

# Cp-AFP3-HB-GFP 之轉基因洋桔梗抗真菌之評估

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## 摘要

台灣地處於亞熱帶及熱帶地區，農作物多元而複雜，洋桔梗是近年來在國內發展快速的新興切花之一。真菌疾病造成洋桔梗產量極大之損失。本研究希望利用轉基因策略，以對抗疾病之危害，以減少農藥之使用造成環境危害的疑慮，因此利用轉基因策略加強植物抗病蟲害特性實為一項不錯的策略。抗真菌蛋白基因之構築體Cp-AFP3-HB-GFP，由中央研究院蕭介夫博士提供，經胺基酸序列比對，發現其結構類似於植物防禦素 (Plant Defensins) 中的AFP3蛋白的基因。本研究室之前已構築出8個具有Cp-AFP3-HB-GFP基因之轉基因洋桔梗株系。經由聚合酵素鏈鎖反應、南方點漬法證明抗真菌蛋白確實併入洋桔梗染色體中。利用立枯病原*Rhizoctonia solani*及白絹病原*Rhizoctonia solani*分別各自接種，結果發現接種四天後，非轉基因植物均已產生病徵或罹病死亡，而轉基因洋桔梗line 1、line 8、line 14及line 17則展現了對*Rhizoctonia solani*高度的抗性，有50%以上仍未染病，而line 17則是超過了70%未出現病徵，而上述對*Rhizoctonia solani*有高度抗性的植株，再接種病原菌*Sclerotium rolfsii*後的實驗結果亦和上述相符合，其餘的轉基因植株與非轉基因植物相較之下，也有較佳的抗性。於Western Blotting蛋白質分析後，亦可發現GFP報導基因的蛋白表現。因感染洋桔梗之真菌種類繁多，故在以立枯病及白絹病接種評估後，將繼續以其他種類真菌進行初步測試。

關鍵詞：抗真菌蛋白、轉基因洋桔梗、立枯病、白絹病。

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## 參考文獻

參考文獻 1.行政院農業委員會農糧署。民98。農業統計年報。 <http://www.coa.gov.tw/view.php?catid=21690> 2.行政院農業委員會動植物防疫檢疫局 [http://pesticide.baphiq.gov.tw/web/Insecticides\\_MenuItem5\\_3.aspx](http://pesticide.baphiq.gov.tw/web/Insecticides_MenuItem5_3.aspx) 3.農業委員會台灣農家要覽增修訂三版策劃委員會。2005。台灣農家要覽 - 農作篇(二)。行政院農業委員會。台北市。4.杜金池、張義璋。1992。作物抗病品種之培育。病蟲害非農藥防治技術研討會專刊。台中。霧峰。1991(11)28-29。 5.余聰安。2001。木瓜微體繁殖與營養器官基因轉殖。國立中興大學植物學系博士論文。 6.陳任芳。1996。花蓮區農業專訊。55:15-16。 7.陳福?。1993。洋桔梗 園藝之友39:32-35。 8.黃達雄。1992。洋桔梗(上)興農 283:45-49。 9.張燕玲。2005。抗真菌轉基因甜瓜之構築。大葉大學分子生物學系研究所碩士論文。 10.王菩提。2008。抗真菌轉基因甜瓜之溫室評估。大葉大學分子生物學系研究所碩士論文。 11.楊秀珠。1999。洋桔梗病害及防治。農業世界雜誌190:32-40。 12.廖家德。1994。臺灣立枯絲核菌 (*Rhizoctonia solani* Kuhn)第四融合群菌株質體狀去氧核糖核酸的歧異性及其核酸定序。國立中興大學植物病理學研究所碩士論文。 13.廖麗雅。1993。洋桔梗涼溫育苗及微體繁殖系統建立。國立中興大學園藝研究所碩士論文。 14.賴宣好。2002。青花菜之抗真菌蛋白基因。私立東海大學食品科學系。 15.曾國欽。2004。植物重要防疫檢疫病害診斷鑑定技術研習會專刊(三)。23-24。 16.蔡雲鵬編。1991。台灣植物病害名彙 修訂3版。419-420頁。中華植物保護學會,中華民國植物病理學會印。604頁。 17.蔡竹固。2007。植物與

病原微生物的交互作用。國立嘉義大學微生物與免疫學系。18.蘇宗振。1999。植物基因轉殖之研究。科學農業47(3,4):112-119。

19.Sacchetti, A., Ciccocioppo R, Alberti S. 2000 The molecular determinants of the efficiency of green fluorescent protein mutants, *Histol. Histopathol.* 15: 101-107. 20.Almeida, M.S., Cabral, K. M. Zingali, R. B. Kurtenbach, E. 2000. Characterization of two novel defense peptides from pea (*Pisum sativum*) seeds. *Arch. Biochem. Biophys.* 378:278-286. 21.Bradley, J.M., Rains, S.R. Manson, J.L. Davies, K.M. 2000. Flower pattern stability in genetically modified *lisianthus* (*Eustoma grandiflorum*) under commercial growing conditions. *N. Z. J. Crop Hort. Sci.* 28, 175-184. 22.Broekaert, W. F., F.R. Terras, B.P. Cammue, R.W. Osborn. 1995. Plant defensins: novel antimicrobial peptides as components of host defense system. *Plant Physiol.* 108:1353-1358. 23.Bull, J., F. Mauch, C. Hertig, G. Regmann, R. Dudler. 1992. Sequence and expression of a wheat gene that encodes a novel protein associated with pathogen defense. *Mol. Plant Microbe Interact.* 5:516-519. 24.Coca, M., C. Bortolotti, M. Rufat, G. Penas, R. Eritja, D. Tharreau, A. Martinez del Pozo, J. Messegueur, B. San Segundo. 2004. Transgenic rice plants expressing the antifungal AFP protein from *Aspergillus giganteus* show enhanced resistance to the rice blast fungus *Magnaporthe grisea*. *Plant Molecular Biology* 54:245-259. 25.Baulcombe D.C., S. Chapman, S. Santa Cruz. 1995. green fluorescent protein as a reporter for virus infections, *Plant J.* 7. 1045-1053. 26.Deroles, S. C., M. J. Bradley, K. E. Schwinn, K. R. Markham, S. J. Bloor, D. G. Manson, K. M. Davies. 1998. An antisense chalcone synthase cDNA leads to novel colour patterns in *lisianthus* (*Eustoma grandiflorum*) flowers. *Molecular Breeding* 4, 59-66. 27.Durner, J., J. Shah, D. F. Klessig. 1997. Salicylic acid and disease resistance in plants. *Trends Plant Sci* 2:266-274. 28.Ecker, R., A. Barzilay, E. Osherenko. 1994. Population means and correlation analysis of growth parameters in *lisianthus* (*Eustoma grandiflorum* Shinn.). *Euphytica* 78(3), 193-197. 29.Ezura, H. 2001. Genetic engineering of melon (*Cucumis melo* L.). *Plant Biotechnology.* 18:1-6. 30.Fang, G., R. Grumet. 1993. *Agrobacterium tumefaciens* mediated transformation and regeneration of muskmelon plants. *Plant Cell Rep.* 9 : 160-164. 31.Fant, F., W. Vranken, W. Broekaert, F. Borremans. 1998. Determination of the three-dimensional solution structure of *Raphanus sativus* antifungal protein 1 by <sup>1</sup>H NMR. *J. Mol. Biol.* 279:257-270. 32.Fulton, T.M., J. Chunwongse, S.D. Tanksley. 1995. Microprep Protocol for Extraction of DNA from Tomato and other Herbaceous Plants. *Plant Molecular Biology Reporter* 13:207-209. 33.Gamborg, O.L., R. A. Miller, K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp.Cell.Res.* 50:151-158. 34.Grenier, J., C. Potvin, A. Asselin. 1993. Barley pathogenesis-related proteins with fungal cell wall lytic activity inhibit the growth of yeasts. *Plant Physiol.* 103:1277-1283. 35.Griesbach R. J., P. Semeniuk, M. Roh, R. H. Lawson. 1988. Tissue Culture in the Improvement of *Eustoma*. *Hortsci.* 23, 790-791. 36.Gun Lee, D., S. Y. Shin, C. Y. Maeng, Z. Z. Jin, K. L. Kim, K. S. Hahm. 1999. Isolation and characterization of a novel antifungal peptide from *Aspergillus niger*. *Biochem. Biophys. Res. Commun.* 263:646-651. 37.Hisamatsu, T., M. Koshika, N. Oyama, L.N. Mander. 1999. The relationship between endogenous gibberellins and resetting in *Eustoma grandiflorum*. *J. Jpn. Soc. Hort. Sci.* 68, 433-527. 38.Hopkins, W. L. 1996. *Global Fungicide Directory*. 148pp. AG Chem Information Services. USA. 39.Hototsugu, Y., M. Masanobu, I. O. Masaki, Ichi. Ken. 2004. Reduced Glutathione is a Novel Regulator of Vernalization-Induced Bolting in the Rosette Plant *Eustoma grandiflorum*. *Plant Cell Physiol.* 45(2):129-137. 40.Kawabata, S., Y. Kusuhara, Y. Li, R. Sakiyama. 1999. The regulation of anthocyanin biosynthesis in *Eustoma grandiflorum* under low light conditions. *J. Jpn. Soc. Hort. Sci.* 68, 519-526. 41.Kitajima, S., F. Sato. 1999. Plant pathogenesis-related proteins: molecular 42.Klement, Z. 1982. Hypersensitivity. In *phytopathogenic prokaryotes*, volume 2 (Mount MS and Lacy GH) New York: Academic Press, pp. 149-177. 43.Kombrink, E., I. E. Somssich. 1995. Defence responses of plants to pathogens. *Adv Bot Res* 21:1-34. 44.Kragh, K.M., J. E. Nielsen, K. K. Nielsen, Dreboldt, S. Mikkelsen, J. D. 1995. Characterization and localization of new antifungal cysteine-rich proteins from *Beta vulgaris*. *Mol. Plant Microbe Interact.* 8: 424-434. 45.Kristensen, A.K., J. Brunsted, J. W. Nielsen, J. D. Mikkelsen, P. Roepstorff. K. K. Nielsen. 1999. Processing, disulfide pattern, and biological activity of a sugar beet defensin, AX2, expressed in *Pichia pastoris*. *Protein Expr. Purif.* 16:377-387. 46.Lacadena, J., A. Martinez del Pozo, M. Gasset, B.Patino, Campos-Olivas, R. C. Vazquez, A. Martinez-Ruiz, J. M. Mancheno, M. Onaderra, J.G. Gavilanes. 1995. Characterization of the antifungal protein secreted by the mould *Aspergillus giganteus*. *Arch. Biochem. Biophys.* 324:273-281. 47.Lamberty, M., S. Ades, S. Uttenweiler-Joseph, G. Brookhart, D. Bushey, J. A. Hoffmann, P. Bulet. 1999. Insect immunity. Isolation from the lepidopteran *Heliothis virescens* of a novel insect defensin with potent antifungal activity. *J. Biol. Chem.* 274:9320-9326. 48.Landon, C., A. Pajon, F. Vovelle, P. Sodano. 2000. The active site of drosomycin, a small insect antifungal protein, delineated by comparison with the modeled structure of Rs-AFP2, a plant antifungal protein. *J. Pept. Res.* 56:231-238. 49.Ledger, S.E., S.C. Deroles, D.G. Manson, J. Marie Bradley, N.K. Given. 1997. Transformation of *lisianthus* (*Eustoma grandiflorum*). *Plant Cell Rep.* 16,853-858. 50.Li, J., Y. Notsu, M. Ogawa, H. Ohno, K. Ohkawa. 2002. Rosetting characteristics based classification of *Eustoma grandiflorum* (Raf.) Shinn. Cultivars sown on different dates. *Environment Control in Biology* 40 (2) : 229-237. 51.Lipke, P., R. Ovalle. 1998. Yeast cell walls: new structures, new challenges. *J. Bacteriol.* 180: 3735-3740. 52.Liu, Y., J. Luo, C. Xu, F. Ren, C. Peng, G. Wu, J. Zhao, 2000. Purification, characterization, and molecular cloning of the gene of a seed specific antimicrobial protein from pokeweed. *Plant Physiol.* 122:1015-1024. 53.Anthony, J. D., L. J. W. Thomas. 1999. Antifungal peptides: novel therapeutic compounds against emerging pathogens. *Antimicrob. Agents Chemother.* 43:1-11. 54.Lumbroso, E., G. Fischbeck, I. Wahl. 1982. Infection of barley with conidia suspensions of *Erysiphe graminis* f. sp. *hordei*. *Phytopathol. Z.* 104:222-223. 55.Mariko, k., T. Yasushi, I. Masaki, M. Mino. 2001. Elevated sensitivity to gibberellin by vernalization in the vegetative rosette plants of *Eustoma grandiflorum* and *Arabidopsis thaliana*. *Plant Sci.* 160:1237-1245. 56.Matsuo, T., T. Shirasaki. 1990. Effect of rate of fertilization on the growth and nutrient uptake of *Eustoma grandiflorum*. *J. Japan. Soc. Hort. Sci.* 59(Suppl. 1), 584-585(in Japanese, with English summary). 57.Murashige, T., F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497. 58.Nawrath, C., J. Metraux. 1999. Salicylic acid induction -deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell.* 11: 1393-1404.

59. Ohkawa, K., T. Yoshizumi, M. Korenaga, K. Kanematsu. 1994. Reversal of heat-induced resetting in *Eustoma grandiflorum* with low temperatures. *HortScience* 29, 165-166. 60. Ohta, K., H. Atarashi, Y. Shimatani, S. Matsumoto, T. Asao, T. Hosoki. 2000. Effects of chitosan with or without nitrogen treatments on seeding growth in *Eustoma grandiflorum* (Raf.) Shinn. cv. Kairyuu wakamurasaki. *J. Jpn. Soc. Hort. Sci.* 69, 63-65. 61. Ryals, J., S. Uknes, E. Ward. 1994. Systemic acquired resistance. *Plant Physiol* 104:1109-1112. 62. Ryals, J.A., U. H. Neuenschwander, M. G. Willits, A. Molina, H. Y. Steiner, M.D. Hunt. 1996. Systemic acquired resistance. *Plant Cell* 8:1809-1819. 63. Salzman, R. A., I. Tikhonova, B. P. P. Bordelon, M. Hasegawa, R. A. Bressan. 1998. Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense response during fruit ripening in grape. *Plant Physiol.* 117:465 – 472. 64. Segura, A., M. Moreno, A. Molina, F. Garcia-Olmedo. 1998. Novel defensin subfamily from spinach (*Spinacia oleracea*). *FEBS Lett.* 435:159 – 162. 65. Selitrennikoff, C. P. 2001. Antifungal protein. *Applied and Environmental Microbiology*. p: 2883-2894. 66. Semeria, L., B. Ruffoni, M. Rabaglio, A. Genga, A. Vaira, G. Accotto. 1996. Genetic transformation of *Eustoma grandiflorum* by *Agrobacterium tumefaciens*. *Plant Cell Tissue Org. Culture* 47, 67-72. 67. Shao, F., Y. M. Xiong, Q. Z. Huang, C. G. Wang, R. H. Zhu, D. C. Wang. 1999. A new antifungal peptide from the seeds of *Phytolacca americana*: characterization, amino acid sequence and cDNA cloning. *Biochim. Biophys. Acta* 1430:262 – 268. 68. Sneh, B., L. Burbee, A. Ogoshi. 1991. Identification of *Rhizoctonia* species. 133pp. ASP press. 69. Shinn, L. 1957. Synopsis of genus *Eustoma* (Gentianaceae). *The Southwestern Naturalist* 2 (1), 38-43. 70. Terras, F. R. G., K. Eggermont, V. Kovaleva, N. V. Raikhel, R. W. Osborn, A. Kester, S. B. Rees, S. Torreken, F. van Leuven, J. Vanderleyden, B. P. A. Cammue, W. F. Broekaert. 1995. Small cysteine-rich antifungal proteins from radish: their role in host defense. *Plant Cell* 7 (5): 573-588. 71. Thevissen, K., A. Ghazi, D. G. W. Samblanx, C. Brownlee, R. W. Osborn, W. F. Broekaert. 1996. Fungal membrane responses induced by plant defensins and thionins. *J. Biol. Chem.* 271:5018 – 5025. 72. Thevissen, K., R. W. Osborn, D. P. Acland, W. F. Broekaert. 1997. Specific, high affinity binding sites for an antifungal plant defensin on *Neurospora crassa* hyphae and microsomal membranes. *Biol. Chem.* 272: 32176 – 32181. 73. Thevissen, K., R. W. Osborn, D. P. Acland, W. F. Broekaert. 2000. Specific binding sites for an antifungal plant defensin from *Dahlia* (*Dahlia merckii*) on fungal cells are required for antifungal activity. *Mol. Plant Microbe Interact.* 13:54 – 61. 74. Thevissen, K., F. T. Terras, W. F. Broekaert. 1999. Permeabilization of fungal membranes by plant defensins inhibits fungal growth. *Appl. Environ. Microbiol.* 65: 5451 – 5458. 75. Tulasi, R. B., S. K. Nadimpalli. 1997. Purification of  $\alpha$ -mannosidase activity from Indian lablab beans. *Biochem. Mol. Biol. Int.* 41:925 – 931. 76. Vila, L., V. Lacadena, P. Fontanet, A. Martinez del Pozo, B. San Segundo. 2001. A Protein from the Mold *Aspergillus giganteus* Is a Potent Inhibitor of Fungal Plant Pathogens. *The American Phytopathological Society* 14:1327-1331. 77. Waniska, R. D., A. Chandrashekar, S. Krishnaveni, F. P. Bejosano, J. Jeoung, J. Jayaraj, S. Muthukrishnan, G.H. Liang. 2001. Antifungal proteins and other mechanisms in the control of sorghum stalk rot and grain mold. *J Agric Food Chem.* 49(10): 4732-4742. 78. Zaccari, M., N. Edri. 2002. Floral transition in *Eustoma grandiflorum*. *Sci. Horti.* 95(4), 333-340.