

Greenhouse Evaluation of Transgenic Melons Expressing Antifungal Protein (AFP3) Conferred Resistance against Fungal ...

王菩提、余聰安

E-mail: 9608166@mail.dyu.edu.tw

ABSTRACT

Melon (*Cucumis melo* L.) is one of the economically important crops on the tropics and subtropics. Fungi disease is often causing serious economy loss of melon and fungicides are used to protect against melon diseases. In consideration of the harmful and dangerous effects to the environment ecosystem, maybe the transgene approach has better to control the fungal diseases. The anti-fungal protein gene constructions, Bo-AFP3-HB-GFP and Cp-AFP3-HB-GFP, were kindly supplied by Dr. Xiao, Jei -Fu of Academia Sinica. Transgenic melon lines carrying Bo-AFP3-HB-GFP or Cp-AFP3-HB-GFP genes were previously generated in our laboratory. Therefore, this study evaluated resistance of independent transgenic lines against *Rhizoctonia solani* under greenhouse condition. The transgene was inserted into the genomic DNA of the regenerates confirmed by PCR and Southern blotting. Line B28、line C14 and line C25 exhibited higher resistance 2days postinoculation R. solani and RT-PCR analysis indicated these lines relatively expressing high levels of afp3 mRNA. Photomicrographs under fluorescence microscopy showing GFP proteins was apparently expressing in the higher resistant transgenic leaves.

Keywords : anti-fungal protein, transgenic melon, *Rhizoctonia solani*

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REFERENCES

1. 行政院農委會。民93。農業統計之農作物產銷統計。 <http://www.coa.gov.tw/8/195/202/894/894.html>
2. 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。
3. 張燕玲。2005。抗真菌轉機因甜瓜之構築。私立大葉大學分子生物學系研究所碩士論文。
4. 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醣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。
5. 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。
6. 蔡尚光。1995。設施洋香瓜與胡瓜的高品質生產。P14-23。淑馨出版社。
7. 蔡雲鵬編。1991。台灣植物病害名彙修訂3版。頁419-420。中華植物保護學會,中華民國植物病理學會印。604頁。
8. 蔡竹固譯。1997。瓜類作物病害的田間診斷。嘉農植保8:6-11。
9. 蔡竹固。1999。甜瓜病害的診斷及其防治。國立嘉義技術學院農業推廣委員會。
10. 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。
11. 蔡竹固、陳瑞祥。2000。本省瓜類作物之重要病害及其管理。農業世界雜誌。200:12-19。
12. 薛聰賢編著

。 2000。 蔬香果樂。 頁71-72。 13. 蘇宗振。 1999。 植物基因轉殖之研究。 科學農業47 (3, 4) : 112 – 119。 14. Ainsworth, G: (ed.). 1973. The fungi, an advance treatise. Academic Press, New York, N.Y. 15. Almeida, M.S., Cabral, K. M., Zingali, R. B. and Kurtenbach, E. 2000. Characterization of two novel defense peptides from pea (*Pisum sativum*) seeds. *Arch. Biochem. Biophys.* 378:278 – 286. 16. Bier i , S. , Potrykus , I . ,and Fut terer1, J. 2003. Effects of combined expression of antifungal barley seed proteins transgenic wheat on powdery mildew infection. *Molecular Breeding.* 11:37 – 48. 17. Bohn, G. W. and T. W. Whitaker. 1964. Genetics of resistance to powdery mildew race 2 in muskmelon. *Phytopathology.* 54:587-591. 18. Broekaert, W. F., Terras, F.R., Cammue, B.P. and Osborn, RW. 1995. Plant defensins: novel antimicrobial peptides as components of host defense system. *Plant Physiol.* 108:1353 – 1358. 19. Bull, J., Mauch, F., Hertig, C., Regmann, G. and Dudler, R. 1992. Sequence and expression of a wheat gene that encodes a novel protein associated with pathogen defense. *Mol. Plant Microbe Interact.* 5:516 – 519. 20. Coca, M. , Bor tolott i , C. , Rufat , M., Penas , G. ,Er it ja,R., Tharreau, D. , Mar tinez del Pozo, A. , Messeguer , J . and San Segundo, B. 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. 21. Durner, J., Shah, J. and Klessig, D. F. 1997. Salicylic acid and disease resistance in plants. *Trends Plant Sci* 2:266-274. 22. Ezura H. 2001. Genetic engineering of melon (*Cucumis melo* L.). *Plant Biotechnology.* 18:1-6. 23. Fang, G. and Grumet, R. 1993. Agrobacterium tumefaciens mediated transformation and regeneration of muskmelon plants. *Plant Cell Rep.* 9 : 160-164. 24. Fant, F., W. Vranken, W. Broekaert, and F. Borremans. 1998. Determination of the three-dimensional solution structure of *Raphanus sativus* antifungal protein 1 by 1H NMR. *J. Mol. Biol.* 279:257 – 270. 25. Fuchs, M., Klas, F. E., McFerson, j. R., and Gonsalves ,D. 1998. Transgenic melon and squash expressing coat protein genes of aphid-borne viruses do not assist the spread of an aphid non- transmissible strain of cucumber mosaic virus in the field. *Transgenic Res.* 7:449-462. 26. Fulton, T.M. Chunwongse J, and Tanksley SD. 1995. Microprep Protocol for Extraction of DNA from Tomato and other Herbaceous Plants. *Plant Molecular Biology Reporter* 13:207-209. 27. Gamborg, O.L., Miller, R. A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp.Cell.Res.* 50:151-158. 28. Grenier, J., Potvin, C. and Asselin, A. 1993. Barley pathogenesis-related proteins with fungal cell wall lytic activity inhibit the growth of yeasts. *Plant Physiol.* 103:1277 – 1283. 29. Gun Lee, D., S. Y. Shin, C. Y. Maeng, Z. Z. Jin, K. L. Kim, and K. S. Hahm. 1999. Isolation and characterization of a novel antifungal peptide from *Aspergillus niger*. *Biochem. Biophys. Res. Commun.* 263:646 – 651. 30. Hopkins、 W. L. 1996. Global Fungicide Directory. 148pp. AG Chem Information Services. USA. 31. Jach, G., Gornhardt, B., Mundy, J., Logemann, J., Pindorf, E., Leah, H., Schell, J., and Maas, C. 1995. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *The Plant J.* 8(1): 79-109. 32. Kim, J. K., Jang1, I. C., Wu, R., Zuo, W. N., Boston, R. S., Lee, Y. H., Ahn4, I. P & Nahm, B. H. 2003. Co-expression of a modified maize ribosome-inactivating protein and a rice basic chitinase gene in transgenic rice plants confers enhanced resistance to sheath blight. *Transgenic Res.* 12:475 – 484. 33. Kitajima, S. and Sato, F. 1999. Plant pathogenesis-related proteins: molecular 34. Klement, Z. 1982. Hypersensitivity. In phytopathogenic prokaryotes, volume 2 (Mount MS and Lacy GH) New York: Academic Press, pp. 149-177. 35. Kombrink, E. and Somssich, I. E. 1995. Defence responses of plants to pathogens. *Adv Bot Res* 21:1-34. 36. Kragh, K.M., J. E. Nielsen, K. K. Nielsen, S. Dreboldt, and J. D. Mikkelsen. 1995. Characterization and localization of new antifungal cysteine-rich proteins from *Beta vulgaris*. *Mol. Plant Microbe Interact.* 8: 424 – 434. 37. Kristensen, A.K., J. Brunsted, J. W. Nielsen, J. D. Mikkelsen, P. Roepstorff, and 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. 38. Lacadena, J., A. Martinez del Poxo, M. Gasset, B. Patino, R. Campos-Olivas, C. Vazquez, A. Martinez-Ruiz, J. M. Mancheno, M. Onaderra, and, and Gavilanes., J.G. 1995. Characterization of the antifungal protein secreted by the mould *Aspergillus giganteus*. *Arch. Biochem. Biophys.* 324:273 – 281. 39. Lamberty, M., S. Ades, S. Uttenweiler-Joseph, G. Brookhart, D. Bushey, J. A. Hoffmann, and 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. 40. Landon, C., Pajon, A., Vovelle, F. and Sodano, P. 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. 41. Lipke, P. and Ovalle, R. 1998. Yeast cell walls: new structures, new challenges. *J. Bacteriol.* 180: 3735 – 3740. 42. Liu, Y., J. Luo, C. Xu, F. Ren, C. Peng, G. Wu, and J. Zhao. 2000. Purification, characterization, and molecular cloning of the gene of a seed specific antimicrobial protein from pokeweed. *Plant Physiol.* 122:1015 – 1024. 43. Lucca, D. A. J. and Walsh, T. J. 1999. Antifungal peptides: novel therapeutic compounds against emerging pathogens. *Antimicrob. Agents Chemother.* 43:1 – 11. 44. Lumbroso, E., G. Fischbeck, and I. Wahl. 1982. Infection of barley with conidia suspensions of *Erysiphe graminis* f. sp. *hordei*. *Phytopathol. Z.* 104:222-223. 45. Martinez-Ruiz, A., Martinez del Pozo, A., Lacadena, J., M. Mancheno,J., Onaderra,M. and G.Gavilanes, J. 1997. Characterization of a nature larger form of the antifungal protein(AFP) from *Aspergillus giganteus*. *Biochimica et Biophysica Acta* 1340:81-87. 46. Mehdy, M. C. 1994. Active oxygen species in plant defense against pathogens. *Plant Physiol* 105:467-47. 47. Menzies, J. G., D. L. Ehret, A. D. M. Glass, T. Helmer, C. Koch, and F. Seywerd. 1991. Effect of soluble silicon on the parasitic fitness of *Sphaerotheca fuliginea* on *Cucumis sativus*. *Phytopathology* 81:84-88. 48. Moravcikova, J., Matusikova, I., Libantova, J., Bauer, M. and Mlynarova, L. 2004. Expression of cucumber class III citinase and *Nicotiana plumbaginifolia* class I glucanase genes in transgenic potato plants. *Plant Cell.* 79:161-168. 49. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497. 50. Nawrath, C. and Metraux, J. 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. 51. Oldach, K.H., Becker. D. and Lorz, H. 2001. Heterologous expression of genes mediating enhanced fungal resistance in transgenic wheat. The American Phytopathological Society. 14:832 – 838. 52. Reeser, P. W., D. J. Hagedorn, and D. I. Rouse. 1983. Quantitative inoculations with *Erysiphe pisi* to assess variation of infection

efficiency on peas. *Phytopathology*. 73:1238-1240. 53. Ryals, J., Uknes, S. and Ward, E. 1994. Systemic acquired resistance. *Plant Physiol* 104:1109-1112. 54. Ryals, J.A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y. and Hunt, M.D. 1996. Systemic acquired resistance. *Plant Cell* 8:1809-1819. 55. Salzman, R. A., Tikhonova, I., Bordelon, B. P. P., Hasegawa, M. and Bressan, R. A. 1998. Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense response during fruit ripening in grape. *Plant Physiol.* 117:465 – 472. 56. Segura, A., Moreno, M., Molina, A. and Garcia-Olmedo, F. 1998. Novel defensin subfamily from spinach (*Spinacia oleracea*). *FEBS Lett.* 435:159 – 162. 57. Selitrennikoff, C. P. 2001. Antifungal protein. *Applied and Environmental MicroBiology*. p: 2883-2894. 58. Shao, F., Y. M. Xiong, Q. Z. Huang, C. G. Wang, R. H. Zhu, and 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. 59. Sivapalan, A. 1993. Effects of water on germination of powdery mildew conidia. *Mycol. Res.* 97:71-76. 60. Sitterly, W. R. 1978. Powdery mildew of cucurbits. *The Powdery Mildews*. Spencer, D. M.ed .359-377. 61. Sneh, B., Burbee, L., and Ogoshi, A. 1991. Identification of *Rhizoctonia* species.133pp. ASP press. 62. Terras, F.R.G., Eggermont, K., Kovaleva, V., Raikhel, N.V., Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., van Leuven, F., Vanderleyden, J., Cammue, B.P.A. and Broekaert, W. F. 1995. Small cysteine-rich antifungal proteins from radish: their role in host defense. *Plant Cell* 7 (5): 573-588. 63. Thevissen, K., Ghazi, A., Samblanx, D. G. W., Brownlee, C., Osborn, R. W. and Broekaert, W. F. 1996. Fungal membrane responses induced by plant defensins and thionins. *J. Biol. Chem.* 271:5018 – 15025. 64. Thevissen, K., Osborn, R. W., Acland, D. P. and Broekaert, W. F. 1997. Specific, high affinity binding sites for an antifungal plant defensin on *Neurospora crassa* hyphae and microsomal membranes. *Biol. Chem.* 272: 32176 – 32181. 65. Thevissen, K., Osborn, R. W., Acland, D. P. and Broekaert, W. F. 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. 66. Thevissen, K., Terras, F. T. and Broekaert, W. F. 1999. Permeabilization of fungal membranes by plant defensins inhibits fungal growth. *Appl. Environ. Microbiol.* 65: 5451 – 5458. 67. Tsay, J. G., and B. K. Tung. 1994. Powdery mildew of cucurbit crops. in: Proceeding of symposium on techniques of cucurbits protection, Plant Protection Society of the Republic of China Press, p.135-146. 68. Tulasi, R.B., and S. K. Nadimpalli. 1997. Purification of a-mannosidase activity from Indian lablab beans. *Biochem. Mol. Biol. Int.* 41:925 – 931. 69. Vellicce, G. R., Ricci, J. C. D., Hernandez, L. and Castagnaro, A. P. 2006. Enhanced resistance to *Botrytis cinerea* mediated by the transgenic expression of the chitinase gene ch5B in strawberry. *Transgenic Res.* 15:57 – 68. 70. Vila, L., Lacadena, V., Fontanet, P., Martinez del Pozo, A. and San Segundo, B. 2001. A Protein from the Mold *Aspergillus giganteus* Is a Potent Inhibitor of Fungal Plant Pathogens. *The American Phytopathological Society* 14:1327-1331. 71. Waniska, R. D., Chandrashekhar, A., Krishnaveni, S., Bejosano, F. P., Jeoung, J., Jayaraj, J., Muthukrishnan, S and Liang, G.H. 2001. Antifungal proteins and other mechanisms in the control of sorghum stalk rot and grain mold. *J Agric Food Chem.* 49(10): 4732-4742. 72. Yang, x., Xiao, Y., Wang, X. and Yan, P. 2007. Expression of a Novel Small Antimicrobial Protein from the Seeds of Motherwort (*Leonurus japonicus*) Confers Disease Resistance in Tobacco. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*. 73(3):939-946. 73. Ziv, O. and T. A. Zitter. 1992. Effects of bicarbonates and filmforming polymers on cucurbit foliar diseases. *Plant Dis.* 75: 513-517.