

轉基因植物和病毒載體表現靈芝的免疫調控蛋白LingZhi-8之構築

曹嘉豪、江主惠

E-mail: 345046@mail.dyu.edu.tw

摘要

靈芝的LZ-8蛋白具有免疫調控能力，為具高價值的蛋白。本研究為了利用植物系統來生產LZ-8蛋白，分別以轉基因植物及病毒載體方式，將LZ-8送入植物以表現此蛋白。在轉基因植物的表現系統中，共設計三種不同LZ-8基因之構築，(1) LZ-8：LZ-8基因來自原來靈芝的核醣核酸序列；(2) LZKD：LZ-8基因與前面相同但其3'端增加KDEL訊號勝之序列；(3) synLZKD：LZ-8基因之設計為將LZ-8密碼子優化(codon optimization)使其適合在番茄和矮南瓜中表現，並且在3'端加上KDEL之序列。這些LZ-8基因分別構築於Ti載體(pGA482G)後，再經由農桿菌轉殖於菸草(*Nicotiana benthamiana*)，並以100 mg L⁻¹ kanamycin及300 mg L⁻¹ carbenicillin進行轉基因植物之篩選。總共得到11個LZ-8擬轉殖株、8個LZKD擬轉殖株和16個synLZKD擬轉殖株。純化擬轉殖植物之總DNA，並以PCR偵測擬轉殖株中nptII及lz-8基因，初步得到5個LZ-8轉殖株、2個LZKD轉殖株和10個synLZKD轉殖株。另外，在利用矮南瓜黃化嵌紋病毒(*Zucchini yellow mosaic virus, ZYMV*)載體攜帶LZ-8基因之表現系統中，共完成兩種LZ-8基因之構築，(1) ZYLZ8：帶有原本來自靈芝的核醣核酸序列之構築；(2) ZYsynLZKD：包含合成的synLZKD基因之構築。此兩種構築分別以基因槍接種到矮南瓜上，約10至14天之後葉片出現嵌紋和葉面向下捲曲的系統性病徵，其中ZYsynLZKD所表現病徵較ZYLZ8嚴重，而野生型病毒ZYMV則僅出現嵌紋的系統性病徵。抽取植物葉片總RNA，以RT-PCR偵測lz-8基因並利用限制酵素PvuII和XhoI剪切，初步確認LZ-8基因確實在植物內表現。本實驗已成功將LZ-8基因構築送入菸草和ZYMV病毒載體，之後將測試此蛋白質在植物系統中的表現情形。

關鍵詞：矮南瓜黃化嵌紋病毒、基因轉殖、菸草、矮南瓜

目錄

封面內頁 簽名頁 中文摘要 英文摘要 誌謝 目錄 圖目錄 表目錄 1. 前言 1.1 靈芝簡介 1.2 真菌免疫調控蛋白 1.3 赤芝免疫調控蛋白相關的免疫調控機制 1.4 利用微生物表現重組赤芝免疫調控蛋白 1.5 利用植物表現有價值外源蛋白 2. 材料與方法 2.1 LZ-8基因的構築 2.2 組織培養藥品 2.2.1 細菌類培養基 2.2.2 植物培養基 2.2.3 植物荷爾蒙 2.2.4 抗生素 2.3 菸草轉殖 2.4 擬轉基因植物總DNA之抽取 2.5 植物總RNA抽取 2.6 聚合酶連鎖反應偵測植物染色體中的轉基因 2.7 基因槍接種含LZ-8的重組病毒 2.8 反轉錄酶-聚合酶連鎖反應偵測重組病毒接種之矮南瓜植株 2.9 病葉接種 3. 結果 3.1 菸草基因轉殖與PCR確認 3.2 含LZ-8重組病毒之接種與RT-PCR確認 4. 討論 5. 結論 參考文獻

參考文獻

1. Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. *Nature* 392, 245-252.
2. Brennan, F.R., Jones, T.D., and Hamilton, W.D. (2001). Cowpea mosaic virus as a vaccine carrier of heterologous antigens. *Mol Biotechnol* 17, 15-26.
3. Canizares, M.C., Nicholson, L., and Lomonosoff, G.P. (2005). Use of viral vectors for vaccine production in plants. *Immunol Cell Biol* 83, 263-270.
4. Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. (1997a). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* 388, 782-787.
5. Cella, M., Sallusto, F., and Lanzavecchia, A. (1997b). Origin, maturation and antigen presenting function of dendritic cells. *Curr Opin Immunol* 9, 10-16.
6. Chen, H.F., Chang, M.H., Chiang, B.L., and Jeng, S.T. (2006). Oral immunization of mice using transgenic tomato fruit expressing VP1 protein from enterovirus 71. *Vaccine* 24, 2944-2951.
7. Chen, H.Y., Chen, C.H., Yeh, M.Y., Wu, J.S., Yazama, K., and Mikami, Y. (1992). In vivo anti-candidal activity induced by traditional Chinese herbal medicine, *Ganoderma lucidum*. *Jpn J Med Mycol* 33, 505-512.
8. Chen, T.C., Hsu, H.T., Jain, R.K., Huang, C.W., Lin, C.H., Liu, F.L., and Yeh, S.D. (2005). Purification and serological analyses of tospoviral nucleocapsid proteins expressed by *Zucchini yellow mosaic virus* vector in squash. *J Virol Methods* 129, 113-124.
9. Chung, B.N., Canto, T., and Palukaitis, P. (2007). Stability of recombinant plant viruses containing genes of unrelated plant viruses. *J Gen Virol* 88, 1347-1355.
10. Giddings, G. (2001). Transgenic plants as protein factories. *Curr Opin Biotechnol* 12, 450-454.
11. Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C., and Amigorena, S. (2002). Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 20, 621-667.
12. Haak-Frendscho, M., Kino, K., Sone, T., and Jardieu, P. (1993). Ling Zhi-8: a novel T cell mitogen induces cytokine production and upregulation of ICAM-1 expression. *Cell Immunol* 150, 101-113.
13. Horner, W.E., Helbling, A., and Lehrer, S.B. (1993). Basidiomycete allergens: comparison of three *Ganoderma* species. *Allergy* 48, 110-116.
14. Hsu, H.C., Hsu, C.I., Lin, R.H., Kao, C.L., and Lin, J.Y. (1997). Fip-vvo, a new fungal immunomodulatory protein isolated from *Volvariella volvacea*. *Biochem J* 323 (Pt 2), 557-565.
15. Hsu, H.Y., Hua, K.F., Wu, W.C., Hsu, J., Weng, S.T., Lin, T.L., Liu, C.Y., Hseu, R.S., and Huang, C.T. (2008). Reishi immuno-modulation protein induces interleukin-2 expression via

protein kinase-dependent signaling pathways within human T cells. *J Cell Physiol* 215, 15-26. 16. Huang, L., Sun, F., Liang, C., He, Y.X., Bao, R., Liu, L., and Zhou, C.Z. (2009). Crystal structure of LZ-8 from the medicinal fungus *Ganoderma lucidum*. *Proteins* 75, 524-527. 17. Jeurink, P.V., Noguera, C.L., Savelkoul, H.F., and Wichers, H.J. (2008). Immunomodulatory capacity of fungal proteins on the cytokine production of human peripheral blood mononuclear cells. *Int Immunopharmacol* 8, 1124-1133. 18. Kadouri, D., Peng, Y., Wang, Y., Singer, S., Huet, H., Raccach, B., and Gal-On, A. (1998). Affinity purification of HC-Pro of potyviruses with Ni²⁺-NTA resin. *J Virol Methods* 76, 19-29. 19. Kino, K., Mizumoto, K., Sone, T., Yamaji, T., Watanabe, J., Yamashita, A., Yamaoka, K., Shimizu, K., Ko, K., and Tsunoo, H. (1990). An immunomodulating protein, Ling Zhi-8 (LZ-8) prevents insulinitis in non-obese diabetic mice. *Diabetologia* 33, 713-718. 20. Kino, K., Yamashita, A., Yamaoka, K., Watanabe, J., Tanaka, S., Ko, K., Shimizu, K., and Tsunoo, H. (1989). Isolation and characterization of a new immunomodulatory protein, ling zhi-8 (LZ-8), from *Ganoderma lucidum*. *J Biol Chem* 264, 472-478. 21. Ko, J.L., Hsu, C.I., Lin, R.H., Kao, C.L., and Lin, J.Y. (1995). A new fungal immunomodulatory protein, FIP-fve isolated from the edible mushroom, *Flammulina velutipes* and its complete amino acid sequence. *Eur J Biochem* 228, 244-249. 22. Lin, J.M., Lin, C.C., Chiu, H.F., Yang, J.J., and Lee, S.G. (1993). Evaluation of the anti-inflammatory and liver-protective effects of *anoectochilus formosanus*, *ganoderma lucidum* and *gynostemma pentaphyllum* in rats. *Am J Chin Med* 21, 59-69. 23. Lin, W.H., Hung, C.H., Hsu, C.I., and Lin, J.Y. (1997). Dimerization of the N-terminal amphipathic alpha-helix domain of the fungal immunomodulatory protein from *Ganoderma tsugae* (Fip-gts) defined by a yeast two-hybrid system and site-directed mutagenesis. *J Biol Chem* 272, 20044-20048. 24. Lin, Y.L., Liang, Y.C., Tseng, Y.S., Huang, H.Y., Chou, S.Y., Hseu, R.S., Huang, C.T., and Chiang, B.L. (2009). An immunomodulatory protein, Ling Zhi-8, induced activation and maturation of human monocyte-derived dendritic cells by the NF-kappaB and MAPK pathways. *J Leukoc Biol* 86, 877-889. 25. Mihich, E. (1986). Future perspectives for biological response modifiers: a viewpoint. *Semin Oncol* 13, 234-254. 26. Miyasaka, N., Inoue, H., Totsuka, T., Koike, R., Kino, K., and Tsunoo, H. (1992). An immunomodulatory protein, Ling Zhi-8, facilitates cellular interaction through modulation of adhesion molecules. *Biochem Biophys Res Commun* 186, 385-390. 27. Monger, W., Alamillo, J.M., Sola, I., Perrin, Y., Bestagno, M., Burrone, O.R., Sabella, P., Plana-Duran, J., Enjuanes, L., Garcia, J.A., et al. (2006). An antibody derivative expressed from viral vectors passively immunizes pigs against transmissible gastroenteritis virus infection when supplied orally in crude plant extracts. *Plant Biotechnol J* 4, 623-631. 28. Nykiforuk, C.L., Boothe, J.G., Murray, E.W., Keon, R.G., Goren, H.J., Markley, N.A., and Moloney, M.M. (2006). Transgenic expression and recovery of biologically active recombinant human insulin from *Arabidopsis thaliana* seeds. *Plant Biotechnol J* 4, 77-85. 29. Okamoto, T., Shimada, T., Hara-Nishimura, I., Nishimura, M., and Minamikawa, T. (2003). C-terminal KDEL sequence of a KDEL-tailed cysteine proteinase (sulfhydryl-endopeptidase) is involved in formation of KDEL vesicle and in efficient vacuolar transport of sulfhydryl-endopeptidase. *Plant Physiol* 132, 1892-1900. 30. Staczek, J., Bendahmane, M., Gilleland, L.B., Beachy, R.N., and Gilleland, H.E., Jr. (2000). Immunization with a chimeric tobacco mosaic virus containing an epitope of outer membrane protein F of *Pseudomonas aeruginosa* provides protection against challenge with *P. aeruginosa*. *Vaccine* 18, 2266-2274. 31. Tanaka, S., Ko, K., Kino, K., Tsuchiya, K., Yamashita, A., Murasugi, A., Sakuma, S., and Tsunoo, H. (1989). Complete amino acid sequence of an immunomodulatory protein, ling zhi-8 (LZ-8). An immunomodulator from a fungus, *Ganoderma lucidum*, having similarity to immunoglobulin variable regions. *J Biol Chem* 264, 16372-16377. 32. van der Hem, L.G., van der Vliet, J.A., Bocken, C.F., Kino, K., Hoitsma, A.J., and Tax, W.J. (1995). Ling Zhi-8: studies of a new immunomodulating agent. *Transplantation* 60, 438-443. 33. Verma, R., Boleti, E., and George, A.J. (1998). Antibody engineering: comparison of bacterial, yeast, insect and mammalian expression systems. *J Immunol Methods* 216, 165-181. 34. Wandelt, C.I., Khan, M.R., Craig, S., Schroeder, H.E., Spencer, D., and Higgins, T.J. (1992). Vicilin with carboxy-terminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants. *Plant J* 2, 181-192. 35. Xue, Q., Ding, Y., Shang, C., Jiang, C., and Zhao, M. (2008). Functional expression of LZ-8, a fungal immunomodulatory protein from *Ganoderma lucidum* in *Pichia pastoris*. *J Gen Appl Microbiol* 54, 393-398. 36. Yeh, C.M., Yeh, C.K., Hsu, X.Y., Luo, Q.M., and Lin, M.Y. (2008). Extracellular expression of a functional recombinant *Ganoderma lucidum* immunomodulatory protein by *Bacillus subtilis* and *Lactococcus lactis*. *Appl Environ Microbiol* 74, 1039-1049. 37. Zhang, X., Buehner, N.A., Hutson, A.M., Estes, M.K., and Mason, H.S. (2006). Tomato is a highly effective vehicle for expression and oral immunization with Norwalk virus capsid protein. *Plant Biotechnol J* 4, 419-432.