

## Expression of a cucumber class III chitinase and *Nicotiana plumbaginifolia* class I glucanase genes in transgenic potato plants

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### Abstract

The genes encoding for a cucumber class III chitinase and *Nicotiana plumbaginifolia* class I glucanase were co-introduced into Slovak potato (*Solanum tuberosum* L.) breeding line 116/86 using *Agrobacterium tumefaciens*. For both transgenes the number of integrated copies and level of RNA expression were determined. These analyses demonstrated low variation and significant correlation in expression of the introduced transgenes. The effect of transgene expression on fungal susceptibility of transformants was evaluated *in vitro*. Hyphal extension assays revealed no obvious differences in the ability of extracts from transformants to inhibit growth of *Rhizoctonia solani* comparing to non-transformed potato.

### Introduction

Elucidation of the cytological, physiological and molecular background of plant-pathogen interactions has great importance and provides the basis for the generation of new disease resistant crop varieties. The processes occurring in plants after pathogen attack are being extensively studied. The best-characterised component of plant defence is the group of so-called pathogenesis-related (PR) proteins (Meins et al., 1993) comprising five subgroups (PR1–PR5) with different functions and activities. The glucanhydrolases, chitinases (PR-3) and  $\beta$ -1,3-glucanases (PR-2) catalyse the hydrolysis of the glucan and chitin polysaccharides that represent the major components of many fungal cell walls. By this way, they are capable of inhibiting the growth and spreading of the pathogen. Many of these enzymes have been shown to be active

against various fungi *in vitro* (Honée, 1999). In plants, at least five classes (I–V) of chitinases and three classes (I–III) of  $\beta$ -1,3-endoglucanases have been identified (Melchers et al., 1993; Neuhaus et al., 1996). For both enzymes, their capacity to inhibit fungal growth varies considerably among different isoforms (Kombrink and Somssich, 1995). Generally, vacuolar glucanhydrolases of class I were shown to be potent inhibitors of fungal growth both *in vitro* and *in vivo*, and their anti-fungal effect was shown to be synergistic (Sela-Buurlage et al., 1993; Jongedijk et al., 1995). In contrast, intercellular glucanhydrolases of class II exhibited limited or no antifungal activity (Sela-Buurlage et al., 1993). Whether the class III glucanhydrolases contribute to plant defence response is not clear yet, since alone they are not fungistatic *in vitro* (Ji and Kuć, 1996). However, they are significantly induced by treatment with fungal

elicitors or wounding (Kim et al., 1999; Rojas-Herrera and Loyola-Vargas, 2002).

In view of the expected synergy between glucanases and chitinases, a combination of tobacco glucanase and cucumber chitinase was introduced into potato. The tobacco  $\beta$ -1,3-glucanase (*gn1*) gene of class I has been isolated from *Nicotiana plumbaginifolia* (De Loose et al., 1988). Studies on transgenic plants containing the *gn1* promoter fused to the  $\beta$ -glucuronidase reporter gene revealed increased gene expression during hypersensitive reaction to pathogen attack (Ashfield et al., 1994; Alonso et al., 1995). The chimeric *gn1* gene has been successfully introduced and expressed in tobacco plants (De Carvalho et al., 1992; Libantová et al., 1998) but the GN1 protein has not been tested for its antifungal potential neither *in vitro* nor *in vivo*.

The cucumber chitinase gene encodes an acidic, extracellular chitinase of class III. It occurs in the cucumber genome as three closely linked genes, only one of which appears active. The activity of this gene increases upon infection with tobacco mosaic virus or fungal pathogen (Mètraux et al., 1989; Zhang and Punja, 1994). The role of this gene in systematic acquired resistance (SAR) has also been proved (Kubota and Abiko, 2000). However, there are only limited reports of the expression of this gene in transgenic plants (Vierheilig et al., 1995; Bauer et al., 1998). Tobacco plants transformed with cucumber chitinase gene under the control of cauliflower mosaic virus (CaMV) 35S promoter showed only a slightly increased total chitinase activity in roots and its expression did not interfere with the development of symbiotic fungi (Vierheilig et al., 1995).

In this work the cucumber class III chitinase and *Nicotiana plumbaginifolia*  $\beta$ -1,3-class I glucanase genes were introduced into fungus-sensitive Slovak potato line 116/86 by *Agrobacterium*-mediated transformation. The glucanase genomic and chitinase cDNA clones were fused to the double CaMV 35S promoter and placed on the one binary vector. In order to achieve correlating expression, plant MARs (Matrix Attachment Regions) were placed at the T-DNA border. The presence of MARs can help to overcome the problem with highly variable and poorly correlating expression of co-introduced genes (Mlynárová et al., 2002). The effect of introduced genes on fungal susceptibility of transformants was analysed *in vitro*.

Crude plant protein extracts were assayed for their ability to inhibit spreading of fungus *Rhizoctonia solani* in *in vitro* assays. This is the first time that the co-expression of class I glucanase and class III chitinase has been studied in transgenic potato plants.

## Materials and methods

### Vector for plant transformation

DNA manipulations were carried out as described by Sambrook et al. (1989). The chimeric chitinase and glucanase were constructed by transcriptional fusion of a cDNA clone encoding a cucumber chitinase of class III (Mètraux et al., 1989) and a genomic clone encoding for a *Nicotiana plumbaginifolia* glucanase of class I (De Loose et al., 1988) with the double enhanced dCaMV 35S promoter (Mlynárová et al., 1995). The chitinase gene was linked to the nopaline synthase (*nos*) transcription termination signal, whereas  $\beta$ -1,3-glucanase contained its natural transcription termination signal. To obtain plant transformation vector pIL12 (Figure 1) the appropriate restriction fragments of both genes were cloned into the variant of the binary vector pBINPLUS (Van Engelen et al.,

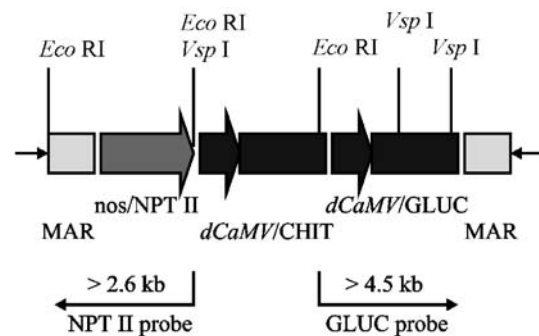


Figure 1. The T-DNA region of the binary vector pIL12. The gene for chitinase (CHIT) and glucanase (GLUC) are driven by double CaMV 35S promoter (*dCaMV*), and the gene for neomycin phosphotransferase (NPT II) by nopaline synthase promoter (*nos*). The transgene cassette is embedded by matrix-associated regions (MAR). Positions of the *Eco* RI and *Vsp* I enzymes recognition sites are indicated as well the size of hybridisation signals when the plant DNA is hybridised to GLUC and NPT II gene fragments as to probes. Transgenic plants are expected to contain a right border (RB) fragment of size at least 2.6 kb (using NPT II probe) and at least 4.5 kb left border (LB) fragment (using GLUC probe).

1995) together with the *nos* promoter driven NPTII gene. At the borders of T-DNA a plant matrix associated regions (MARs) (Nap, unpublished) were placed.

The recombinant binary vector pIL12 was conjugated to *Agrobacterium tumefaciens* LBA 4404 (Hoekema et al., 1983) in a biparental mating (Simon et al., 1983) using *Escherichia coli* S 17.1 as a donor strain.

#### Potato transformation and regeneration

*In vitro* plantlets of potato line 116/86 (*Solanum tuberosum* L.) were provided by The Potato Research and Breeding Institute, Veľká Lomnica, Slovak Republic. The plantlets were cultivated at  $20 \pm 2$  °C with a day length of 16 h under  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity. Shoots were subcultured monthly as a nodal segments on basal MS medium (Murashige and Skoog, 1962) with 2% (w/v) sucrose and 0.8% (w/v) agar. Leaf segments of 4–6 weeks old plantlets were transformed with *Agrobacterium* inoculum according to Moravčíková et al. (2003).

#### Southern hybridisation

DNA was isolated from 1.5 g of leaf material using a urea-phenol extraction procedure as described by Mlynárová et al. (1995). Total DNA (10  $\mu\text{g}$ ) was digested with the restriction enzyme *Vsp* I (in case of NPT II probe) or *Eco* RI (GLUC probe), separated on 0.8% (w/v) agarose gel and blotted onto a Hybond N<sup>+</sup> membrane (Amersham). The restriction fragment of 1.4 kb of NPT II gene and the 2 kb fragment of glucanase gene were radioactively labelled (Amersham Megaprime DNA labelling Kit) and used as probes. The hybridisation was performed in hybridisation solution containing 10% (w/v) dextran sulfate, 1% (w/v) SDS, 1 mol l<sup>-1</sup> NaCl and 100  $\mu\text{g ml}^{-1}$  of salmon sperm DNA at 65 °C. Hybridisation signals were visualised by autoradiography using a BAS2000 PhosphorImager (Fuji).

#### Northern hybridisation

Total RNA was extracted from potato leaves as described by Békésiová et al. (1999). The samples (10  $\mu\text{g}$  of RNA per line) were separated on 1.5% (w/v) formaldehyde agarose gel and blotted onto a

Hybond N<sup>+</sup> membrane (Amersham). The 0.7 kb restriction fragment of *gnl* gene, 1.1 kb fragment of the cucumber chitinase gene and 0.8 kb fragment of the tobacco tubulin gene were radioactively labelled (Amersham Megaprime DNA labelling Kit) and used as probes.

The hybridisation was performed as described above at 60 °C. Hybridisation signals were quantified by Phosphor imaging, using a FUJI BAS2000 Phosphorimager with BasReader and TINA software. Signals were expressed as pixels per mm<sup>2</sup>. Those for chitinase and glucanase genes were normalised for the amount of RNA loaded relative to the tubulin signal.

#### Antifungal assays

The phytopathogenic fungus *Rhizoctonia solani* was obtained from The Potato Research and Breeding Institute, Veľká Lomnica, Slovak Republic. Fungi were cultured on Czapek-Dox agar (Duchefa) at 21 °C in the dark.

Crude protein extracts were isolated from leaves of *in vitro* grown plants under sterile conditions. The extraction buffer contained 0.05 mol l<sup>-1</sup> sodium acetate (pH 5.2) and 0.02% (v/v)  $\beta$ -mercaptoethanol. Plant tissue (0.5 g) was ground in a mortar using liquid nitrogen, transferred into extraction buffer and homogenised by vortexing. Insoluble material was removed from the homogenate by centrifugation at 14,000 rpm at 4 °C for 15 min. The supernatant was removed, centrifuged at 14,000 rpm at 4 °C for 10 min. Protein concentration was determined according to Bradford (1976). A square (1 cm<sup>2</sup>) of agar with growing fungus was placed into the middle of a Petri dish ( $\varnothing$  90 mm) with Czapek-Dox agar. When the fungal mycelium area reached a diameter of 3–5 cm, sterile filter paper discs of Whatmann 3MM ( $\varnothing$  50 mm) were positioned adjacent to the fungal colony margin. The discs were saturated with 100 and 150  $\mu\text{g}$  (50  $\mu\text{l}$ ) of plant crude protein extracts (the amount optimised in preliminary experiments). Sterile extraction buffer and/or bovine serum albumin (BSA) were used as controls. The plates were incubated at 21 °C in the dark. After 15–24 h the ability of extracts to inhibit fungal growth around discs were scored and evaluated visually by comparing with controls. All experiments were repeated at least 3 times and data were pooled.

## Results and discussion

In this work the set of transgenic plants with over-expressed *Nicotiana plumbaginifolia* class I glucanase and a cucumber class III chitinase genes was obtained by *Agrobacterium*-mediated transformation with binary vector IL12 (Figure 1). The transgenic nature of transformants was studied.

In 15 out of 16 different transgenic plants the presence of T-DNA region was analysed by hybridisation using left border (GLUC gene) and right border (NPT II gene) fragments as probes (Figure 2). The data are summarised in Table 1. In most of the transformants the presence of one or two glucanase (GLUC) transgene copies was proven. Two plants did not carry GLUC transgene, and based on mRNA expression data they probably lack also the CHIT transgene (Table 1). The number of NPT II gene copies varied from one to five and was higher (except plants T11, T12) than the number of GLUC copies in the same transgenic plants. Such unequal number of left and right border T-DNA fragments in transformed genomes is observed relatively frequently and it is a result of processes comprising transfer and integration of T-DNA (Zupan et al., 2000).

More copies of the transgene have been previously proposed to result also in higher gene expression level. However, many authors have shown that multiple genes can be silenced and the expression levels may vary widely among individual transformants (Fagard and Vaucheret, 2000). We have studied the expression of intro-

Table 1. RNA data, transgene copy number and fungal resistance tests of potato 116/86(IL12)

Plants <sup>a</sup>	Natural logarithm <sup>b</sup>		Copy number <sup>c</sup>	
	Ln (GLUC/ TUB)	Ln (CHIT/ TUB)	NPT II	GLUC
T1	3.39	4.20	3	2
T2	1.94	0.79	1	0
T3	1.49	0.48	1	0
T4	3.25	3.59	4	2
T5	2.37	3.38	2	1
T6	3.60	3.44	3	2
T7	4.08	4.44	3	2
T8	3.69	4.22	4	1
T9	2.53	2.84	2	1
T10	2.97	3.85	2	1
T11	2.55	3.34	1	2
T12	2.94	3.64	1	1
T13	5.11	1.99	5	2
T14	4.98	2.13	4	3
T15	2.48	3.27	nd	nd
T16	2.95	2.11	4	2
C	1.44	1.49	0	0

<sup>a</sup> Transgenic plants tested (T1-T16), (K) – non-transformed control 116/86.

<sup>b</sup> Natural logarithms of glucanase (GLUC) and chitinase (CHIT) hybridisation signals normalised with respect to the tubulin signal.

<sup>c</sup> Transgene copy number determined by DNA blot analyses; nd – not-determined.

duced glucanase and chitinase genes in transgenic plants by quantification of corresponding mRNA

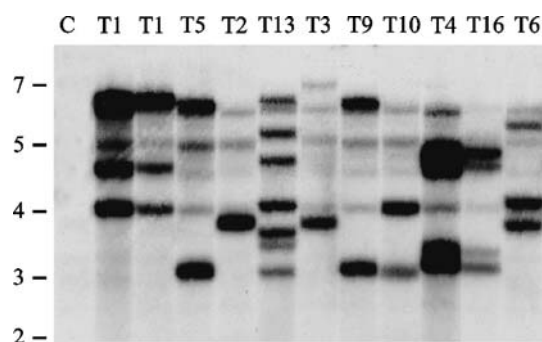


Figure 2. Autoradiograph of Southern blot with *VspI*-digested DNA of transgenic plants 116/86(IL12) (lane 2-11) and non-transformed control 116/86 (C). The blot was hybridized with a 1.4 kb <sup>32</sup>P-labelled fragment containing the NPTII gene as the probe. The numbers on the left indicate the positions of size markers in kb.

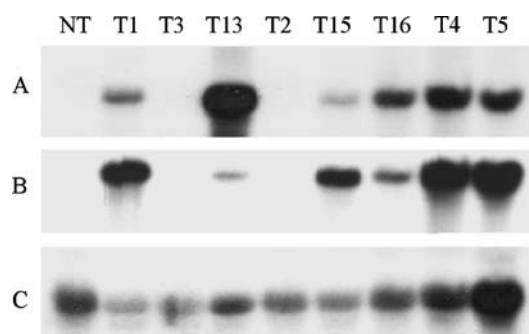


Figure 3. Autoradiograph of Northern blot with total RNA extracted from leaves of some transgenic plants 116/86(IL12) (lanes 2-9 marked with T) and non-transformed control plant (lane 1 marked with NT). The blot was hybridised with <sup>32</sup>P-labelled DNA fragments encoding *Nicotiana plumbaginifolia* glucanase (A), cucumber chitinase (B) and potato tubulin (C).

(Figure 3, Table 1). Correlation analysis indicated that levels of gene expression in case of GLUC transgene did not depend on the number of integrated transgene copies (at  $p < 0.05$ ), as it was observed by others (Allen et al., 2000). In 2 out of 16 transformants (T2, T3) the mRNA hybridisation signals for both GLUC and CHIT transgenes were comparable on the background level of untransformed control. Indeed, in these plants only NPT II gene was detected by Southern analysis (Table 1). As mentioned earlier, some defects during transformation process might have occurred. Two transformants (T13, T14) showed large differences between chitinase and glucanase expression level. Because the plants contained the GLUC and CHIT genes according to Southern analyses, there was no biological reason to exclude them from further analysis. However, they were very influential (correlation coefficient  $-0.29$ ), and we have omitted them from statistical analyses. In the remaining twelve plants the expression levels of both GLUC and CHIT transgenes were correlating (correlation coefficient  $0.62$ ,  $p < 0.05$ ) (Figure 4). Generally, no such correlation between nearby transgenes, though driven by the same (CaMV 35S) promoter, has been observed (Jach et al., 1995; Jongedijk et al., 1995).

The quasi-constitutive expression of CaMV 35S promoter in transgenic plants has made this promoter the choice for many biotech applications. On the other hand, its high level of expression (especially when contains enhancing

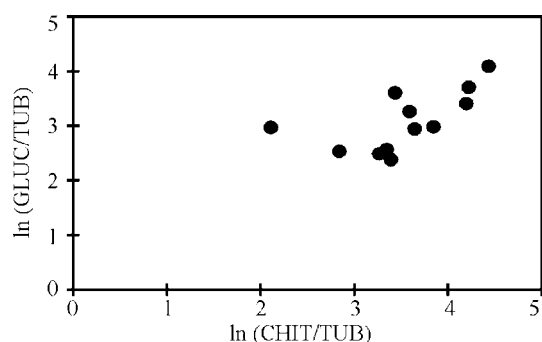


Figure 4. The relationship between expression levels of glucanase (GLUC) and chitinase (CHIT) transgenes. The natural logarithms (ln) of glucanase hybridisation signals in transformed plants are plotted against the corresponding natural logarithms (ln) of chitinase hybridisation signals. All hybridisation signal values were normalised with respect to the tubulin (TUB) signals.

sequences) is often associated with gene silencing (Covey and Al-Kaff, 2000). In our population of transgenic plants, no significant differences in GLUC and CHIT expression levels depending on the number of transgene copies integrated were observed. It appears that the presence of two enhanced CaMV promoters in a single T-DNA did not increase the incidence of gene silencing. The coordinated transgene(s) expression in population of 116/86(IL12) was expected as a consequence of MAR elements at the T-DNA borders (Mlynárová et al., 2002). It has been shown that the MAR elements can contribute to more stable expression of transgenes in transgenic plants. In addition, the close linkage between genes of interest on the same binary vector could also support the correlating expression of transgenes (Allen et al., 2000). Results of others show that when multiple genes of interests are combined, but not embedded by MAR(s), the expression levels are highly variable and poorly correlating (Jach et al., 1995; Jongedijk et al., 1995; Leech et al., 1998). So far, there are limited data on coordinated expression of two plant transgenes linked on a single MAR delimited chromatin loop. Dean et al. (1988a, b) showed a reasonable coordinated expression (quantified as RNA) when transgenes were flanked with large similar, but not identical, genomic regions of well expressed petunia *rbcS* gene regions and *rbcS* promoters. This may have been due to the presence of MAR elements in those regions of petunia DNA. Similarly, in plants carrying chicken lysozyme A elements, mRNA levels of both  $\beta$ -glucuronidase and firefly luciferase genes, driven by two different promoters, showed high correlation (Mlynárová et al., 2002). However, the presence of MAR does not necessarily protect against the influence of strong silencing loci (Vaucheret et al., 1998). This may have occurred also in transformants T13 and T14.

Correlating expression between cucumber chitinase and tobacco glucanase genes may support the synergistic activity of transgene products in transgenic plants. Consequently it can lead to more effective limitation of fungal growth. To test this, the crude protein extracts from leaves of transformants were examined in hyphal extension assays with the fungus *Rhizoctonia solani*. The 100 and 150  $\mu$ g of protein extracts of individual transgenic plants of line 116/86(IL12) and non-transformed plants 116/86 were applied on sterile

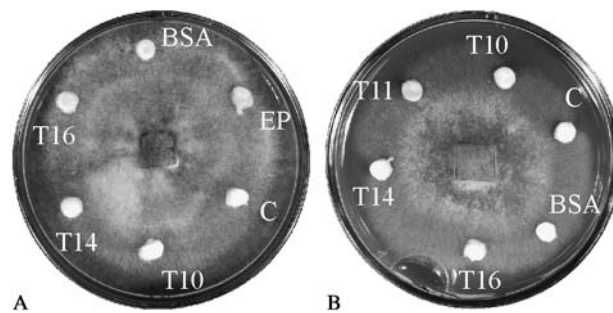


Figure 5. The effect of crude protein extracts from the leaves of potato on the growth of *Rhizoctonia solani*. Crude protein extracts from transgenic plants (T10, T11, T14, T16) and nontransformed plants (C) were applied in amount of 100  $\mu\text{g}$  (A) and 150  $\mu\text{g}$  (B) on sterile filter paper discs. Extraction buffer (EB) and BSA (100 and 150  $\mu\text{g}$ ) were used as negative controls.

filter paper discs. BSA and extraction buffer were used as negative controls (Figure 5). The results showed that there was no obvious difference in antifungal activity of leaf extracts from transgenic plants compared to extracts from untransformed control plant. Similar results were observed with fungi *Fusarium sambucinum* and *Fusarium oxysporum* spp. *lini* (data not shown). This suggests that the co-introduction of a tobacco glucanase and cucumber chitinase genes did not significantly contribute to antifungal properties of crude protein extracts from transformed potato plants. There are several possible explanations for the unaltered antifungal activity of crude extracts from potato transformants. The products of transgenes are not sufficiently toxic to fungus tested. Another fungi could be much more susceptible. Further, effective concentration of proteins desired is much lower in crude extracts than would be required. Ji and Kuć (1996) showed, that purified  $\beta$ -1,3 glucanase and chitinase isolated from pathogen-induced cucumber plants inhibited fungal growth more effectively than the compatible amount of these enzymes present in crude intercellular wash fluid. Since crude protein extract is a mixture containing many compounds, it is possible that some of such compound(s) can act as a nutrient or factor benefiting the fungus and negating the role of the two enzymes. Therefore the positive effect of both transgene products together with another defence proteins might be masked in inhibiting of fungal growth under our test conditions. It should also be realised, that the results of *in vitro* fungal tests with plant extracts are not always relevant for the *in planta* situation (Paxton et al., 1991).

In summary, transgenic potato plants carrying *Nicotiana plumbaginifolia* class I glucanase and a cucumber class III chitinase genes were obtained with low variability and coordinated expression of transgenes. The over-expression of these PR genes did not result in changed sensitivity of transformed potato plant extracts against fungi tested in *in vitro* tests. However, greenhouse or field trials may provide much more relevant conclusions on the antifungal properties of transgenic plants with respect to introduced PR genes.

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#### References

- Allen G, Spiker S & Thompson W (2000) Use of matrix attachment regions (MARs) to minimize transgene silencing. *Plant Mol. Biol.* 43: 241–256

- Alonso E, De Carvalho NF, Obregon P, Cheysen G, Inze D, Van Montagu M & Castresana C (1995) Differential DNA binding activity to a promoter elements of the *gn1* beta-1,3-glucanase gene in hypersensitivity reacting tobacco plants. *Plant J.* 7: 309–320
- Ashfield T, Hammond-Kosack KE, Harrison K & Jones JDG (1994) *Cf* gene-dependent induction of a  $\beta$ -1,3-glucanase promoter in tomato plants infected with *Cladosporium fulvum*. *Mol. Plant-Microbe Interact.* 7: 645–657
- Bauer M, Libantová J, Moravčíková J & Békésiová I (1998) Transgenic tobacco plants constitutively expressing acidic chitinase from cucumber. *Biologia* 53: 749–758
- Békésiová I, Nap JP & Mlynárová L (1999) Isolation of high quality DNA and RNA from leaves of the carnivorous plant *Drosera rotundifolia*. *Plant Mol. Biol. Rep.* 17: 267–277
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254
- Covey SN & Al-Kaff NS (2000) Plant DNA viruses and gene silencing. *Plant Mol. Biol.* 43: 307–322
- De Carvalho F, Gheysen G, Kushnir S, Van Montagu, Inzé D & Castresana C (1992) Suppression of beta-1,3-glucanase transgene expression in homozygous plants. *EMBO J.* 11: 2595–2602
- De Loose M, Alliotte T, Gheysen G, Genetello C, Gielen J, Soetaert P, Van Montagu M & Inze D (1988) Primary structure of a hormonally regulated  $\beta$ -1,3-glucanase of *Nicotiana plumbaginifolia*. *Gene* 70: 13–23
- Dean C, Favreau M, Tanmaki S, Bond-Nutter D, Dunsmuir P & Bedbrook J (1988a) Expression of tandem gene fusions in transgenic tobacco plants. *Nucleic Acids Res.* 16: 7601–7617
- Dean C, Jones J, Favreau M, Dunsmuir P & Bedbrook J (1988b) Influences of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. *Nucleic Acids Res.* 16: 9267–9283
- Fagard M & Vaucheret H (2000) Systemic silencing signal(s). *Plant Mol. Biol.* 43: 285–293
- Hoekema A, Hirsch PR, Hooykaas PJJ & Schilperoort RA (1983) A binary plant vector strategy based on separation of *vir* and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* 30: 179–184
- Honée G (1999) Engineered resistance against plant pathogens. *Europ. J. Plant Pathol.* 105: 319–336
- Jach G, Görnhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J & Maas C (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *Plant J.* 8: 97–109
- Ji C & Kuć J (1996) Antifungal activity of cucumber  $\beta$ -1,3-glucanase and chitinase. *Physiol. Mol. Plant Pathol.* 49: 257–265
- Jongedijk E, Tigelaar H, Van Roekel JSC, Bres-Vloemans SA, Dekker I, Van Den Elzen PJM, Cornelissen BJC & Melchers L (1995) Synergistic activity of chitinases and  $\beta$ -1,3-glucanases enhances fungal resistance in transgenic tomato plants. *Euphytica* 85: 173–180
- Kim MG, Lee KO, Cheong NE, Choi YO, Jeong JH, Cho MJ, Kim SCH & Lee SY (1999) Molecular cloning and characterization of class III chitinase in pumpkin leaves, which strongly binds to regenerated chitin affinity gel. *Plant Sci.* 147: 157–163
- Kombrink E & Somssich IE (1995) Defence responses of plants to pathogens. *Adv. Bot. Res.* 21: 2–26
- Kubota M & Abiko K (2000) Induced resistance in cucumbers against *Fusarium oxysporum* f. sp. *cucumerinum* and *Rhizoctonia solani* AG/2 by infection of the cotyledons. *J. Phytopat.* 149: 297–300
- Leech MJ, May K, Hallard D, Verpoorte R, De Luca V & Christou P (1998) Expression of two constitutive genes of a secondary metabolic pathway in transgenic tobacco: molecular diversity influences levels of expression and product accumulation. *Plant Mol. Biol.* 38: 765–774
- Libantová J, Bauer M, Mlynárová L, Moravčíková J & Békésiová I (1998) Transgenic tobacco and potato plants expressing basic vacuolar  $\beta$ -1,3-glucanase from *Nicotiana plumbaginifolia*. *Biologia* 53: 739–748
- Meins FJR, Neuhaus JM, Sperisen C & Ryals J (1993) The primary structure of plant pathogenesis-related glucanohydrolases and their genes. In: Fritig B & Legrand M (eds) *Mechanisms of Plant Defense Responses* (pp. 245–273). Kluwer Academic Publishers, Dordrecht
- Melchers LS, Ponstein AS, Sela-Buurlage MB, Vloemans SA & Cornelissen BJC (1993) *In vitro* antimicrobial activities of defense proteins and biotechnology. In: Fritig B & Legrand M (eds) *Mechanisms of Plant Defense Responses* (pp. 401–410). Kluwer Academic Publishers, Dordrecht
- Métraux JP, Burkhardt W, Moyer M, Dichner S, Middlesteadt W, Williams S, Payne G, Carnes M & Ryals J (1989) Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. *Proc. Natl. Acad. Sci. USA* 86: 896–900
- Mlynárová L, Jansen RC, Conner AJ, Stiekema WJ & Nap JP (1995) The MAR-mediated reduction in position effect can be uncoupled from copy number-dependent expression in transgenic plants. *Plant Cell* 7: 599–609
- Mlynárová L, Loonen A, Mietkiewska E, Jansen RC & Nap JP (2002) Assembly of two transgenes in an artificial chromatin domain gives highly coordinated expression in tobacco. *Genetics* 160: 727–740
- Moravčíková J, Libantová J, Matušíková, Libiaková G, Nap JP & Mlynárová L (2003) Genetic transformation of Slovak cultivar of potato (*Solanum tuberosum* L.): efficiency and the behaviour of the transgene. *Biologia* 6, in press
- Murashige T & Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plantarum* 15: 473–497
- Neuhaus JM, Fritig B, Linthorst HJM, Meins F, Mikkelsen JD & Ryals J (1996) A revised nomenclature for chitinase genes. *Plant Mol. Biol. Rep.* 14: 102–104
- Paxton DJ (1991) Assays for antifungal activity. *Meth. Plant Biochem.* 6: 33–46
- Rojas-Herrera R & Loyola-Vargas VM (2002) Induction of class III chitinase in foliar explants of *Coffea arabica* L. during somatic embryogenesis and wounding. *Plant Sci.* 4: 705–711
- Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York
- Sela-Buurlage MB, Ponstein AS, Bres-Vloemans SA, Melchers LS, Van Den Elzen PJM & Cornelissen BJC (1993) Only specific tobacco (*Nicotiana tabacum*) chitinase and  $\beta$ -1,3-glucanase exhibit antifungal activity. *Plant Physiol.* 101: 857–863

- Simon R, Priefer U & Pühler A (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Tech.* 1: 784–791
- Van Engelen A, Molthoff JW, Conner AJ, Nap JP, Pereira A & Stiekema WJ (1995) pBINPLUS: an improved plant transformation vector based on pBIN19. *Transg. Res.* 4: 288–290
- Vaucheret H, Elmayan T, Thierry D, Van der Geest A, Hall T, Conner AJ, Mlynarova L & Nap JP (1998) Flank matrix regions (MARS) from chicken, bean, yeast or tobacco do not prevent homology dependent trans-silencing in transgenic tobacco plants. *Mol. Gen. Genet.* 259: 388–392
- Vierheilig H, Alt M, Lange J, Gut-Rella M, Wiemken A & Boller T (1995) Colonization of transgenic tobacco constitutively expressing pathogenesis-related proteins by the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. *Envir. Microbiol.* 8: 3031–3034
- Zhang YY & Punja ZK (1994) Induction and characterization of chitinase isoforms in cucumber (*Cucumis sativum* L.) effect of elicitors, wounding and pathogen inoculation. *Plant Sci.* 2: 141–150
- Zupan J, Muth TR, Draper O & Zambryski P (2000) The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *Plant J.* 23: 11–28