to the top of the gel, indicating that 53-kDa protein exists as polymeric forms in nature. Previous studies showed that 53-kDa protein is one of the most immunogenic antigens as revealed by western blot analysis of sera from periodontitis patients (Kokeguchi et al. 1990; Kurihara et al. 1991). Besides, it has been recently reported that along with major fimbriae (FimA), minor fimbriae (Mfa1) are also abundant surface appendages of *P. gingivalis*, and 53-kDa protein is another major subunit of minor fimbriae, which had been known to be comprised of homopolymers of 75-(or 67-) kDa protein (Arai et al. 2000; Nagano et al. 2015). In the present study, the plant-produced monoclonal antibodies recognized the native polymeric forms of 53-kDa protein. Therefore, the monoclonal antibodies produced by the rice suspension culture system in the present study can be used for passive immunization against *P. gingivalis*-induced periodontitis.

Since the production of monoclonal antibodies was firstly reported in transgenic tobacco (1.3 % of total soluble protein) by crossing transgenic plants expressing each heavy and light chain in 1989 (Hiatt et al. 1989), many monoclonal antibodies were produced in several plant expression systems. The plant-derived monoclonal chimeric secretory IgA/G antibody CaroRx against *S. mutans* (200–500 μg g^{-1} fresh weight material) is one of the first clinically tested plantibodies (Ma et al. 1995). Recently, transient expression systems using agroinfiltration with a viral vector system (MagnICON system) was developed to rapidly and highly produce recombinant vaccines in *Nicotiana benthamiana* (Gleba et al. 2005). Two different non-competing viruses, tobacco mosaic virus and potato virus X, were shown to produce full-size monoclonal antibodies at levels as high as 0.5 mg g^{-1} fresh weight (Giritch et al. 2006). The heavy and light chain subunits of IgG monoclonal antibody, mAb 6D8 against Ebola virus GP1 was produced at 0.5 mg g^{-1} fresh weight using the multi-replicon single vector based on bean yellow dwarf virus (Huang et al. 2010). Although plant cell suspension culture is more expensive than production in plants, secreted antibody in the suspension medium allows simple recovery and downstream purification (Huang and McDonald 2012). Transgenic rice cell suspension culture using rice amylase 3D promoter system is used to produce highly recombinant proteins (Shin et al. 2003). This system has been improved by reducing secreted amylase and cysteine proteinase by RNA interference to increase recombinant proteins accumulated in rice cell suspension culture (Kim et al. 2013). The expression levels of monoclonal antibodies against FimA of *P. gingivalis* were 17.3 mg L^{-1} in rice cell suspension culture under the control of rice amylase 3D promoter (Kim et al. 2014). In this study, the production of monoclonal antibody against 53-kDa protein of *P. gingivalis* was 14.9 mg L^{-1} in rice cell suspension culture under the control of rice amylase 3D promoter.

In the present study, we cloned cDNA of heavy and light chain genes from hybridoma cell lines expressing

monoclonal antibodies to 53-kDa protein of *P. gingivalis*, and produced and accumulated the monoclonal antibodies in transgenic rice cell suspension culture system. The plant-derived monoclonal antibodies showed immunoreactivity with the protein and demonstrated their feasibility for passive immunization against *P. gingivalis*. The overall results suggest that transgenic rice cell suspension culture can be employed for production of monoclonal antibodies with biological activity, and the monoclonal antibodies to 53-kDa protein produced in the suspension culture may be used for periodontal protection from *P. gingivalis*-induced periodontitis. It is possible to expect that concomitant use of the plant-produced monoclonal antibodies against FimA major fimbriae and 53-kDa minor fimbriae of *P. gingivalis* we have generated may be able to provide more effective protection of periodontal tissue from invading *P. gingivalis*.

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