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Plant-derived PAP proteins fused to immunoglobulin A and M Fc domains induce anti-prostate cancer immune response in mice

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In this study, recombinant Fc-fused Prostate acid phosphatase (PAP) proteins were produced in transgenic plants. PAP was fused to immunoglobulin (Ig) A and M Fc domain (PAP-IgA Fc and PAP-IgM Fc), which were tagged to the ER retention sequence KDEL to generate PAP-IgA FcK and PAP-IgM FcK. *Agrobacterium*-mediated transformation was performed to produce transgenic tobacco plants expressing four recombinant proteins. Genomic PCR and RT-PCR analyses confirmed the transgene insertion and mRNA transcription of PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK in tobacco plant leaves. Western blot confirmed the expression of PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK proteins. SEC-HPLC and Bio-TEM analyses were performed to confirm the size and shape of the plant-derived recombinant PAP-Fc fusion proteins. In mice experiments, the plant-derived IgA and IgM Fc fused proteins induced production of total IgGs including IgG1 against PAP. This result suggests that IgA and IgM Fc fusion can be applied to produce recombinant PAP proteins as a prostate cancer vaccine in plant expression system. [BMB Reports 2023; 56(7): 392-397]

INTRODUCTION

Prostate cancer is a common cancer in men and the second leading cause of cancer deaths (1-3). Existing cancer treatment strategies such as surgery, chemotherapy, and radiation therapy (4-6) have been helpful in treating prostate cancer; however, new effective treatments are needed to overcome limitations such as side effects and recurrence rates (7).

Therapeutic Fc-fusion proteins have been successfully used to treat various diseases, including cancer, viral infections, and immune diseases (8-12). The Fc-fusion proteins are composed of an immunoglobulin (Ig) Fc domain linked to another peptide or antigen. Immunoglobulin IgG1 Fc-fusion proteins have emerged as promising therapeutic drugs in immunology (9). In addition, the Fc fusion can be applied to generate dimeric and hexameric structures, likely in a star shape (9, 13-15). Among diverse tissues, PAP highly exists in prostate tissues compared to other organs (16). PAP is a prostate tumor antigen, and the target of the FDA-approved anti-prostate cancer vaccine. In this study, thus, PAP Fc fusion proteins were produced as a prostate cancer vaccine candidate through the fusion of immunoglobulin A (IgA) and immunoglobulin M (IgM) Fc domains. IgA exists primarily as a monomer or dimer, while IgM primarily exists as a pentamer. The polymer structure is thought to increase antibody avidity for the antigen and is required for functions such as complement activation by IgM and epithelial secretion of IgA and IgM (13, 17, 18).

Plants have generally been used to obtain therapeutic recombinant proteins and monoclonal antibodies for diseases such as cancers, viral infections, and immune diseases (19-22). In previous studies, immunotherapeutic proteins have been produced using transgenic and transient expression systems including plant suspension cell cultures (23, 24). One advantage of using the plant expression system for immunotherapeutic proteins such as vaccines and antibodies is economical plant biomass production. In addition, there is no risk of contaminating therapeutic proteins with human pathogens, and proteins can be produced with similar glycosylation patterns as eukaryotic cells (25-27). In this study, plant was utilized to express PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK. These proteins were then compared to determine the immunogenic effects of the IgA and IgM Fc domains.

RESULTS

Production of transgenic tobacco plants expressing PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK

Transgenic tobacco plant lines expressing PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK were obtained through *Agrobacterium*-mediated plant transformation. PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK genes were placed downstream of an untranslated leader sequence from AMV and the duplicated CaMV 35S promoter (E/35S-P) (Fig. 1). PCR analyses

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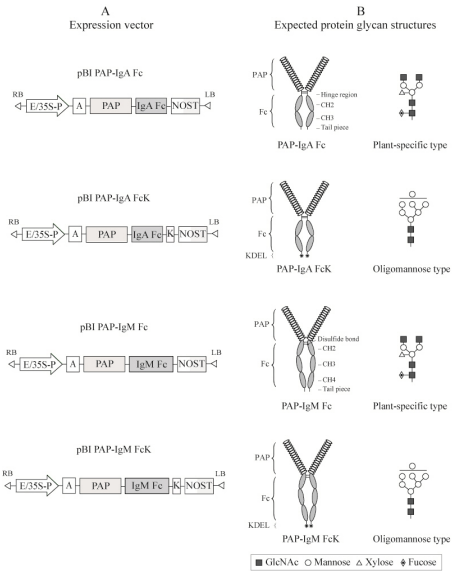

Fig. 1. Schematic diagram of expression cassettes for PAP-Fc fusion proteins, and their protein and glycan structures. (A) PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK gene expression cassettes were designed for plant binary vectors pBI PAP-IgA Fc, pBI PAP-IgM Fc, pBI PAP-IgA FcK, and pBI PAP-IgM FcK, respectively. E/35S-P, duplicated 35S promoter; A, AMV RNA4 untranslated leader sequence; NOST, nopaline synthase gene terminator; K, KDEL ER retention motif. (B) The expected recombinant protein and glycan structures of PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK.

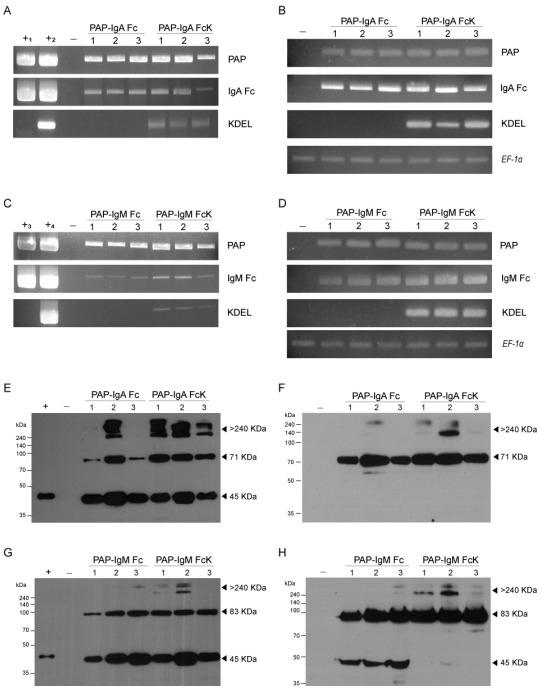

Fig. 2. PCR, RT-PCR, and immunoblot analyses to confirm transgenic plant expressing PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK. The PCR and RT-PCR products from tobacco plant leaf tissues were visualized by ethidium bromide staining. (A, C) PCR of PAP-IgA Fc (1,905 bp), PAP-IgM Fc (2,208 bp), PAP-IgA FcK (1,917 bp), and PAP-IgM FcK (2,220 bp) $+_1$, $+_2$, $+_3$, and $+_4$ = positive controls (PAP-IgA Fc, PAP-IgA FcK, PAP-IgM Fc, and PAP-IgM FcK in vector, respectively). (B, D) RT-PCR analysis of PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK. Total mRNA isolated from tobacco leaves of three randomly selected PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK transgenic plants. − = negative control (non-transgenic tobacco plant); EF-1α = endogenous control. The PAP-IgA Fc (71 kDa), PAP-IgM Fc (82.8 kDa), PAP-IgA FcK (71.5 kDa), and PAP-IgM FcK (83.3 kDa) protein bands were observed in blot with anti-PAP IgG conjugated to HRP (1:5,000) (E, G) anti-IgA Fc IgG conjugated to HRP (1:5,000) (F) and goat anti-IgM Fc IgG conjugated to HRP (1:5,000) (H) respectively. +, positive control (mammalian-derived recombinant PAP, 50 ng); −, negative control (non-transgenic plant). PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK were extracted from 20-μl leaf samples.

were conducted to confirm the presence of PAP, IgA Fc, IgM Fc, and KDEL domain genes in PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK transgenic tobacco plant leaves, respectively (Fig. 2A, C). The Semi-quantitative RT-PCR was conducted to determine the expression of PAP, IgA Fc domain, IgM Fc domain, and KDEL in PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK transgenic plants (Fig. 2B, D). Transgenic tobacco plant lines with PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK had the PCR bands at 1,905, 2,208, 1,917, and 2,220 bp, respectively (Fig. 2A, C). The RT-PCR bands of PAP, IgA Fc, IgM Fc, and KDEL were observed in the transgenic plant leaves (Fig. 2B, D). The PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK transgenes were not detected in non-transgenic plant material (Fig. 2). The EF-1α band was used as the standard control and was detected at 67 bp.

Expression of PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK in plants

Leaf samples of PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK transgenic plant lines were obtained for immunoblot (Fig. 2). Transgenic lines with PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK showed approximately 71 kDa (Fig. 2E, F) and 83 kDa (Fig. 2G, H) protein bands, respectively. The positive control PAP was observed at 45 kDa (Fig. 2E, G). In PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK protein samples, 45 kDa and >240 kDa protein bands were observed in addition to the 71 and 83 kDa protein bands (Fig. 2E, G). In each of the four protein samples treated with anti-human Fcγ antibody, protein bands were detected at 71 kDa and >240 kDa. However, no 45 kDa protein band was observed in the PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK transgenic lines (Fig. 2F, H, anti-IgG Fc). No protein band was observed in non-transgenic plant (Fig. 2E-H).

Purification of PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK fusion proteins

In SDS-PAGE, transgenic plants expressing PAP-IgA Fc (71 kDa), PAP-IgM Fc (82.8 kDa), PAP-IgA FcK (71.5 kDa), and PAP-IgM

FcK (83.3 kDa) showed the expected recombinant fusion protein sizes (Supplementary Fig. 2). The purified bands of PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK were clear, showing that the plant-derived recombinant proteins were properly purified. The 50-kDa protein band was observed in PAP-IgA Fc, but not in PAP-IgA FcK (Supplementary Fig. 2A, B). In PAP-IgM Fc, <50 kDa protein was detected, but not in PAP-IgM FcK (Supplementary Fig. 2C, D).

Size-exclusion chromatography-high-performance liquid chromatography (SEC-HPLC) analysis of plant-derived recombinant fusion proteins

SEC-HPLC was performed to determine the size distribution of the fusion proteins PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK. All proteins migrated on HPLC as double or triple peaks between 5 and 8 min (Fig. 3B-E). The three peaks corresponded to >150, 300, and 1,500 kDa similar to the molecular weight (MW) of a monomer, dimer, trimer, and polymer. When IgA and IgM Fc domains were fused to PAP, peaks were observed at 5 min, predicting the MW of more than 1,500 kDa (Fig. 3B-E). On HPLC with PAP-IgA Fc and PAP-IgA FcK, a peak was observed between 5.7 and 7 min. The polymer (P) on HPLC corresponded to a MW greater than 670 kDa. Both PAP-IgA Fc and PAP-IgA FcK formed dimers, while PAP-IgM Fc and PAP-IgM FcK formed polymers. The smaller complexes of PAP-IgA Fc and PAP-IgA FcK appeared as broad peaks in the calculated MW ranging at 250-670 kDa between 5.7 and 7 min.

Bio-electron microscopy (EM) to visualize plant-derived PAP-Fc fusion proteins

The EM confirmed structures of PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK (Fig. 3F-I). The recombinant proteins showed the complex forms with monomer assembly. The complexes were identified either as a dimer, in which two monomers were assembled, or a polymer, in which three or more monomers were assembled. This different shape may be due to the recombinant protein orientation bound to the chip.

Immune response in mice injected with PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK

After confirming the molecular size and shape of the PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK fusion proteins, their immune responses were tested in BALB/c mice. The mice were injected three times in total, and blood samples were collected for serum IgG response analysis after each vaccination. As shown in Fig. 4A, immune response was not observed in the first bleeding samples in any of the experimental groups. However, in the second bleeding samples, IgG was observed in all the groups except for the mice injected with 1 × PBS. ELISA was performed on the immune serum, and the highest response observed was in the PAP-IgA FcK protein (Fig. 4B). The IgG_1 subtype induction in the IgG response indicated that the immune responses were related to Th_2 responses. As shown in Fig. 4C, the IgG_1 absorbance values were

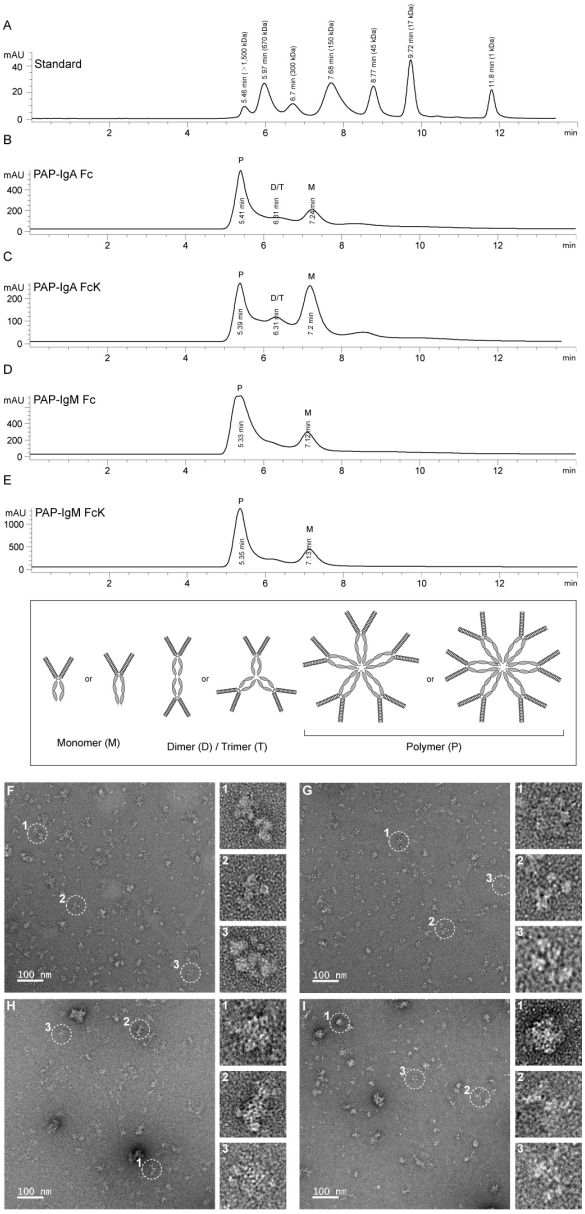

Fig. 3. SEC-HPLC and Bio-EM analyses of PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK proteins. In SEC-HPLC chromatogram, the indicated retention times were applied to determine the molecular weights of proteins, according to the AdvanceBio SEC 300 Å Protein (A) Standard, (B) PAP-IgA Fc, (C) PAP-IgA FcK, (D) PAP-IgM Fc, and (E) PAP-IgM FcK. SEC-HPLC of Bio-EM was performed to analyze the protein structure of (F) PAP-IgA Fc, (G) PAP-IgA FcK, (H) PAP-IgM Fc, and (I) PAP-IgM FcK recombinant proteins. The white circles indicate the selected proteins for magnification. The PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK were dimers and polymers with two or more in a Y-shape.

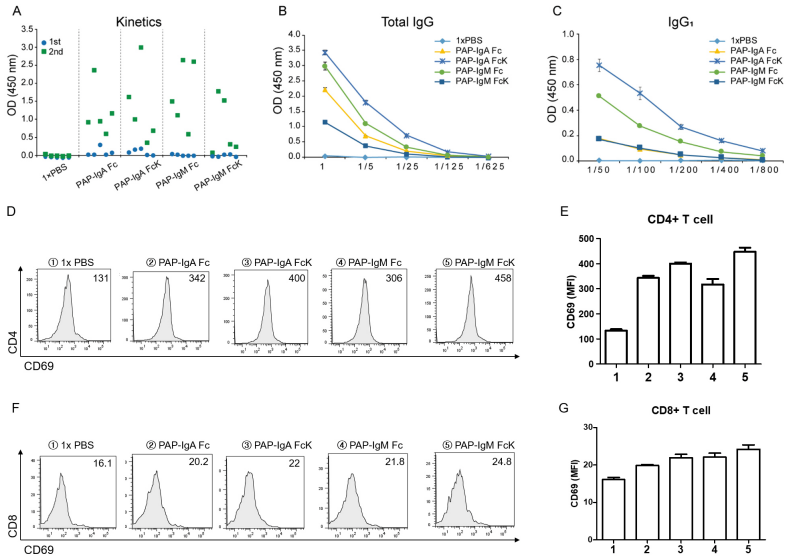

Fig. 4. Immune responses to PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK proteins in BALB/c mice. (A) Anti-PAP IgG kinetics in serum of immunized mice. The plot shows the means ± standard error (SE) for five mice per group. (B) Endpoint titer of anti-PAP IgG in pooled sera. (C) Endpoint titer of anti-PAP IgG_1 in pooled sera. Flow cytometry histograms were used to analyze expression of CD69 on both $CD4^+$ T cells (D, E) and $CD8^+$ T cells (F, G) in mice immunized with 1 × PBS, PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK. Mean fluorescence intensity (MFI) of CD69 in $CD4^+$ T cell (D, E) and $CD8^+$ T cell (F, G) were presented.

lower than the total IgG response. The IgG_1 response was the highest in the PAP-IgA FcK protein.

Immune response to PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK

The spleens were harvested to determine the $CD4^+$ and $CD8^+$ T cellular responses from the mice injected with PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK proteins (Fig. 4D-G). The $CD4^+$ and $CD8^+$ T cell number and activation state were measured in the splenocyte. The $CD4^+$ and $CD8^+$ T cells were assessed to determine the $CD4^+$ and $CD8^+$ T cell induction activity of PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK proteins. This was conducted using the gating strategy shown in Supplementary Fig. 3. In the mean fluorescence intensity (MFI) values of both $CD4^+$ and $CD8^+$ T cells, PAP-IgA FcK (400 MFI) and PAP-IgM FcK (458 MFI) proteins had higher values compared to PAP-IgA Fc (342 MFI) and PAP-IgM Fc (306 MFI) proteins, respectively (Fig. 4D-G). Notably, for $CD4^+$ and $CD8^+$ T cells, the IgM Fc type with KDEL had the highest MFI values among the others (Fig. 4D-G).

DISCUSSION

This study demonstrated that the plant-derived PAP-Fc fusion vaccine protein had a potential of preventive and therapeutic agents for prostate cancer. PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK were glycoproteins with PAP fused to the human immunoglobulin IgA Fc and IgM Fc domains. The ER signal peptide was fused to the N-terminus of PAP, and the ER retention signal motif was fused to the C-terminus of the IgA Fc and IgM Fc domains. The PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK genes were expressed under the control of the enhanced CaMV 35S promoter (28, 29). This study hypothesized that the expression and immunogenicity of PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK proteins would vary depending on the immunoglobulin (Ig) Fc domain type in transgenic plants. Thus, the goal of the current research was to determine the effect of the IgA and IgM Fc domain types on the expression of the recombinant protein as well as the efficacy of their immune responses. This would then determine their potential use in a recombinant Fc fusion vaccine expressed in plants.

Both PCR and RT-PCR analyses demonstrated that the PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK transgenes were inserted and expressed in transgenic plant DNA genomes. Western blot revealed the PAP-Fc fusion protein expression in plants. Expression of both PAP-IgA FcK and PAP-IgM FcK with ER retention signal peptide was higher compared to PAP-IgA Fc and PAP-IgM Fc. PAP- IgA Fc (3 mg/kg), PAP-IgM Fc (5.6 mg/kg), PAP-IgA FcK (3.5 mg/kg), and PAP-IgM FcK (6.2 mg/kg) proteins were obtained from fresh leaves of transgenic plants. These results suggest that the ER retention signal motif enhanced production level of the KDEL tagged proteins. This high expression of PAP-IgA FcK and PAP-IgM FcK can be explained by the localization of ER (22, 29-31).

Typically, dimeric or trimeric structures are formed for IgA,

and pentameric or hexameric structures are formed for IgM (32, 33). In addition, the Fc domain of Ig activates the effector function by binding to FcRs or mediating other immune responses such as the complement system (32, 34). The Fc fusion protein can significantly induce the immune response for cancer. Therefore, in this study, IgA and IgM Fc domains were fused to PAP to generate vaccine proteins with polymeric structures for prostate cancers in transgenic plants. In the SEC-HPLC analysis, polymer and monomer-sized peaks were identified in all of the plant-derived proteins, and dimer or trimer-sized peaks were identified in the PAP-IgA Fc and PAP-IgA FcK proteins. Likewise, Bio-TEM analysis identified dimer and polymer shapes of proteins. These results suggest that proteins fused with IgA Fc and IgM Fc domains can form their polymeric shapes in plant cells.

In the mice experiment, plant-based recombinant PAP-Fc fusion proteins induced PAP-specific antibody responses in mice. The PAP-IgA FcK recombinant protein showed a much higher response in terms of total IgG and IgG_1 compared to the other recombinant proteins, suggesting a Th2 bias. Notably, the IgA protein form showed a higher IgG response in the PAP-IgA FcK protein, which had the ER retention signal peptide KDEL.

Recent studies have shown that T-cell responses are a crucial component of an effective therapeutic cancer vaccine (35, 36). The PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK proteins induced $CD4^+$ and $CD8^+$ T cell responses in mice. The activation of $CD4^+$ and $CD8^+$ was measured based on the MFI value of CD69. PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK vaccine proteins induced higher activity of $CD4^+$ T cells. $CD4^+$ T cells promote anti-cancer immunity through various mechanisms such as T cell activation, T cell homing, antigen presenting enhancement, effector function, and co-stimulation (37). These results were attributed to have immunogenic effects from dimeric and pentameric formation in PAP-IgA Fc, PAP-IgM Fc, PAP-IgA FcK, and PAP-IgM FcK. The PAP-IgA FcK and PAP-IgM FcK proteins were more efficient at inducing $CD4^+$ T cell response compared to the PAP-IgA Fc and PAP-IgM Fc proteins. It is speculated that vaccines may be more effective with high mannose glycan structure modification caused by the ER retention signal peptide KDEL (29-31).

Unlike other microorganisms which do not have subcellular organisms such as endoplasmic reticulum and golgi complex, plants express and assemble PAP polymeric IgA and IgM Fc fusion antigenic proteins increasing immunogenicity with and without adjuvant (38). In addition, plant expression system has several advantages for recombinant protein production in cost, safety, and scalability compared to microorganism system (39).

In this study, the immunotherapeutic vaccine candidate proteins with IgA and IgM Fc fusions for prostate cancer were produced in transgenic plants using a stable expression system, and those PAP-Fc fusion proteins exhibited polymeric shapes to induce immune response for prostate cancer.

MATERIALS AND METHODS

See supplementary information.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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