



Expression and in vitro function of anti-CTLA-4 human antibody expressed in plants

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Abstract

Biosimilars produced in plant systems encompass a range of molecules, including antibodies, vaccines, enzymes, receptors, and other therapeutic molecules. In the present study, we aimed to express the immune checkpoint-inhibitor anti-CTLA-4 antibody, Ipilimumab, in *Nicotiana tabacum* plant. We generated transgenic tobacco plants using *Agrobacterium*-mediated transformation and purified an anti-CTLA-4 monoclonal antibody (mAb^P CTLA-4) using protein A affinity chromatography. This purified mAb^P CTLA-4 showed specific binding activity to CTLA-4-positive human tonsil tissues, as confirmed by immunoblot, indirect ELISA, and immunohistochemistry analyses. Our study confirmed that mAb^P CTLA-4 effectively targeted CTLA-4 in human tonsil tissues. These findings suggest that our plant-derived anti-CTLA-4 antibody retains its original functionality, underscoring the promising potential of plant-based therapeutic expression systems for producing high-value medicinal biosimilars.

Keywords Anti-CTLA-4 antibodies · Biosimilars · Immune system · Ipilimumab · Plant expression system · Therapeutic protein

Introduction

Immunotherapy has recently gained attention as a practical and effective alternative for treating many types of cancers. In particular, monoclonal antibody-based immune checkpoint-blockade (ICB) therapies have shown highly promising results in clinical studies (Hargadon et al. 2018). Blocking immune checkpoint receptors, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death receptor 1 (PD-1), programmed death-ligand 1 (PD-L1), killer immunoglobulin-like receptors, lymphocyte-activation gene 3 (LAG-3), and T cell immunoglobulin and mucin domain-containing 3 (TIM-3), can help overcome immune escape and promote an anti-tumor immune response (Marin-Acevedo et al. 2018). Further, in recent years, monoclonal antibody-based immune checkpoint inhibitors, such as anti-CTLA-4, anti-PD-1, and anti-PD-L1 antibodies, have emerged as primary treatment modalities in cancer immunotherapy. Therefore, immune-checkpoint inhibition using monoclonal antibodies can serve as a highly effective approach for the treatment and diagnosis of various forms of cancer (Gravbrot et al. 2019).

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Originally known as an antigen expressed in T-lymphocytes, CTLA-4 was recently found to be expressed in hematopoietic cells and their derivatives (monocytes, macrophages, dendritic cells, plasma cells, mast cells, and neutrophils), stromal cells of mesodermal (mesenchymal) origin, and reticular epithelial cells of ectodermal origin. This indicates that CTLA-4 is not limited to the lymphoid cell lineage but can exert broader effects on immune regulation (Tiemann et al. 2021).

Research aimed at producing therapeutic proteins, such as antibodies and vaccine candidates, has recently attracted significant attention. Indeed, ongoing efforts to produce recombinant therapeutic proteins are using various eukaryotic systems, including not only mammalian cell systems, but plant (Jin et al. 2023a, b; Kang et al. 2023; Lee et al. 2023a, b; Park et al. 2024; Yang et al. 2024), insect (Lee et al. 2023a, b, 2024), and yeast systems (Schütz et al. 2023) as well. Owing to the similarities in post-translational modifications, eukaryotic heterologous expression systems have proven to be suitable for producing high-molecular-weight glycoproteins, such as antibodies. Notably, plant systems can now reportedly produce biosimilars, such as anti-breast and prostate cancer therapeutic antibodies (Jin et al. 2023a, b; Kang et al. 2023; Lee et al. 2023a, b; Park et al. 2024; Yang et al. 2024).

In the present study, we aimed to express the anti-human CTLA-4 antibody ipilimumab, in *N. tabacum* plant and confirm its binding activity in human tonsil tissues to investigate the potential of this experimental plant system as a platform for the production of biosimilars.

Materials and methods

Plant-based protein expression vector

The plant protein-expression vector was constructed according to previously described methods (Lee et al. 2023a, b). The amino-acid sequences encoding the heavy chain (HC) and light chain (LC) of ipilimumab (anti-human CTLA-4 antibody) were obtained from the DrugBank database (<https://go.drugbank.com/drugs/DB06186>). The HC and LC amino-acid sequences were then converted to a plant-specific codon-optimized cDNA sequence (<https://www.idtdna.com>).

Plant transformation

Plant transformation was conducted according to previously described methods (Lim et al. 2022). Transgenic plants were selected based on their growth on Murashige and Skoog medium containing B5 vitamins (Duchefa Biochemie, Haarlem, Netherlands), kanamycin (100 µg/mL),

cefotaxime (250 µg/mL) in Magenta GA-7 vessels (Sigma, St. Louis, MO).

Plant genomic DNA isolation and PCR analysis

For PCR analysis, 200 mg of leaf samples were first harvested from both the transgenic and non-transgenic plants. Leaf genomic DNA was isolated using a DNA-extraction kit (RBC, Taipei, Taiwan), following the manufacturer's recommendations. To confirm the insertion of the HC- and LC-encoding genes, PCR analysis of genomic DNA was performed using the following primer pairs: HC, forward primer 5'-ATG GCT ACT CAA CGA AGG GCA A-3' and reverse primer 5'-CAT AAT TCG TCC TTT TTC CCA GG-3'; LC, forward primer 5'-ATG GGC ATC AAG ATG GAA TCA CAG-3' and reverse primer 5'-TTA ACA TTC CCC TCG ATT AAA AGA T-3'; Elongation factor 1- α (EF-1 α), forward primer 5'-GTG GAA TTG GTA CTG TCC CTG-3' and reverse primer 5'-CCA ACA TTG TCA CCA GGA AGT G-3'. The PCR analysis was performed with 30 cycles at 94 °C for 20 s of denaturation, 62 °C for 10 s of annealing, and 72 °C for 45 s of extension, using the Maxime PCR PreMix i-Taq kit (Intron Biotechnology, Daejeon, Korea).

Immunoblot analysis

The preparation of immunoblot samples to confirm the expression of plant-derived anti-CTLA-4 was conducted in accordance with the protocol described in a study by Lee et al. (2023a, b). The samples were loaded onto 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. The separated total soluble proteins were transferred to a nitrocellulose (NC) membrane, which was incubated with rabbit anti-human IgG Fc γ and rabbit anti-human IgG F(ab')₂ fragment-specific antibodies conjugated to horseradish peroxidase (Jackson Immunolab, West Grove, PA) to recognize the HC and LC of mAb^P CTLA-4, respectively. For band detection, a chemiluminescent substrate (Bio-Rad, Hercules, CA) was used, and the NC membranes were exposed to X-rays (Agfa, Mortsel, Belgium). Non-transgenic plants were used as a negative controls and mammalian cell-derived anti-CTLA-4 antibodies (mAb^M CTLA-4) (Invivogen, Toulouse, France) were used as a positive control.

Purification of mAb^P CTLA-4

The mAb^P CTLA-4 protein was purified from transgenic *N. tabacum* leaves according to previously described methods (Lee et al. 2023a, b). An equal volume of 1 × binding buffer (20 mM of sodium phosphate, pH 7.0) was mixed with the reserved plant leaf extract. The mixture was centrifuged at 15,000 × *g* at 4 °C for 30 min, after which

the supernatant was filtered using a 0.45- μ m Millex filter (Merck Millipore, Burlington, MA). The filtered supernatants were then placed in a HiTrap Protein A HP column (Cytiva, Uppsala, Sweden) and eluted with elution buffer (0.1 M glycine-HCl, pH 2.7). The purified protein samples eluted from the separation column were resolved by 10% SDS-PAGE and stained with Coomassie brilliant blue R250 (Thermo Fisher Scientific, Waltham, MA). The ipilimumab biosimilar (Invivogen, Toulouse, France) was used as a positive control.

PCR and western blotting analyses of human tonsil tissues

Tonsil tissues were obtained from Chung-Ang University Hospital, Seoul, Korea. Total RNA was isolated using a FavorPrep™ Blood/Cultured Cell Total RNA mini kit (Favorgen, Pingtung, Taiwan), according to the manufacturer's recommendations. Isolated total RNA was applied to synthesize cDNA using an AMPIGENE® cDNA Synthesis Kit (Enzo Life Science, Farmingdale, NY). To confirm the transcription levels of CTLA-4 in the human tonsil tissues, the PCR for CTLA-4 was performed as follows: 30 cycles involving 20 s of denaturation at 95 °C, 20 s of annealing at 57 °C, and 30 s of extension at 72 °C. PCR for 18S rRNA was performed as follows: 25 cycles involving 20 s of denaturation at 95 °C, 20 s of annealing at 60 °C, and 30 s of extension at 72 °C using AccuPower® PCR PreMix (Bioneer, Daejeon, Korea).

Western blotting was performed to identify the expression levels of CTLA-4 in human tonsil tissues using previously described methods (Chung et al. 2018). Briefly, the tissue samples were first treated with cell lysis buffer (10 mM of Tris, 1% SDS, 1 mM of Na₃CO₄), followed by incubation for 1 h on ice. Subsequently, 30 μ g of the cell lysate was separated using 12% SDS-PAGE; the protein bands were electro-transferred onto an NC membrane. Then, the blots were incubated overnight with primary antibodies (1:2,000) at 4 °C. The primary antibodies used for immunoblotting were anti-GAPDH antibodies (Bethyl Laboratories, Montgomery, TX) and anti-CTLA-4 antibodies (Santa Cruz Biotechnology, Dallas, TX). The membranes were then incubated with secondary antibodies (1:5,000) labelled with horseradish peroxidase. Signals were detected using the enhanced chemiluminescence immunoblot kit from the ATTO Corporation (Motoasakusa, Tokyo, Japan) and analyzed using the ImageQuant LAS 4000 mini-machine (Cytiva, Uppsala, Sweden). All experiments were reviewed and approved by the Institutional Review Board of the Chung-Ang University College of Medicine (approval number: 2090-006-434).

Enzyme-linked immunosorbent assay (ELISA)

The human CTLA-4 recombinant protein (50 ng/well) (Abcam, Cambridge, UK) diluted in a coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) was coated onto Nunc 96-well immunoplates (Thermo Fisher Scientific, Waltham, MA) for 16 h at 4 °C. Then, the plates were blocked with 5% skim milk in 1 $\times$ PBS-T for 2 h at 37 °C. The primary antibodies [mAb^P CTLA-4; mAb^M CTLA-4 (Ipilimumab, Invivogen); and human IgG isotype antibody (as negative control, Enzo life science)] (100 μ L/well) were diluted to obtain concentrations ranging from 0.1 to 10 ng/mL in 5% skim milk in 1 $\times$ PBS-T and added to each well. After incubation at 37 °C for 2 h, the plate was treated with peroxidase-conjugated rabbit anti-human IgG H + L (Jackson Immunolab, West Grove, PA; 1:5,000 dilution) for 2 h at 37 °C. After all the reactions were completed, a soluble TMB peroxidase substrate (SeraCare, Milford, MA) was added to the plate (100 μ L/well), and the reaction was terminated using the TMB stop solution (SeraCare). Finally, the absorbance of the samples at 450 nm was measured using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT).

Florescent immunohistochemistry (IHC) analysis of human tonsil tissues

Expression of CTLA-4 in human tonsil tissue samples was examined as described previously (Lee et al. 2016). Briefly, the slides with normal tonsil tissue sections were dewaxed in xylene for at least 20 min and then sequentially rehydrated in 100%, 95%, 90%, and 80% ethanol solutions. After a 5-min rinse in water, the slides were pretreated with 1 $\times$ Tris-EDTA buffer (pH 9.0) and heated for 15 min at 100 °C and then cooled for another 15 min for antigen retrieval. After the slides were rinsed, endogenous peroxidase activity was blocked by treatment with 3% H₂O₂ for 30 min. Then, the slides were treated with the following primary antibodies: murine anti-human CTLA-4 (1:300, Santa Cruz Biotechnology) and mAb^P CTLA-4 (1:300) antibodies. The sections were incubated for 1 h in a moist chamber at room temperature. After the slides were rinsed with PBS, they were incubated with secondary antibodies (FITC-conjugated goat anti-rabbit IgG H + L and FITC-conjugated rabbit anti-human IgG H + L) for 30 min at room temperature and then rinsed in PBS. Sections for the negative control were incubated with the same reagents; these sections were subjected to the same epitope-retrieval protocol without the primary antibodies. All experiments were reviewed and approved by the Institutional Review Board of the Chung-Ang University College of Medicine (approval number: 2090-006-434).

Statistical analysis

All experiments were conducted in triplicate, and all data are expressed as means $\pm$ standard deviation (SDs). Statistical significance was assessed using two-tailed Student's T-tests for comparisons between two groups. P-values of < 0.05 were considered statistically significant.

Results

Generation of transgenic plants expressing mAb CTLA-4

A plant binary vector harboring the mAb CTLA-4 HC and LC gene-expression cassettes was used to transfer the mAb CTLA-4-expression cassette into plant genomic DNA through *Agrobacterium*-mediated plant transformation (Fig. 1). Plant genomic DNA was isolated from randomly selected transgenic plant leaves. The sizes of the PCR products for the HC and LC were approximately 1400 and 700 bp, respectively. EF-1 α was used as an internal control gene of *N. tabacum*, and the size of the PCR product was approximately 157 bp. The mAb CTLA-4 gene inserted into pBINPLUS vectors was used as a positive control, while non-transgenic plant genomic DNA isolated from leaves was used as a negative control (Fig. 2A). The sizes of the HC and LC proteins were approximately 53 and 26 kDa, respectively

(Fig. 2B). The results showed that *Agrobacterium*-mediated transformation successfully generated transgenic plants expressing the mAb CTLA-4 protein.

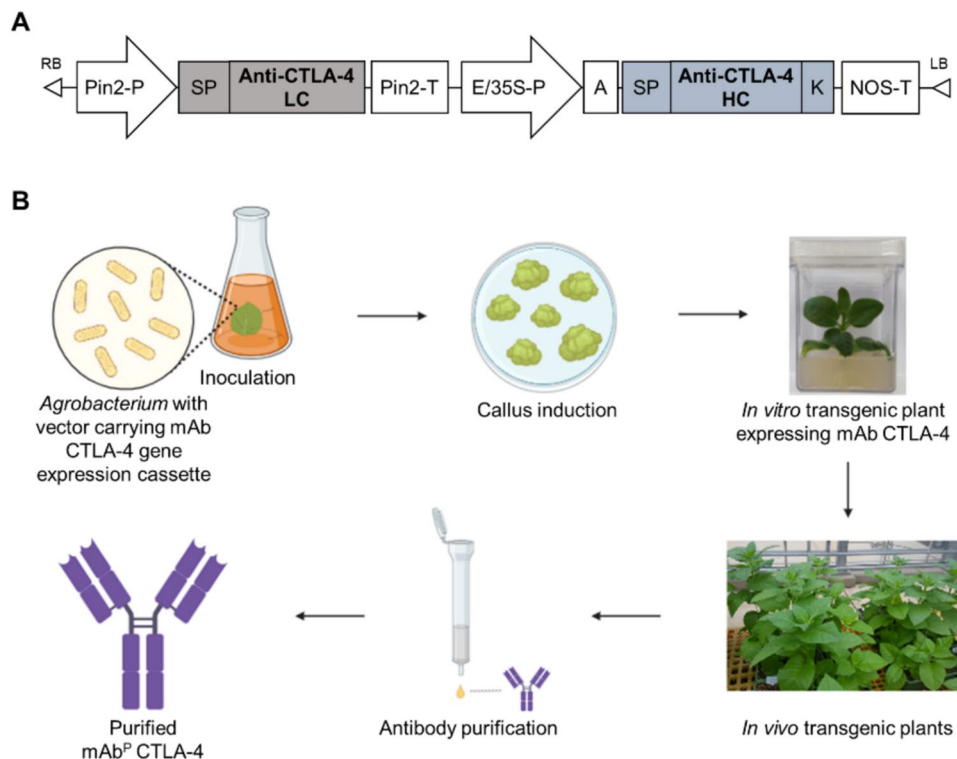
Purification of mAb^P CTLA-4

To purify mAb^P CTLA-4, plants expressing mAb^P CTLA-4 were cultivated in potted soil, and the leaves were harvested (Fig. 2C). The mAb^P CTLA-4 protein was then purified from the harvested leaves using protein A affinity chromatography. Purification of the mAbs were confirmed by SDS-PAGE under non-reducing and reducing conditions (Fig. 2C, Fig. S1). The sizes of the HC and LC proteins of mAb^P CTLA-4 in reducing condition were approximately 53 and 26 kDa, respectively (Fig. 2C). The sizes of mAb^M and purified mAb^P in non-reducing conditions were approximately 158 kDa (Fig. S1).

Recognition of CTLA-4 in human tonsil tissues by mAb^P CTLA-4

Human tonsil tissues were collected from two patients with sleep apnea, in compliance with IRB regulations. The expression of CTLA-4 mRNA in the collected tonsils was confirmed (Fig. 3A) by RT-PCR analysis, which enabled the CTLA-4 PCR band to be observed in both tonsil tissue samples (Fig. 3B). Further, using western blotting, the CTLA-4 protein band (~45 kDa) was found to be

Fig. 1 Schematic diagram of the plant mAb CTLA-4 expression cassette and the plant transformation process. **A** Expression cassette design of plant binary vector to express the heavy (HC) and light chains (LC) of mAb CTLA-4. *RB* right border, *Pin2-P* promoter of *Pin2* gene from potato, *SP* signal peptide, *Pin2-T* terminator of *Pin2* gene from potato, *E/35S-P* the cauliflower mosaic virus 35S promoter with duplicated enhancer region; *A*, untranslated leader sequence of alfalfa mosaic virus; *K*, KDEL (endoplasmic reticulum retention motif); *NOS-T*, terminator of nopaline synthase gene; *LB*, Left border. **B** Overview of *Agrobacterium*-mediated plant transformation and purification for mAb^P CTLA-4 antibody. This figure was created with BioRender.com



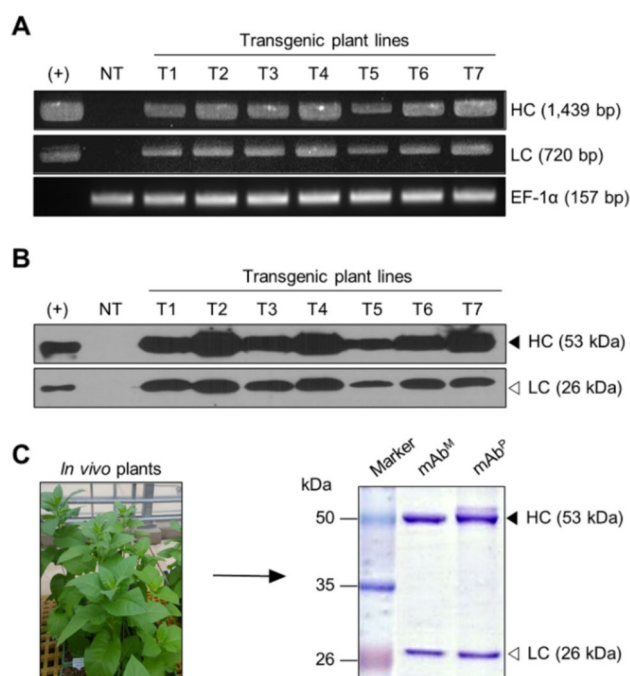


Fig. 2 Confirmation of gene insertion and expression of protein mAb^P CTLA-4, and purification of mAb^P CTLA-4 from transgenic plant leaves. **A** PCR analysis of transgenic tobacco plant lines used to confirm transgene insertion. HC is 1,439 bp; LC is 720 bp. Elongation factor1-alpha (EF-1α) is used as internal positive control. EF-1α is 157 bp. (+) positive control, *pBI mAb CTLA-4* vector, *NT* genomic DNA isolated from a non-transgenic tobacco plant, *T1-7* transgenic plant lines, *HC* heavy chain, *LC* light chain. **B** Western blot analysis used to confirm mAb^P CTLA-4 protein expression levels. HC is 53 kDa; LC is 26 kDa. (+) mAb^M CTLA-4, *NT* non-transgenic tobacco plant, *T* transgenic plant lines, *HC* heavy chain, *LC* light chain. **C** Purification of mAb^P CTLA-4 antibody. Plants expressing anti-CTLA-4 were cultivated in potted soil. Leaves were harvested for the extraction and purification of mAb CTLA-4. SDS-PAGE was conducted to confirm the purification of mAb CTLA-4 from the leaves of the transgenic lines. For SDS-PAGE, mAb^M CTLA-4 as a positive control (1 μg) and purified mAb^P CTLA-4. *Marker* protein marker, *mAb^M* mammalian-derived anti-CTLA-4 antibody, *mAb^P* plant-derived anti-CTLA-4 antibody, *HC* heavy chain, *LC* light chain

recognized by both mAb^M CTLA-4 and mAb^P CTLA-4 (Fig. 3C). Additionally, confocal analysis was conducted to confirm whether mAb^P CTLA-4 could recognize CTLA-4 in the tonsil tissues (Fig. 4). Green dots were observed in the tonsil tissue samples treated with both mAb^M CTLA-4 and mAb^P CTLA-4 (Fig. 4). The results showed that the green dot signals in mAb^P CTLA-4 were slightly stronger than those in mAb^M CTLA-4. Conversely, no green dots were detected in the NC group (Fig. 4). Meanwhile, ELISA was performed using the recombinant human CTLA-4 protein, and comparative analysis showed that the absorbance of mAb^M CTLA-4 at 450 nm was slightly higher than that of mAb^P CTLA-4 (Fig. 5).

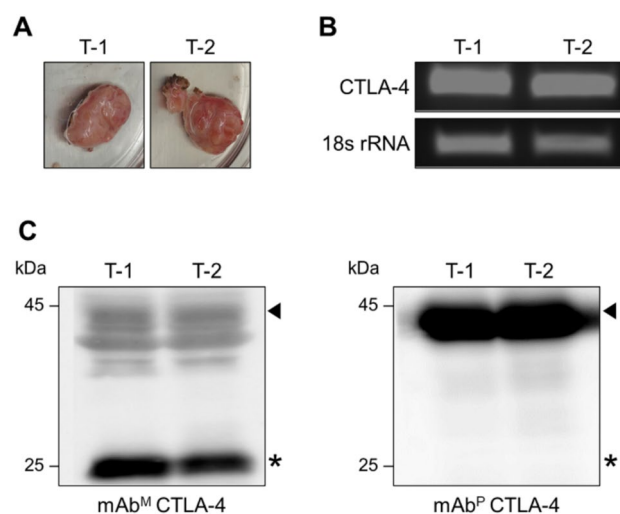


Fig. 3 Confirmation of the binding activity of mAb^P CTLA-4 to CTLA-4 in human tonsil tissues. **A** Human tonsil tissues used in this study were obtained in compliance with the appropriate IRB procedures. **B** PCR was performed with gene-specific primers using 40 ng of cDNA, loaded onto 2% agarose gels. **C** A total of 10 μg of tonsil proteins was loaded onto 12% acrylamide gels to assess the expression levels of CTLA-4. For detection purposes, mAb^M CTLA-4 (left) and mAb^P CTLA-4 (right) were administered separately. The symbol (◀) represents human CTLA-4, and the symbol (*) indicates nonspecific binding

Discussion

The diversity of plant-derived biologics is rapidly gaining increasing recognition in various fields, such as human and animal health, industry, and agriculture (Zahmanova et al. 2023). In the present study, we expressed the immune checkpoint-inhibitor anti-CTLA-4 antibody ipilimumab in tobacco plant (*N. tabacum*). For this purpose, we generated transgenic tobacco plants using *Agrobacterium*-mediated plant transformation and purified the mAb^P CTLA-4 using protein A affinity chromatography. An approximate yield of 0.804 mg/kg was obtained based on the weight of the harvested leaves (data not shown). The purified mAb^P CTLA-4 showed specific binding activity to CTLA-4-positive human tonsil tissues, as per the immunoblotting and IHC assays. Indirect ELISA using recombinant human CTLA-4 indicated that mAb^P CTLA-4 showed binding activity to recombinant human CTLA-4. Meanwhile, the results of confocal analysis suggested that mAb^P CTLA-4 effectively recognized CTLA-4 expressed in the tonsil tissues in a different manner from that of mAb^M CTLA-4.

In a previous study, plant-derived anti-CTLA-4 2C8 antibodies were produced in a transient expression system using *N. benthamiana* (Bulaon et al. 2023). This study showed that the plant-produced anti-CTLA-4 2C8 mAb elicited strong antitumor responses and established similar degree of tumor growth inhibition with the commercially

Fig. 4 Confocal analysis used to confirm the binding activity of mAb^M CTLA-4 and mAb^P CTLA-4 to CTLA-4 in human tonsil tissues. Nuclei were stained with DAPI (blue, 405 nm). Both mAb^M CTLA-4 and mAb^P CTLA-4 were stained with FITC (green, 525 nm). NC denotes a negative control, which was treated only with the secondary antibody. Scale bars = 100 μ m

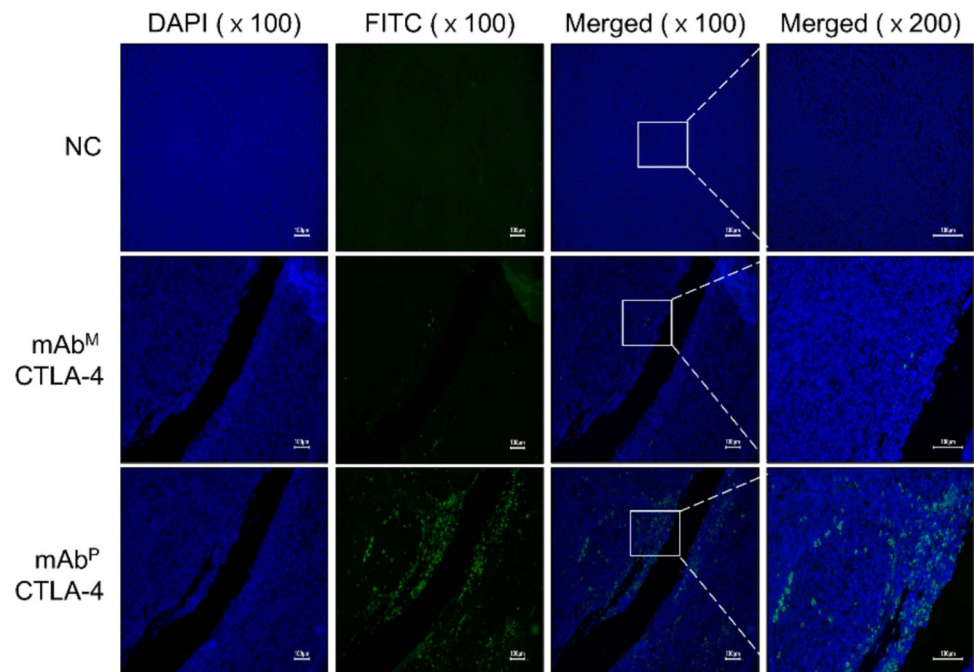
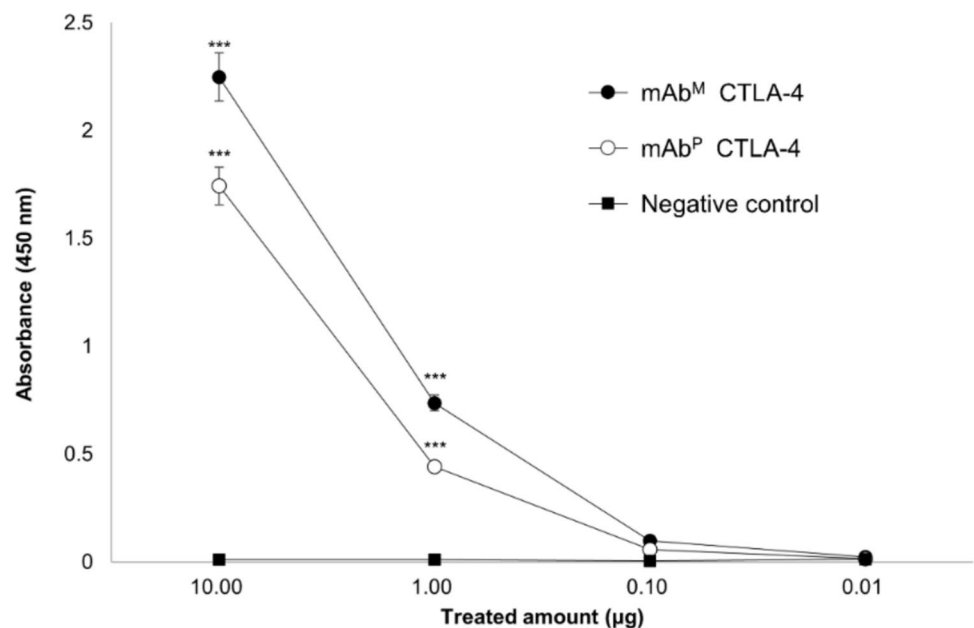


Fig. 5 Indirect ELISA analysis used to confirm the binding activity of purified mAb^P CTLA-4 to the recombinant CTLA-4 protein. An indirect ELISA was used to compare the binding activity of mAb^M CTLA-4 and purified mAb^P CTLA-4 to the recombinant human CTLA-4 protein. Human IgG isotype (hIgG) was used as a negative control. Error bars represent means $\pm$ SEMs (***) P < 0.001, two-tailed Student's T-tests)



available anti-CTLA-4 drug. However, our study differs from the previous study in that we established transgenic plants for antibody production. In a previous study using a transient expression system, the expression level was 39.65 ± 8.42 μ g/g fresh weight. In this study, the transgenic plant expression system showed an expression level of 0.804 μ g/g fresh leaf, which is lower compared to the transient expression system. However, the transgenic plant system offers several advantages, such as stable expression across generations and no requirement for the

time-consuming *Agrobacterium* solution infiltration process. Thus, we propose in this study that transgenic plants can be applied to the production of valuable therapeutic recombinant proteins.

In our previous studies, we investigated recombinant protein expression and glycosylation depending on plant age and leaf position (Lim et al. 2015). Protein expression was not significantly affected by age or leaf position, whereas glycosylation patterns differed before and after flowering. Therefore, it is important to properly choose

the leaf harvesting time for the purification of recombinant proteins.

Plant-based systems for expressing therapeutic proteins are associated with various advantages, such as their inherent safety profile and reduced unwanted immunogenicity, which can improve patient tolerance and minimize the incidence of adverse effects (Jin et al. 2023a, b; Kang et al. 2023; Lee et al. 2023a, b; Park et al. 2024; Rup et al. 2017; Yang et al. 2024). Notably, in clinical trials involving healthy human volunteers, no overt adverse effects related to plant-specific *N*-glycan residues were observed and no anti-drug antibodies were detected (Aviezer et al. 2009). Therefore, we verified that plant-derived anti-human CTLA-4 biosimilars successfully recognized CTLA-4 in human tonsil tissues. These results demonstrate that plant-based systems for therapeutic protein expression represent a promising technology for producing anti-CTLA-4 antibodies.

When producing therapeutic immune proteins using plants, the most frequently reported limitations include low productivity, high downstream processing costs, and a slow transition to practical applications (Schillberg and Finfern 2021). Another controversial issue is that plant-specific glycosylation can alter the biological activity of the proteins (Grabowski et al. 2014). However, in the present study, we confirmed that the binding activity of mAb^P CTLA-4 relies on the recombinant human CTLA-4 protein as well as human tonsillar tissue. Nonetheless, we observed that the binding activity to either the recombinant protein or the protein present in the tonsil tissues varied between antibodies. This suggests that the binding activities of plant-derived antibodies or mammalian cell-derived antibodies can differ for two proteins with different forms or structures.

One of the primary tissues with CTLA-4 expression is the tonsil (Tiemann et al. 2021). The data presented in this study show that the binding affinity of the mAb^P CTLA-4 is higher than that of the mAb^M CTLA-4 for CTLA-4 expressed in human tissue, indicating the potential biological functionality of the mAb^P CTLA-4.

In conclusion, in the present study, the biologically active mAb^P CTLA-4 protein was successfully expressed in tobacco leaves, suggesting that the plant-based protein expression system developed herein is a promising platform to produce recombinant proteins for cancer immunotherapy.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11816-025-00976-y>.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent to publish Not applicable.

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