J. Plant Biotechnology(1999) *Vol. 1(2). pp.85~90*

Journal of Plant Biotechnology

Expression of Chitinase Gene in *Solanum tuberosum* L.

Kyung Hwa Choi, Deok Chun Yang[1], Jae Heung Jeon, Hyun Soon Kim, Young Hee Joung, Hyouk Joung*

Plant Tissue Culture Research Unit, KRIBB, Taejon, 305-600, Korea: [1]Korea Ginseng & Tobacco Research Institute, Taejon, 305-345, Korea.

Key words: chimeric gene, PCR, northern blot analysis

Abstract

In order to protect fungal diseases, leaf disc explants of *Solanum tuberosum* cultivar, Belchip, was infected with an *Agrobacterium* MP90 strain containing chimeric gene construct, consisting of antibiotic resistance and chitinase gene driven by the CaMV 35S promoter, for transformation. Regenerated multiple shoots were selected on a medium containing kanamycin and carbenicillin after exposure to *Agrobacterium*. The presence and integration of the *npt* II and chitinase gene were confirmed by polymerase chain reaction(PCR). Northern blot analysis indicated that the genes coding for the enzyme could be expressed in potato plants. The chitinase activity of transgenic potato plants was higher than the control potato.

Introduction

Fungal disease has been one of the principal causes of crop losses. Higher plants generally defend themselves from invasion by pathogenic organisms by various mechanisms. As one of these mechanisms, plants lead to the induction of defence related genes. A number of defense related genes such as phenylalanine ammonia lyase(PAL) gene(Lois and schulz, 1989; Ward et al., 1989), chalcone synthase gene(Wienand et al., 1986; Sommer and Saedler, 1986), PR proteins genes(Somssich et al., 1986), chitinase gene(Yanai et al., 1992) etc., have been cloned. We decided to investigate chitinase gene for its potential importance in general defense. In many plants, chitinase rapidly accumulate following pathogen attack and hydrolyze the polymer chitin, major constituents of the cell walls of many fungi. As a result, the growth of fungi is inhibited. The identification of chitinase has first been shown in tobacco (Legrand et al., 1987) and confirmed in several other dicots(Kurosaki et al., 1987; Jutidamrongphan et al., 1991). Since then, considerable efforts have been made for determining the molecular basis for its enhancement of pathogenic tolerance. For last 5 years, fungal resistance genes were transferred to the several plants including various cultivars of tobacco (Terakawa et al., 1997), cucumber(Tabei et al., 1998) and barley(Jutidamrongphan et al., 1991).

Potato(*Solanum tuberosum* L.) is a major source of food in many parts of the world. In Korea, potato is the major crop, and its production has been effected due to diseases. Potatoes are frequently damaged by fungi from seedlings to tubers, resulting in not only decreasing the production but also deteriorating the quality. For this reasons much efforts have been devoted to the breeding of fungal resistant potato cultivar. However, potato cultivars, in general, are highly heterozygous which show significant difficulties to breeding programs. Recently, advances in genetic engineering have made it possible to introduce foreign genes into various plant species to improve their resistance beyond the limit of conventional breeding.

The aim of this study is to develop antifungal potato cultivar by using gene manipulation technology. Therefore, we tried to introduce chitinase gene into the potato genome by using leaf disc transformation mediated by *A. tumefaciens* MP90.

* Corresponding author, E-mail ; choikh@kribb4680.kribb.re.kr
Received Dec. 3, 1998 ; accepted Mar. 30, 1999

Materials and Methods

Plant material

Growing apices and auxillary buds from *in vitro* maintained plants of the cultivar, Belchip, was subcultured every two weeks in MS solidified medium without growth regulators. These plants were grown at 22±2℃ and exposed 16 h to 3000 Lux illumination. Under these conditions, leaf discs were excised by trimming their borders after 14 days.

Bacterial growth

The disarmed *Agrobacterium tumefaciens* MP 90(Koncz et al., 1986) carrying a binary vector pRD 400(Datla et al., 1992) were used to infect potato explants. This plasmid carries two genes that could be expressed in plant cells, such as antifungal gene (chitinase, Lamb et al., 1992), and neomycin phosphotransferase(*npt* II) gene which confers kanamycin resistance. *Agrobacterium* was obtained from Dr. Yang (Korea Ginseng & Tobacco Research Institute, Taejon) and was maintained on AB medium (An, 1987) containing the appropriate antibiotics: 50 μg/mL kanamycin and 25 μg/mL streptomycin.

Transformation and selection

Leaf discs were co-cultivated with *Agrobacterium* cells of exponential phase. Co-cultivation was carried out in an AB liquid medium containing antibiotics in Petri dishes for 10 minutes. Leaf discs were then dried on sterile filter paper to remove excess bacterial culture medium. For an effective transformation, the explants were placed in the co-culture medium containing 2 mg/L of 2,4-D. Two days later, the explants were transferred to the regeneration medium (MS medium, supplemented with 2.0 mg/L Zeatin, 0. 01 mg/L NAA and 0.1 mg/L GA_3, 3% sucrose at pH 6.0, solidified with 0.8% agar) supplemented with 100 μg/mL kanamycin for transformant selection and 500 μg/mL carbenicillin for *Agrobacterium* elimination. The explants were transferred to the regeneration medium every two weeks. Induced shoots were then transferred to the MS medium supplemented with 0.25 mg/L BA and 0.1 mg/L GA_3. Antibiotics were added as above.

Polymerase chain reaction for npt II *and chitinase genes*

Genomic DNA from 200 mg each of control(non-transgenic) plants and all putative kanamycin resistant plants were extracted from leaves as described by Edwards et al. (1991). The sequence information derived from chitinase, and designed degenerated oligonucleotides of corresponding genes from *S. tuberosum* genomic DNA for PCR amplification were utilized. The primer for the *npt* II gene fragment, 5'-GAGGCTATTCGGCTATGACTG-3' as the sense primer and 5'-ATCGGGAGCGGCGA TACCGTA-3' as the antisense primer were used for PCR screening of kanamycin resistant plants. The primers, 5'-GGAAG CTACGGAGAGCAGTG-3' as the sense primer and 5'-CCGTTGATGATGTTCGTCAC-3' as the antisense primer, were used to yield a 746 bp internal chitinase fragment. Gene Amp PCR system 9600(Perkin Elimer Cetus, Norwalk, USA) was used in a reaction mixture of 20 μL containing 0.1-0.5 μg of genomic potato DNA using a Pre-mix Top(Bioneer, Korea). Mineral oil were overlaid with the mixture, and heated to 94℃ for 5 min. The PCR was performed with 35 cycles, each consisting of 1 min at 94℃, 1 min at 60℃ and 1 min 30 s at 72℃. PCR products were electrophoresed in 1% agarose gel and detected by ultraviolet light.

Northern blot analysis

Total RNA was extracted by the Ultraspec™-II RNA isolation system(Bio Tecx, Houston, USA). Total RNA(30 μg) was fractionated by electrophoresis in 1.0% agarose gels at 4 V/cm in the presence of 2.2 M formaldehyde as a denaturant. After electrophoresis, the RNA was transferred to nylon membranes(Schleicher and Schuell, Keene, USA), and fixed by cross-linking in a UV Spectrolinker (Spectronics Corporation, New York, USA). The RNA gel-blot filter was added to the hybridization buffer and the hybridization reaction was carried out at 42℃. RNA blot analysis was performed with the DIG labeled insert of chitinase.

Assays for chitinase

About 500 mg of plant leaves were ground in liquid nitrogen with a mortor and pestle. The fine powder was extracted with 1 mL of 0.1M sodium citrate buffer(pH 5. 0). The extract was centrifuged at 6000×g for 10 min and the supernatant was recentrifuged at 12,000×g for 15 min. Protein concentrations were determined by the method of Lowry et al. (1951) with albumin as the standard. Chitinase activity was assayed spectrofluorometrically by the method of Watanabe et al. (1993) with 4-methylumbelliferyl-N,N′N″-triacetylchitotriose(4-Mu-$(GlcNAc)_3$) as an assay substrate. A reaction mixture contained, in 50 μL enzyme, 50 μL of 1M phosphate buffer(pH 6.0), 10 μL substrate and 390 μL distilled water. It was incubated for 1h at 37℃. The amount of 4-Mu released was measured using a Hoofer

Tko100 Fluorometer(Tokyo, Japan). All assays were performed in triplicate.

Results and Discussion

Transformation, regeneration and selection

Belchip cultivar of in vitro potato leaf discs were trimmed and co-cultivated with *A. tumefaciens* MP90 containing the binary vector for 10 min without agitation. Leaf discs were transferred to the selection medium(Figure 1A). After one week of culture, explants were enlarged twice. Calli formed on the cut edges of the explants were observed in the presence of 100 μg/mL kanamycin after three weeks of co-cultivation. Control leaf discs, that is, discs not infected with *Agrobacterium* became chlorotic and did not produce any calli on medium containing 100 μg/mL kanamycin. After placing these calli on fresh selection medium, many of them formed shoots within 6 weeks(Figure 1B). Putatively transformed shoots were excised when they were about 1 cm tall and transferred to shoot elongation medium containing carbenicillin and kanamycin(Figure 1C). Eleven shoots were selected in the presence of 100 μg/mL kanamycin. After this first selection, stem and leaf fragments from each excised shoot were taken

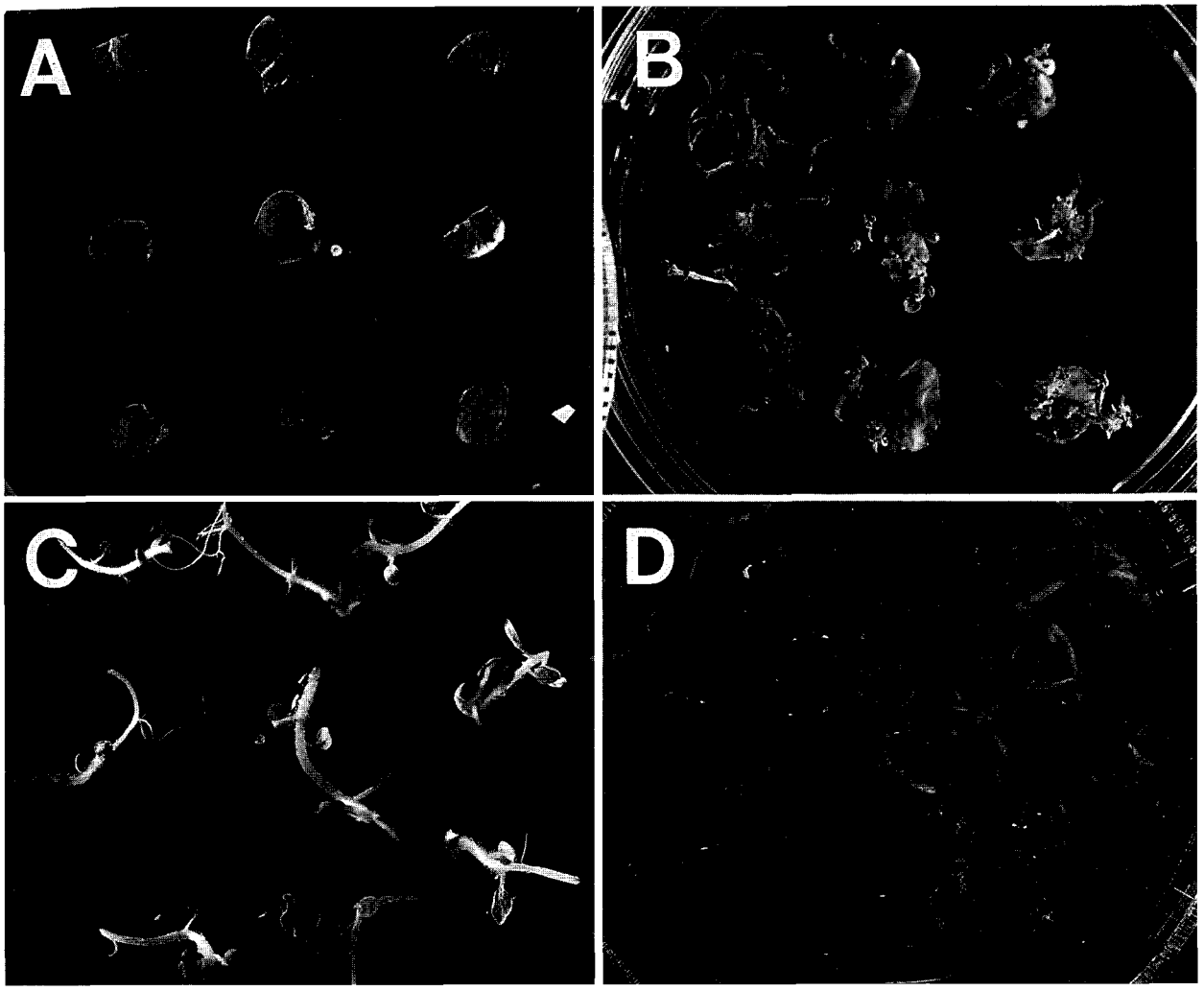

Figure 1. Shoot regeneration from *Solanum tuberosum* leaf discs. (A) Leaf discs were cultured on MS selection medium after co-cultivation with *Agrobacterium tumefaciens*. (B) Kanamycin resistant calli developed on leaf discs and numerous shoots formed on the explant. (C) Transformed plantlets transferred to medium containing 100 μg/mL kanamycin, 500 μg/mL carbenicillin, 0.25 mg/L BA and 0.1 mg/L GA. (D) Mature transformants in a propagation medium. The plants are vigorous and phenotypically normal.

for identification of transgenic plants. Rooted transformants were transferred to propagation medium after one to two weeks(Figure 1D).

Polymerase chain reaction analysis for selection of transgenic plants

The introduction of genes into transformed plant was demonstrated by PCR analysis. DNA amplification of all fifteen plants regenerated in the presence of kanamycin was analysed by PCR for the co-integration of the *npt* II (Figure 2A) and chitinase (Figure 2B). The expected 700bp *npt* II and 746bp chitinase gene bands were found in the transformants. Six among eleven km^r Belchip transformants showed introduction of *npt* II and chitinase genes.

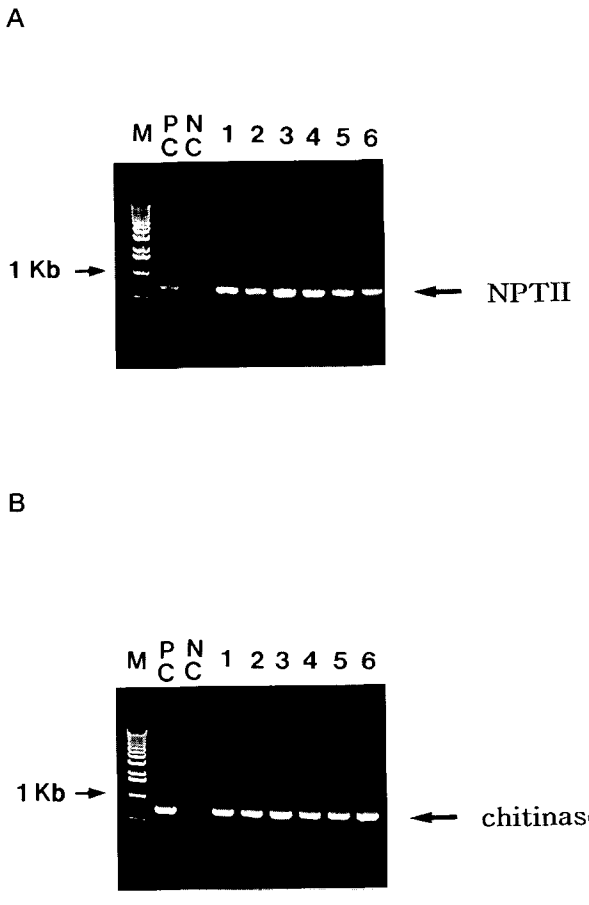

Figure 2. PCR detection of *npt* II (A) and chitinase(B) gene from genomic DNA in transgenic and control potato plants. M:1Kb size marker(Gibco BRL, Life Technologies, New York, USA), lane PC: Positive control(using cloned chitinase gene), lane NC: Non-transformed potato plant (cv. Belchip), lanes 1-6: Transgenic potato plants(cv. Belchip). The size of the amplified DNA fragments are 700 bp(*npt* II) and 746 bp(chitinase), respectivly.

Northern blot analysis

Transgenic potatoes producing chitinase mRNA transcripts were detected by northern blot analysis (Figure 3). No signal could be detected in the control potatoes(lane C). In the three lines of transgenic potatoes, similar levels of transcripts were observed from number 2 and number 3 lines, whereas lower level of transcripts were detected in number 1 line. Three other lines among six transformants that confirmed by PCR, showed dim signals, so it could be hardly detected from northern blot analysis (data not shown).

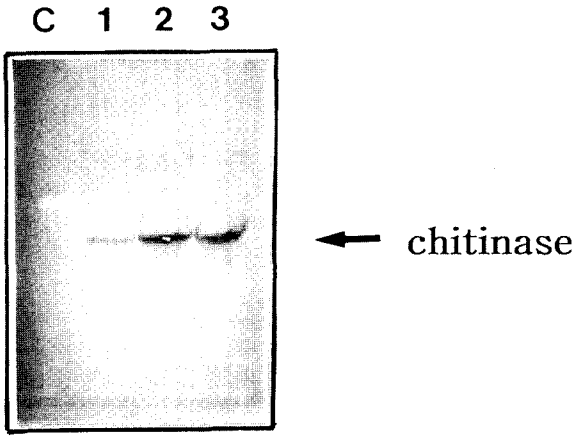

Figure 3. Northern blot hybridization of total RNA isolated from transgenic and control potato plants. Thirty micrograms of total RNA was electrophoresed, blotted and then hybridized with DIG labeled chitinase as a probe. Lane C, control potato; lanes 1-3, transgenic plants(cv. Belchip).

Assays for chitinase

Three transgenic potatoes that had been verified in a northern blot analysis were checked for chitinase activity by spectrofluorometer. Chitinase activity was shown both in transgenic and control potatoes but that of transgenic potatoes were higher than the control. Chitinase is generally found at low or basal levels in healthy plants, its expression is increased during pathogen attack. These results demonstrate that there is a substantial variation among independent transformants in the level of expression and potatoes containing the chimeric gene coding for chitinase lead to enhanced exochitinase activity in the transformed potatoes.

Table 1. Chitinase enzyme activity in transgenic potatoes measured spectrofluorometrically

Plant line[a]	Chitinase activity[b] (Fu)
Control	36.2±1.6[c]
T1	47.9±1.0
T2	56.7±1.5
T3	46.2±1.0

[a]Explants were transgenic line and control potatoes.
[b]Kinetic studies of control and transgenic chitinase were performed using 4-methylumbelliferyl-N, N′, N″-triacetylchitotriose(4-Mu-$(GlcNAc)_3$) as an assay substrate. The amount of 4-Mu released was measured. The results were means of 3 replicates each.
[c]Mean values standard error.

Recently, transgenic plants are used widely to study gene expression. For transformation of potato, tuber discs(Sheerman and Bevan, 1988; Stiekema et al., 1988), leaf discs(De Block, 1988; Ooms et al., 1987), stem segments of explants(Twell and Ooms, 1987) were used. In this experiment, leaf discs gave the most uniform and rapid response and produced the largest number of transgenic shoots compared to tuber discs and stem explants(data not shown). This result agree with Horsch et al. (1984). Therefore, the leaf discs have been successfully utilized to regenerate transgenic plants from potato. There is difference in shoot regeneration ability amongst different cultivars. According to our previous study (Choi et al., 1996), Desiree cultivar showed over 85% shoot regeneration frequency while Jaju showed no regeneration from leaf disc. In this study, Belchip cultivar showed 10% regeneration frequency. These results indicate that shoot regeneration ability is influenced by genotype. One et al. (1994) reported the same conclusion in *Brassica spp.* For Belchip cultivar, it may be necessary to attempt protocols to increase transformation efficiency.

In a historical event of the world potato cultivation, destruction of potato crops in nineteenth centry in Europe by the fungus *Phytophthora infestans* caused severe famine and emigration. Today, fungal disease still remains as one of the major factors limiting the crop productivity. Newly developed technologies in plant science such as gene transfer methods can be used to develop new cultivars. Through this technique, we were successful to produce transgenic potatoes that integrated an antifungal gene for protection against fungi. Among eleven putative transformants grown on 100 μg/mL kanamycin, six independently transformed lines were obtained that contained chitinase gene confirmed by PCR. However, only three lines were confirmed for chitinase gene expression by northern blot in which the other 3 lines seemed to show transgene inactivation. The way in which plants inactivate foreign DNA is unknown but several mechanisms such as gene silencing, co-suppression have been postulated(Schmulling and Rohrig, 1995). Researchers working with transgenic plants have been confronted persistently with problems such as weak(or no) expression of transgenes. The problem of transgene inactivation still remains as one of the major challenges faced for plant molecular biologists. A strong hybridizing signals were observed in three transformed lines. The data suggest that these three lines may have the ability of fungal resistance activity. However, nothing can be concluded with these transgenic lines in terms of antifungal resistance at present time. Therofore, further analysis is necessary to evaluate possible changes and the fungal resistance. Experiments are now in progress to assay for the tolerance of transgenic potatoes against fungus *Phytophthora infestans* exposure.

References

An GH (1987) Binary Ti vector for plant transformation and promoter analysis. Methods in Enzymology 153: 292-305

Choi KH, Jeon JH, Kim HS, Joung YH, Cho SJ, Lim YP, Joung H (1996) Development of herbicide-resistant transgenic potato. Korean J Plant Tissue Culture 23: 161-165

Datla RSS, Hammerlindl JK, Panchuk B, Pelcher LE, Keller W (1992) Modified binary plant transformation vectors with the wild type gene encoding NPT II. Gene 211: 383-384

De Block M (1988) Genotype-independent leaf disc transformation of potato(*Solanum tuberosum*) using *Agrobacterium tumefaciens*. Theor Appl Genet 76: 767-774

Edwards K, Johnston C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19: 1349

Horsch RB, Fraley RT, Rogers SG, Sanders PR, Lloyd A, Hoffmann N (1984) Inheritance of functional foreign genes in plants. Science 223: 496-498

Jutidamrongphan W, Andersen JB, Mackinnon G, Manners JM, Simpson RS, Scott KJ (1991) Induction of β-1,3-glucanase in barley in response to infection by fungal pathogens. Mol Plant-Microbe Interactions 4: 234-238

Koncz C, Schell J (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet 204: 383-396

Kurosaki F, Tashiro N, Nishi A (1987) Induction, purification and possible function of chitinase in cultured carrot cells. Physiol Mol Plant Path 31: 201-210

Lamb CJ, Ryals JA, Ward ER, Dixon RA (1992) Emerging strategies for enhancing crop resistance to microbial pathogens. Biotechnology 10: 1436-1445

Legrand M, Kauffmann S, Geoffroy P, Fritig B (1987) Biological function of pathogenesis-related proteins:Four tobacco pathogenesis related proteins are chitinase. Proc Natl Acad Sci USA 84: 6750-6754

Lois R, Schulz W (1989) A phenylalanine ammonia-lyase

gene from parsley: structure, regulation and identification of elicitor and light responsive cis-acting elements. The EMBO J 8: 1641-1648

Lowry OH, Roseborough HJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275

Ooms G, Burrel MM, Karp A, Bevan M, Hille J (1987) Genetic transformation in two potato cultivars with T-DNA from disarmed *Agrobacterium*. Theor Appl Genet 73: 744-750

One Y, Takahata Y, Kaizuma N (1994) Effect of genotype on shoot regeneration from cotyledonary explants of rapeseed(*Brassica napus* L.). Plant Cell Rep 14: 13-17

Schmulling T, Rohrig H (1995) Gene silencing in transgenic tobacco hybrids: frequency of the event and visualization of somatic inactivation pattern. Mol Gen Genet 249: 375-390

Sheerman S, Bevan MW (1988) A rapid transformation method for *Solanum tuberosum* using binary *Agrobacterium tumefaciens* vectors. Plant Cell Rep 7: 13-16

Sommer H, Saedler H (1986) Structure of the chalcone synthase gene of *Antirrhinum majus*. Mol Genet 202: 429-434

Somssich IE, Schmelzer E, Bollmann J, Hahlbrock K (1986) Rapid activation by fungal elicitor of genes encoding "pathogenesis - related" proteins in culture parsley cells. Proc Natl Acad Sci USA 83: 2427-2430

Stiekema WJ, Heidekamp F, Louwerse JD, Verhoeven HA, Dijkhuis P (1988) Introduction of foreign genes into potato cultivars Bintje and Desiree using an *Agrobacterium tumefaciens* binary vector. Plant Cell Rep 7: 47-50

Tabei Y, Kitade S, Nishizawa Y, Kikuchi N, Kayano T, Hibi T, Akutsu K (1998) Transgenic cucumber plants harboring a rice chitinase gene exhibit enhanced resistance to gray mold(*Botrytis cinerea*). Plant Cell Rep 17: 159-164

Terakawa T, Takaya N, Horiuchi H, Koike M, Takagi M (1997) A fungal chitinase gene from *Rhizopus oligosporus* confers antifungal activity to transgenic tobacco. Plant Cell Rep 16: 439-443

Twell O, Ooms G (1987) The 5 flanking DNA of a patatin gene directs tuber specific expression of a chimaeric gene in potato. Plant Mol Biol 9: 365-375

Ward EWB, Cahill D, Bhattacharyya MK (1989) Abscisic acid suppression of phenylalanine ammonia-lyase activity and mRNA and resistance of soybeans to *Phytophthora megasperma*. Plant Physiol 91: 23-27

Watanabe T, Kobori K, Miyashita K, Fujii T, Sakai H, Uchida M, Tanaka H (1993) Identification of glutamic acid 204 and aspcrtic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. J Biol Chem 268: 18567-18572

Wienand U, Weydemann U, Niesbach-Klosgen U, Peterson PA, Saedler H (1986) Molecular cloning of the c2 locus of *Zea mays*, the gene coding for chalcone synthase. Mol Gen Genet 203: 202-207

Yanai K, Takaya N, Kojlma N, Horiuchi H, Ohta A, Takagi M (1992) Purification of two chitinase from *Rhizopus oligosporus* and isolation and sequencing of the encoding genes. J Bacteriology 174: 7398-7406