

Transgenic Watermelon Lines Expressing Chimeric Construct Containing Antifungal Protein and Chitinase Confer Resistance

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ABSTRACT

Watermelon (*Citrullus lanatus*) is an economically important crop of worldwide. Fungal diseases often causes serious economy loss of watermelon and people is usually spray tons of agricultural chemicals or bacteria inhibitor to protect against watermelon fungal diseases. In consideration of the harmful and dangerous effects to the environment ecosystem, we are trying to introduced antifungal protein and chitinase genes into watermelons to control watermelon diseases. This investigation tried to set up an approach of *Agrobacterium*-mediated transformation of watermelon carrying with chimeric gene, cp-AFP3-CHI. The anti-fungal protein genes, cp-AFP3 and PR-protein genes cp-CHI chimeric gene from *Caria papaya* L., were kindly provide by Dr. Xiao, Chiehfu of Academia Sinica. Seed was through pretreatment for 3 days, and cotyledons were cut into six part segments as explants. The explants co-cultured with *Agrobacteria* for 3 days and then transferred to the selection medium. There were 7 putative cp-AFP3-CHI transgenic lines were obtained. PCR analysis confirmed that the foreign gene was present in the regenerates. RT-PCR analysis indicated using primers specific for the mRNA sequence of cp-CHI-AFP to monitor expression of the gene at the mRNA level and showed 0.85 kb and 0.25 kb. Three transgenic lines(F27, F47, F49) conferred better degrees against *Rhizoctonia solani*, and we confirmed protein express were increase after *Rhizoctonia solani* by inoculation in vitro in western blot. One transgenic watermelon (F47 line) from cp-AFP3-CHI lines exhibited resistance to infection fungi under greenhouse conditions.

Keywords : antifungal protein、 chitinase、 transgenic watermelon

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REFERENCES

- 行政院農業委員會農糧署。農業統計之農作物產銷統計。2007。(<http://www.afa.gov.tw/>)李豐在。1985。花蓮區農業專訊 54:14-15。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。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。陳任芳。1996。花蓮區農業專訊 55:15-16。陳玉婷。1993。木瓜熟變相關基因之研究。國防醫學院生命科學研究所博士論文。黃怡萍。2004。木瓜幾丁質?之選殖與分析。私立東海大學食品科學研究所食品科技組碩士論文。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) 23-34。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。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。楊秀珠。1991。植物病原真菌抗藥性問題探討。中華民國雜草學會會刊12:135-154。蘇宗振。1999。植物基因轉殖之研究。科學農業47(3, 4): 112-119。Anand, A., Zhou T., Trick H N., Gill G S., Bockus W.W. and Muthukrishnan, S. 2002 Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against *Fusarium graminearum* Journal of Experimental Botany. 54: 1101-1111. Bieri, S., Potrykus I and Fütterer, J. 2000. Expression of active barley seed ribosome-inactivating protein in transgenic wheat. Theoretical and Applied Genetics 100: 755-763. Bliffeld, M., Mundy J., Potrykus I. and Fütterer J. 1999. Genetic engineering of wheat for increased resistance to powdery mildew disease. Theoretical and Applied Genetics 98: 1079-1086. 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 patho-gen defense. Mol. Plant Microbe Interact. 5: 516 – 519. Durner, J., Shah, J. and Klessig, D. F. 1997. Salicylic acid and disease resistance in plants. Trends Plant Sci 2: 266-274. Florman A.L. .1946. "False Positive" Hemoagglutination by Allantoic Fluids of Embryonated Eggs Inoculated with Unfiltered Throat Washings. J Bacteriol 52(3): 307-310. 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. 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. 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. Hopkins, W. L. 1996. Global Fungicide Directory. pp148. AG Chem Information Services. USA. Jansen, C., Wettstein., D.V., Schafer, W., Kogel, K.H., Felk, A. and

Frank, J.M. 2005. Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Plant Physiology* 88: 936-942.

Jayaraj, J. and Punja, Z. K. 2007. Combined expression of chitinase and lipid transfer protein genes in transgenic carrot plants enhances resistance to foliar fungal pathogens. *Plant Cell Rep* (2007) 26:1539-1546.

Kelley KM, Johnson TR, Gwatkin RB, Ilan J (1993) Transgenic strategies in reproductive endocrinology. *Mol Reprod Dev* 34(3): 337-347

Kitajima, S. and Sato, F. 1999. Plant pathogenesis-related proteins: molecular. Kirubakaran, S.I. and Sakthivel N. 2006. Cloning and overexpression of antifungal barley chitinase gene in *Escherichia coli*. *Protein Expression and Purification* 52: 159-166.

Klement, Z. 1982. Hypersensitivity. In *Phytopathogenic Prokaryotes*, volume 2 (Mount MS and Lacy GH) New York: Academic Press, pp. 149-177.

Kombrink, E. and Somssich, I. E. 1995. Defence responses of plants to pathogens. *Adv Bot Res* 21: 1-34.

Lipke, P. and Ovalle, R. 1998. Yeast cell walls: new structures, new challenges. *J. Bacteriol.* 180: 3735 – 3740.

Mauch, F., Mauch-Mani, B. and Boller, T. 1988. Antifungal Hydrolases In Pea Tissue: II. Inhibition of Fungal Growth by Combinations of Chitinase and β -1,3-Glucanase. *Plant Physiol* 88 (3):936-942.

Mehdy, M. C. 1994. Active oxygen species in plant defense against pathogens. *Plant Physiol* 105: 467-47.

Moreno AB, Del Pozo AM, Borja M, Segundo BS . 2003. Activity of the Antifungal Protein from *Aspergillus giganteus* Against *Botrytis cinerea*. *Phytopathology* 93(11): 1344-1353.

Moreno AB, Penas G, Rufat M, Bravo JM, Estopa M, Messeguer J, San Segundo B . 2005. Pathogen-induced production of the antifungal AFP protein from *Aspergillus giganteus* confers resistance to the blast fungus *Magnaporthe oryzae* in transgenic rice. *Mol Plant Microbe Interact* 18(9): 960-972

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.

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.

Oldach KH, Becker D, Lorz H. 2001. Heterologous expression of genes mediates enhanced fungal resistance in transgenic wheat. *Molecular Plant Microbe Interactions* 14: 832-838.

Runton N (1993) Ribavirin. *Neonatal Netw* 12(8): 75-77

Ryals, J., Uknes, S. and Ward, E. 1994. Systemic acquired resistance. *Plant Physiol* 104: 1109-1112.

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.

Schenk, R. U. and A. C. Hildebrandt, 1972, *Medium and Techniques for Induction and Growth of Monocotyledonous and Dicotyledonous Plant Cell Cultures*, *Can. J. Bot.* 50 : 199-204.

Schweizer, P., Christoffel, A., Dudler R. 1999. Transient expression of members of the germin-like gene in epidermal cells of wheat confers disease resistance. *The Plant Journal* 20: 541-552.

Segura, A., Moreno, M., Molina, A. and Garcia-Olmedo, F. 1998. Novel defensin subfamily from spinach (*Spinacia oleracea*). *FEBS Lett.* 435: 159 – 162.

Selitrrennikoff, C.P. 2001. Antifungal protein. *Applied and Environmental Microbiology*. p: 2883-2894.

Singh, A., Kirubakaran S.I, Sakthivel N, Heterologous expression of new antifungal chitinase from wheat . *Protein expression and purification* 56 :100 – 109.

Sneh, B., Burbee, L., and Ogoshi, A. 1991. Identification of *Rhizoctonia* species. pp 133. ASP press.

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.

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: 5018-5025.

Thevissen, K., Osborn, R. W., Acland, D. P. and Broekaert, W. F. 1999. Specific, high affinity binding sites for an antifungal plant defensin on *Neurospora crassa* hyphae and microsomal membranes. *Biol. Chem.* 272: 32176-32181.

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.

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.

VAN LOON, L. C. & VAN KAMMEN, A. (1970). Polyacrylamide disc electro-phoresis of the soluble leaf proteins from *Nicotiana tabacum* var. 'Samsun' and 'Samsun NN' II. Changes in protein constitution after infection with tobacco mosaic virus. *Virology* 40, 199-211.