Molecular Farming

Subjects: Medicine, Research & Experimental

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Molecular farming is the production of recombinant proteins in plants and has gained immense interest in the biotechnology sector since it offers a novel platform that is straightforward, rapid and scalable. In addition, plants have the ability to be propagated indefinitely, providing low-cost biomass production that can be used for the large-scale manufacturing of mAbs.

immune checkpoint inhibitors pembrolizumab nivolumab molecular farming

1. Vector Construction

The design and subsequent construction of expression vectors for agrobacterium-mediated transformation is imperative to ensure the optimum transient expression of the genes encoding the HC and LC of pembrolizumab or nivolumab [1][2]. The gene fragments of the HC and LC first undergo codon optimization using the in silico GeneArt® software supplied by Invitrogen (ThermoFisher Scientific, Waltham, MA, USA) and are subsequently synthesized and amplified by means of polymerase chain reaction (PCR) [1][2][3]. Thereafter, the HC and LC genes of pembrolizumab and nivolumab are digested with XbaI and SacI, and ligated into a pBYR2e geminiviral vector harboring a T-DNA region [1][2][3]. The vectors are then transformed into Agrobacterium tumefaciens strain GV3101 via electroporation, which involves the use of high-voltage electric shocks to create pores within the bacterial cell membrane through which the expression vectors can pass [1][3][4]. Subsequently, the cells are grown overnight, followed by centrifugation and finally resuspension in the agroinfiltration buffer containing 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) and 10 mM MgSO4 at a pH 5.5 [1][2][3].

2. Agroinfiltration and Plant Growth

To successfully agroinfiltrate expression vectors into Nicotiana benthamiana leaf cells, it is crucial to first complete an optical density (OD) measurement to ensure that the correct number of Agrobacterium tumefaciens cells are present within the agroinfiltration buffer [5][6]. The most frequent way to perform an OD measurement is by determining the absorbance of the cell suspension at a wavelength of 600 nm using a spectrophotometer, which allows for the rapid and precise quantification of cell number [6][7]. Furthermore, a final OD_{600} of 0.2, which equates to 1.6×10^8 cells/mL, is required for the successful agroinfiltration and delivery of genes encoding the HC and LC of pembrolizumab or nivolumab into plant cells [1][2][3][8]. The agrobacterium cell suspension is then infiltrated into the leaves of 6–8-week-old wild-type Nicotiana benthamiana plants [1][3][9]. Finally, the plants are either grown in greenhouses or indoor growing facilities, which are under strictly controlled environmental conditions to ensure that the correct temperature, humidity, light exposure and sterility are maintained for optimal protein yields [1][10].

Additionally, the ideal environmental conditions for the mass production of pembrolizumab and nivolumab in plants is at a temperature of 28 °C, a 70% humidity, and a 16 h light/8 h dark cycle at a light intensity between 80–100 µmol m^{-2} s^{-1} [1][10][11][12][13]. The plants are grown under these conditions for a period of 4 days following agroinfiltration and thereafter undergo the extraction and purification process to obtain purified pembrolizumab and nivolumab [1][2][3].

3. Purification

The purification of pembrolizumab and nivolumab from plants is initiated by first removing the agroinfiltrated leaves from the plant, followed by the homogenization of the leaves with 1X phosphate buffer solution (PBS) in an electronic blender [1][2][14]. Blending disrupts the plant cell wall by shear force, leading to the release of the intracellular contents, resulting in the formation of homogenate [14]. Subsequently, the homogenate is centrifuged at approximately 26,000× *g* for 40 min at 4 °C to remove cell debris and is further filtered through a membrane filter with a pore size of 0.45 µm [1][2]. The supernatant is then purified using protein A affinity chromatography, which is a highly efficient purification technique used to capture and purify IgG mAbs due to the high affinity of the protein A bead column for the Fc region [1][2][15][16]. Finally, the recombinant anti-PD-1 mAbs are removed from the column by washing with 1X PBS, and further eluted and neutralized with 0.1 M glycine and 1.5 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), respectively. Finally, the concentrations of pembrolizumab and nivolumab are determined using enzyme-linked immunosorbent assay (ELISA) and are quantified as µg/g of fresh leaf weight (FLW) [1][2][3]. Previous studies have shown that the transient expression of pembrolizumab and nivolumab in wild-type Nicotiana benthamiana leaves produce more than 340 µg/g and 140 µg/g FLW, respectively [1][2]. Overall, this is equivalent to a total of 340 mg/kg and 140 mg/kg FLW, which equates to approximately USD 18,000 and 4200 of pembrolizumab and nivolumab in 1 kg of leaves, respectively. Ultimately, this demonstrates that the utilization of molecular farming for the production of pembrolizumab and nivolumab is a viable platform that can be potentially implemented in LMICs to increase the accessibility of these ICIs. However, before the commercialization of plant-produced pembrolizumab and nivolumab can take place, it is crucial to complete both in vitro and in vivo testing to ensure that the same structure and activity are exhibited in those that are already commercially available [1][2].

4. Structural and Functional Assays

Following the purification and quantification of plant-produced pembrolizumab and nivolumab, multiple structural (physicochemical) and functional in vitro assays need to be conducted [1][2]. The physicochemical assays required to ensure that the structural characteristics of the purified anti-PD-1 mAbs are similar to the commercially available pembrolizumab and nivolumab include sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), western blot, circular dichroism (CD) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) [1][2]. Furthermore, SDS-PAGE and western blot are conducted to determine whether the plant-produced mAbs are correctly assembled and have a similar MW compared to the commercial pembrolizumab and nivolumab produced in CHO cells [1][2][3]. In addition,

the secondary and tertiary structures of the anti-PD-1 mAbs are determined using CD and NMR spectroscopy, respectively [1][2][17][18]. Finally, LC-ESI-MS is used to determine the N-glycosylation profile of the plant-produced pembrolizumab and nivolumab and to confirm whether this profile is similar to that commercially produced in CHO cells [1][2][19]. Altogether, Phakham et al. revealed that transiently expressing pembrolizumab and nivolumab in Nicotiana benthamiana leaves leads to anti-PD-1 mAbs that assemble into the correct tetrameric form, have similar secondary and tertiary structures, have slightly different N-glycosylation profiles, and have remarkably similar MWs. For instance, plant-produced pembrolizumab had a MW of 150 kDa, which is only 1 kDa different compared to commercial pembrolizumab (149 kDa) [1][2]. Moreover, the assays required to determine the functional characteristics of the anti-PD-1 mAbs include ELISA, surface plasmon resonance (SPR) and luciferase reporter assay, which are used to investigate the PD-1 binding affinity, kinetics and PD-1/PD-L1 inhibitory activity, respectively. Phakham et al. reported that there were no significant differences between the binding affinity and kinetics of the plant-derived mAbs to PD-1 when compared to the commercial mammalian-produced pembrolizumab and nivolumab [1][2]. Finally, the plant-produced pembrolizumab and nivolumab demonstrated the crucial ability to inhibit the binding of PD-1 to PD-L1 in a dose-dependent manner with a half-maximal effective concentration (EC50) of 147.2 ng/mL and 496 ng/mL, respectively, when compared to commercial pembrolizumab and nivolumab, which had EC_{50s} of 146.7 ng/mL and 544 ng/mL, respectively [1][2]. On the whole, Phakham et al. were the first to successfully demonstrate that the transient expression of pembrolizumab and nivolumab in Nicotiana benthamiana leaves is a rapid, simple and cost-effective production platform capable of producing mAbs that exhibit the correct assembly, molecular weight, structure, binding affinity, kinetics and PD-1/PD-L1 inhibitory activity [1][2]. However, significant research is still required to optimize the N-linked glycosylation of these plant-derived mAbs.

References

1. Phakham, T.; Bulaon, C.J.I.; Khorattanakulchai, N.; Shanmugaraj, B.; Buranapraditkun, S.; Boonkrai, C.; Sooksai, S.; Hirankarn, N.; Abe, Y.; Strasser, R.; et al. Functional Characterization of Pembrolizumab Produced in Nicotiana Benthamiana Using a Rapid Transient Expression System. Front. Plant Sci. 2021, 12, 1956.
2. Rattanapisit, K.; Phakham, T.; Buranapraditkun, S.; Siriwattananon, K.; Boonkrai, C.; Pisitkun, T.; Hirankarn, N.; Strasser, R.; Abe, Y.; Phoolcharoen, W. Structural and In Vitro Functional Analyses of Novel Plant-Produced Anti-Human PD1 Antibody. Sci. Rep. 2019, 9, 15205.
3. Diamos, A.G.; Hunter, J.G.L.; Pardhe, M.D.; Rosenthal, S.H.; Sun, H.; Foster, B.C.; DiPalma, M.P.; Chen, Q.; Mason, H.S. High Level Production of Monoclonal Antibodies Using an Optimized Plant Expression System. Front. Bioeng. Biotechnol. 2020, 7, 472.
4. Young, J.L.; Dean, D.A. Chapter Three—Electroporation-Mediated Gene Delivery. In Advances in Genetics; Huang, L., Liu, D., Wagner, E., Eds.; Academic Press: Cambridge, MA, USA, 2015; Volume 89, pp. 49–88. ISBN 0065-2660.

5. Prudhomme, N.; Pastora, R.; Muselius, B.; McLean, M.D.; Cossar, D.; Geddes-McAlister, J. Exposure of Agrobacterium Tumefaciens to Agroinfiltration Medium Demonstrates Cellular Remodelling and May Promote Enhanced Adaptability for Molecular Pharming. Can. J. Microbiol. 2020, 67, 85–97.
6. Beal, J.; Farny, N.G.; Haddock-Angelli, T.; Selvarajah, V.; Baldwin, G.S.; Buckley-Taylor, R.; Gershater, M.; Kiga, D.; Marken, J.; Sanchania, V.; et al. Robust Estimation of Bacterial Cell Count from Optical Density. Commun. Biol. 2020, 3, 512.
7. Myers, J.A.; Curtis, B.S.; Curtis, W.R. Improving Accuracy of Cell and Chromophore Concentration Measurements Using Optical Density. BMC Biophys. 2013, 6, 4.
8. Couto, R.A.S.; Chen, L.; Kuss, S.; Compton, R.G. Detection of Escherichia Coli Bacteria by Impact Electrochemistry. Analyst 2018, 143, 4840–4843.
9. Leuzinger, K.; Dent, M.; Hurtado, J.; Stahnke, J.; Lai, H.; Zhou, X.; Chen, Q. Efficient Agroinfiltration of Plants for High-Level Transient Expression of Recombinant Proteins. JoVE 2013, e50521.
10. Fujiuchi, N.; Matoba, N.; Matsuda, R. Environment Control to Improve Recombinant Protein Yields in Plants Based on Agrobacterium-Mediated Transient Gene Expression. Front. Bioeng. Biotechnol. 2016, 4, 23.
11. Davis, K.; Gkotsi, D.S.; Smith, D.R.M.; Goss, R.J.M.; Caputi, L.; O'Connor, S.E. Nicotiana Benthamiana as a Transient Expression Host to Produce Auxin Analogs. Front. Plant Sci. 2020, 11, 581675.
12. Goulet, M.-C.; Gaudreau, L.; Gagné, M.; Maltais, A.-M.; Laliberté, A.-C.; Éthier, G.; Bechtold, N.; Martel, M.; D'Aoust, M.-A.; Gosselin, A.; et al. Production of Biopharmaceuticals in Nicotiana benthamiana—Axillary Stem Growth as a Key Determinant of Total Protein Yield. Front. Plant Sci. 2019, 10, 735.
13. Mathur, J.; Griffiths, S.; Barton, K.; Schattat, M.H. Chapter Eight—Green-to-Red Photoconvertible MEosFP-Aided Live Imaging in Plants. In Methods in Enzymology; Conn, P.M., Ed.; Academic Press: Cambridge, MA, USA, 2012; Volume 504, pp. 163–181. ISBN 0076-6879.
14. Moussavou, G.; Ko, K.; Lee, J.-H.; Choo, Y.-K. Production of Monoclonal Antibodies in Plants for Cancer Immunotherapy. BioMed Res. Int. 2015, 2015, 306164.
15. Grom, M.; Kozorog, M.; Caserman, S.; Pohar, A.; Likozar, B. Protein a Affinity Chromatography of Chinese Hamster Ovary (CHO) Cell Culture Broths Containing Biopharmaceutical Monoclonal Antibody (MAb): Experiments and Mechanistic Transport, Binding and Equilibrium Modeling. J. Chromatogr. B 2018, 1083, 44–56.
16. Liu, H.F.; Ma, J.; Winter, C.; Bayer, R. Recovery and Purification Process Development for Monoclonal Antibody Production. mAbs 2010, 2, 480–499.

17. Rogers, D.M.; Jasim, S.B.; Dyer, N.T.; Auvray, F.; Réfrégiers, M.; Hirst, J.D. Electronic Circular Dichroism Spectroscopy of Proteins. Chem 2019, 5, 2751–2774.
18. Jang, A.; Cheon, D.; Hwang, E.; Kim, Y. Structural Stability of Cutibacterium Acnes Acyl Carrier Protein Studied Using CD and NMR Spectroscopy. J. Anal. Sci. Technol. 2022, 13, 1.
19. Castilho, A.; Gattinger, P.; Grass, J.; Jez, J.; Pabst, M.; Altmann, F.; Gorfer, M.; Strasser, R.; Steinkellner, H. N-Glycosylation Engineering of Plants for the Biosynthesis of Glycoproteins with Bisected and Branched Complex N-Glycans. Glycobiology 2011, 21, 813–823.