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Summary

Simultaneous expression of a tobacco class I chitinase and a class I β-1,3-glucanase gene in tomato resulted in increased fungal resistance, whereas transgenic tomato plants expressing either one of these genes were not protected against fungal infection. After infection with *Fusarium oxysporum* f.sp. *lycopersici*, a 36% to 58% reduction in disease severity was observed in resistant tomato lines. Two transgenic lines largely recovered from the initial infection by the time wild-type tomato plants had died.

The overall results are consistent with the observation that class I chitinases and class I β-1,3-glucanases synergistically inhibit the growth of fungi *in vitro* and provide the first experimental support to the hypothesis that such synergy can contribute to enhanced fungal resistance *in planta*.

Introduction

Most agricultural and horticultural crop species suffer from a vast array of fungal diseases which cause severe yield losses all over the world. In addition to polygenes which confer only moderate levels of partial resistance against specific fungi, single dominant genes providing immunity to specific races of fungi have been identified for many crop species (Poehlman, 1987; Kalloo & Bergh, 1993). Although such race-specific resistance genes are often easily overcome by rapidly evolving new fungal races, today, continuous accumulation of race-specific resistance genes in commercial varieties, remains the major method of achieving sufficient resistance to a wide range of fungal pathogens.

Agronomically-viable levels of durable resistance in crop plants against a relatively broad range of fungi might be achieved by recently described molecular approaches, which include expression of fungal avirulence genes to provoke non-specific, hypersensitive resistance when combined with the corresponding plant resistance genes, expression of genes involved in the synthesis of phytoalexins toxic to fungi, and expression of genes encoding inhibitors of fungal enzymes or known antifungal proteins (reviewed by Lamb et al., 1992; Cornelissen & Melchers, 1993; Strittmatter & Wegener, 1993). The latter include chitinases and β-1,3-glucanases. These hydrolytic enzymes catalyze the degradation of chitin and β-1,3-glucan and, since these compounds are abundantly present in the cell wall of many filamentous fungi (Wessels & Sietsma, 1981), they are thought to be capable of inhibiting fungal growth *in planta*.

In plants, five classes (I–V) of endochitinases and three classes (I–III) of β-1,3-endoglucanases have been identified (Ward et al., 1991; Collinge et al., 1993; Melchers et al., 1994). In contrast to the intercellular class II isoforms, the vacuolar class I chitinases and class I β-1,3-glucanases have been shown to be potent inhibitors of fungal growth and to act synergistically *in vitro* (Mauch et al., 1988; Leah et al., 1991; Sela-Buurlage et al., 1993). Such synergy has also been demonstrated to occur between the intracellular class V chitinases and class I β-1,3-glucanases (Melchers

et al., 1994) and between a recently purified chitin-binding protein and both the class I β-1,3-glucanases and class I chitinases (Ponstein et al., 1993). Class III chitinases seem to lack antifungal activity (Vogelsang & Barz, 1993). Whether the class IV hydrolases inhibit fungal growth *in vitro* is not yet known.

In planta, increased tolerance to fungal infection has recently been observed in transgenic tobacco plants expressing a barley ribosome-inhibiting protein gene (Logemann et al., 1992), a groundnut stilbene synthase gene (Hain et al., 1993) or a tobacco gene encoding the pathogenesis-related protein PR-1a (Alexander et al., 1993). In the case of chitinases and β-1,3-glucanases, *in planta* resistance data are limited to increased tolerance to infection by *Rhizoctonia solani* in transgenic tobacco plants, which constitutively expressed a bean class I chitinase gene (Broglie et al., 1991) or an exochitinase gene of bacterial origin (Logemann et al., 1993). In contrast, transgenic tobacco plants expressing a tobacco class I chitinase gene did not show enhanced resistance to infection with the fungus *Cercospora nicotianae* (Neuhaus et al., 1991), despite the fact that the fungus proved very sensitive to chitinases *in vitro*.

In our attempts to increase fungal resistance in tomato by introducing multiple genes encoding class I and class II endochitinases and β-1,3-endoglucanases, we found that transgenic tomato plants constitutively expressing both a tobacco class I chitinase and a tobacco class I β-1,3-glucanase gene showed substantially enhanced resistance against infection with *Fusarium oxysporum* f.sp. *lycopersici*, whereas transgenic tomato plants constitutively expressing either one of these genes are not significantly protected.

Materials and methods

Gene constructs and tomato transformation

Chimeric endochitinase and β-1,3-endoglucanase genes were constructed by transcriptional fusion of a cDNA clone encoding a 32 kD class I endochitinase (Chi-I) and genomic clones encoding a 28 kD class II endochitinase (Chi-II, PR-3a), a 33 kD class I β-1,3-endoglucanase (Glu-I) and a 40 kD class II β-1,3-endoglucanase (Glu-II, PR-2b) from tobacco (*Nicotiana tabacum* cv. Samsun NN) with a double-enhanced cauliflower mosaic virus (CaMV) 35S promoter (Melchers et al., 1993). The chitinase genes were linked to the *Agrobacterium tumefaciens* nopaline synthase (*nos*) transcription termination signal, whereas the β-1,3-glucanase constructs contained their natural transcription termination signals. Two single-gene constructs encoding either the class I chitinase (pMOG198) or the class I β-1,3-endoglucanase (pMOG412) and a four-gene construct encoding both the class I and class II chitinases and β-1,3-glucanases (pMOG539) were made by assembly of the respective genes next to the neomycin phosphotransferase II (*nptII*) gene in the binary plasmid pMOG402 (Fig. 1). The binary plasmid pMOG402 was obtained through replacing the mutant *nptII* gene in pMOG23 (Sijmons et al., 1990) by the wild-type NPTII coding region (Yenofsky et al., 1990).

The binary plasmids pMOG198, pMOG412, pMOG539 and pMOG402 (empty vector control) were mobilized from *Escherichia coli* $DH5_{\alpha}$ into *Agrobacterium tumefaciens* strain MOG101 (Hood et al., 1993) and used to transform *Lycopersicon esculentum* cv. 'Moneymaker', a diploid (2n = 2x = 24) pure-breeding tomato line. Transgenic tomato plants were obtained by a standard cotyledon transformation method using kanamycin selection (Van Roekel et al., 1993).

Characterization of transgenic lines

Diploid kanamycin-resistant primary transformants were selected by establishing the mean number of chloroplasts in stomatal guard cells (Koorneef et al., 1989), analysed for expression of the respective transgenes and self-pollinated. The number of T-DNA loci present in primary transformants was established on the basis of segregation for kanamycin resistance in S1 progenies using a non-destructive kanamycin spraying assay (Weide et al., 1989). Chi square tests (α = 0.05) were used to assess the goodness of fit to expected Mendelian segregation ratios assuming one (3:1), two (15:1) and three (63:1) independently segregating loci.

Analyses of transgene expression

Protein expression levels were quantified by immunoblot analyses using a range (12.5, 25, 50 and 100 ng) of the respective purified class I and class II chitinases and β-1,3-glucanases as standards. Leaf samples containing 5 μg of soluble protein were electrophoresed on 12.5% SDS-polyacrylamide gels. Following blotting onto nitrocellulose membranes, the transgene-encoded proteins were visualized by enhanced chemiluminescence (ECL, Amersham). Pro-

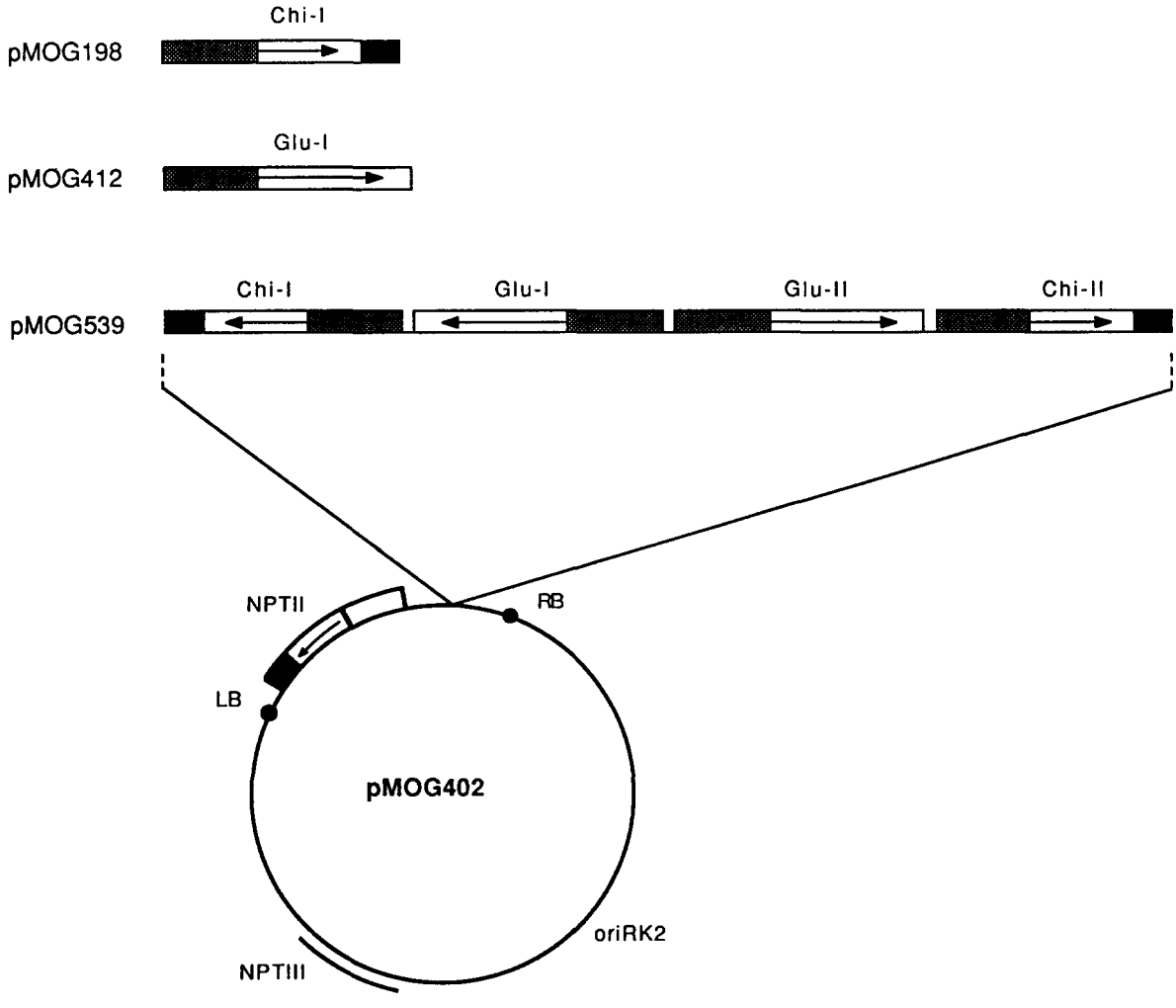

Fig. 1. Schematic representation of chimeric gene constructs pMOG198, pMOG412 and pMOG539 contained in the binary plasmid pMOG402. The chitinase and β-1,3-glucanase genes are transcriptionally regulated by the 35S CaMV promoter (hatched box) and by the *nos*-terminator region (black box) or their natural transcription termination signal. The arrows indicate the orientation of the respective genes.

tein extraction and Western blot analyses were essentially performed as described previously (Melchers et al., 1993). Polyclonal antibodies raised against PR-3a (detection of both the 32 kD class I and the 28 kD class II chitinase) and PR-2b (detection of the 40 kD class II β-1,3-glucanase) were used in a 10^4-fold dilution and that raised against the 33 kD class I β-1,3-glucanse in a 2×10^3 fold dilution.

Fusarium *resistance screening*

Transgenic tomato lines were screened for *Fusarium* resistance in randomized complete block experiments with 3 replicates and 7 plants per plot, using 25-day old S1 seedlings, which had been selected for kanamycin resistance using a non-destructive kanamycin spraying assay (Weide et al., 1989) and thus contained the respective transgene loci in either hemizygous or homozygous condition. Selection of NPTII-expressing seedlings by kanamycin spraying was confirmed to be very reliable (< 2% escapes) and did not induce any expression of chitinases and β-1,3-glucanases in tomato (unpublished results).

S1 seedlings were inoculated by dipping their root system in a freshly-prepared spore suspension from *Fusarium oxysporum* f.sp. *lycopersici* (race 1, 10^6 micro-conidia/ml), subsequently transplanted into 8 litre polystyrene plant trays (60% black peat/40%

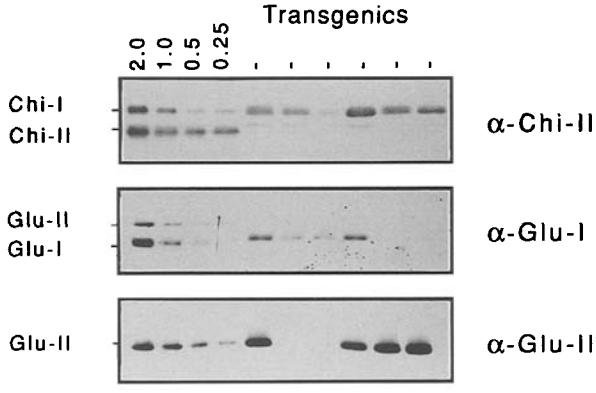

Fig. 2. Western blot quantification of class I and class II chitinase and β-1,3-glucanase gene (Chi-I, Chi-II, Glu-I and Glu-II) expression in transgenic tomato lines harbouring T-DNA contained in the binary plasmids pMOG539 (top and bottom panel) and pMOG412 (middle panel). The first lanes contain standard quantities of the respective chitinases and β-1,3-glucanases representing 2.0, 1.0, 0.5 and 0.25 percent of total soluble protein.

white peat containing 6 gl^{-1} CaCO3 and 1 gl^{-1} NPK 12/14/24 + spore elements, 21 plants/tray) and grown in a phytotron (18–20° C, relative humidity 80%, daylength 15 hrs, light intensity 2000 Lux). Microconidial inoculum of *Fusarium oxysporum* f.sp. *lycopersici* race 1 was harvested from fungal cultures freshly grown on potato dextrose agar (PDA) plates for 25 days (18° C, 18 hrs daylength, light intensity 300 Lux).

Disease severity was scored on a standard graduated scale by the time wild-type 'Moneymaker' had developed severe wilting and chlorosis (25–30 days post inoculation): 1 – slight browning of vascular tissue, no wilting of leaves; 3 – browning of vascular tissue, slight wilting of leaves; 5 – browning of vascular tissue, wilting of leaves, some slight chlorosis of leaves; 7 – strong browning of vascular tissue, severe wilting of plants, strong chlorosis of leaves; 9 – necrosis of leaves, dead plants. For each transgenic line, mean levels of disease severity per plot were calculated and compared by oneway Analysis of Variance according to Duncan's multiple range procedure (α = 0.05).

Results

Characterization of transgenic lines

Kanamycin-resistant 'Moneymaker' plants that were regenerated following transformation with the two single-gene constructs (pMOG198 and pMOG412), the four-gene construct (pMOG539) and the empty vector (pMOG402) were grown in the greenhouse and screened for ploidy level. About 20% of all independent transgenic plants proved tetraploid and were discarded. The remaining diploid plants showed no obvious aberrant phenotype when compared to wild-type 'Moneymaker' and were screened for expression of the respective chitinase and β-1,3-glucanase genes by immunoblot analysis (Fig. 2). Expression of the class II chitinase gene proved very low ($\leq$ 0.1% of total soluble protein) and in most cases was undetectable. Expression levels of the class I chitinase and β-1,3-glucanase genes and the class II β-1,3-glucanase gene differed between independent transgenic plants and ranged from 0.0–4.0%, 0.0–2.0% and 0.0–3.0% of total soluble protein, respectively. Some 54% of the single-gene 'Moneymaker' plants (MM198 and MM412 plants) had reasonable levels of transgene expression ($\geq$ 0.3% of total soluble protein). Among the four-gene 'Moneymaker' plants (MM539 plants), some 33%, 29% and 25% showed reasonable expression for 1, 2 and 3 of the respective transgenes, respectively. Total soluble protein isolated from wild-type 'Moneymaker' and from kanamycin-resistant empty vector control plants, did not show any cross reaction in the immunoblot analyses.

Based on the immunoblot analyses, six single-gene and twelve four-gene, transgenic 'Moneymaker' plants with varying but reasonably high levels of expression of the respective chitinase and β-1,3-glucanase genes were selected, self-pollinated and allowed to set seed. The resulting S1 progenies were subsequently characterized with regard to the number of (independently) segregating T-DNA loci. Except for two lines that segregated for two independent T-DNA loci (MM198-01 containing the single gene chitinase construct and MM539-20 containing the four gene chitinase/β-1,3-glucanase construct), all transgenic 'Moneymaker' lines were shown to segregate for a single T-DNA locus (Tables 1 and 2).

Table 1. Number of segregating T-DNA loci, mean chitinase and β-1,3-glucanase expression levels and mean disease rating 26 days after inoculation with *Fusarium oxysporum* f.sp. *lycopersici* in kanamycin-resistant S1 progenies from transgenic tomato lines expressing the four-gene chitinase/β-1,3-glucanase construct

Tomato lines	Construct		Disease severity[a]		Expression levels[b]			
	Genes	Loci	Mean	σ	Chi-I	Chi-II	Glu-I	Glu-II
Non-inoculated								
'Moneymaker' (MM)	–	–	0.00 a	0.00	0.0	0.0	0.0	0.0
Inoculated								
'Belmondo'	I-1,I-2[c]	–	0.00 a	0.00	0.0	0.0	0.0	0.0
'Dombito'	I-1,I-2[c]	–	0.00 a	0.00	0.0	0.0	0.0	0.0
MM-539-61	Chi-I,II/Glu-I,II	1	2.90 b	0.46	2.0	0.0	1.0	2.0
MM-539-18	Chi-I,II/Glu-I,II	1	3.05 b	0.67	4.0	0.05	1.5	2.0
MM-539-51	Chi-I,II/Glu-I,II	1	3.19 bc	0.97	4.0	0.0	1.0	3.0
MM-539-31	Chi-I,II/Glu-I,II	1	3.52 bcd	0.71	1.5	0.0	2.0	3.0
MM-539-10	Chi-I,II/Glu-I,II	1	3.66 bcd	1.00	4.0	0.1	0.1	0.0
MM-539-20	Chi-I,II/Glu-I,II	2	3.86 bcde	1.51	1.5	0.05	1.0	3.0
MM-539-16	Chi-I,II/Glu-I,II	1	4.38 bcde	1.58	4.0	0.1	0.1	0.0
MM-539-19	Chi-I,II/Glu-I,II	1	4.95 cdef	1.64	1.0	0.05	1.0	3.0
MM-539-60	Chi-I,II/Glu-I,II	1	5.24 defg	1.62	0.5	0.0	1.0	1.0
MM-539-34	Chi-I,II/Glu-I,II	1	5.34 defg	1.28	1.0	0.0	1.0	0.0
MM-539-02	Chi-I,II/Glu-I,II	1	5.63 efg	0.67	0.5	0.0	1.0	3.0
MM-402-09	Vector	1	6.45 fg	0.04	0.0	0.0	0.0	0.0
MM-539-50	Chi-I,II/Glu-I,II	1	6.67 fg	1.53	0.5	0.0	0.5	3.0
'Moneymaker' (MM)	–	–	6.88 g	1.12	0.0	0.0	0.0	0.0
'Planet'	–	–	6.91 g	0.44	0.0	0.0	0.0	0.0

[a] Different letters denote a significant difference (Duncan's multiple range test, $\alpha = 0.05$).
[b] Percentage of total soluble protein.
[c] Host gene-mediated immunity to *Fusarium oxysporum* f.sp. *lycopersici* races 1 and 2.

Table 2. Number of segregating T-DNA loci, mean chitinase and β-1,3-glucanase expression levels and mean disease rating 30 days after inoculation with *Fusarium oxysporum* f.sp. *lycopersici* in kanamycin-resistant S1 progenies from transgenic tomato lines expressing either the single-gene chitinase or the single-gene β-1,3-glucanase construct

Tomato lines	Construct		Disease severity[a]		Expression levels[b]	
	Gene	Loci	Mean	σ	Chi-I	Glu-I
Non-inoculated						
'Moneymaker' (MM)	–	–	0.00 a	0.00	0.0	0.0
Inoculated						
MM-412-04	Glu-I	1	4.94 b	1.58	0.5[c]	0.75
MM-412-03	Glu-I	1	5.38 bcd	1.01	0.0	0.75
MM-402-09	Vector	1	6.14 bcd	0.52	0.0	0.0
MM-412-06	Glu-I	1	6.38 bcd	0.80	0.0	1.5
MM-412-02	Glu-I	1	6.67 cd	0.91	0.0	1.5
MM-198-02	Chi-I	1	7.21 d	0.13	4.0	0.0
'Moneymaker' (MM)	–	–	7.29 d	0.14	0.0	0.0
MM-198-01	Chi-I	2	7.48 d	0.52	4.0	0.0

[a] Different letters denote a significant difference (Duncan's multiple range test, $\sigma = 0.05$).
[b] Percentage of total soluble protein.
[c] Induced expression of endogenous chitinase gene.

Fig. 3. Resistance in transgenic tomato (cv. 'Moneymaker') plants expressing the four-gene chitinase/β-1,3-glucanase construct, 40 days after inoculation with *Fusarium oxysporum* f.sp. *lycopersici* (race 1). Wild-type 'Moneymaker' non-inoculated (A), wild-type 'Moneymaker', inoculated (B) and inoculated transgenic 'Moneymaker' lines MM-539-18 (C) and MM-539-61 (D).

Screening for Fusarium *resistance*

To establish whether expression of the respective chitinase and/or β-1,3-glucanase genes enhanced fungal resistance, the selected single-gene and four-gene 'Moneymaker' plants were tested for resistance to infection with *Fusarium oxysporum* f.sp. *lycopersici* race 1. To enable relative resistance ratings to be assigned to transgenic lines, wild-type 'Moneymaker', empty vector control plants and, occasionally, sensitive and resistant control cultivars were included.

Between the twelve different transgenic tomato lines expressing the four-gene chitinase/β-1,3-glucanase construct, considerable variation in disease severity was observed. As expected, disease symptoms were consistently absent in control cultivars with host gene-mediated immunity to *Fusarium oxysporum* f.sp. *lycopersici* race 1 ('Belmondo' and 'Dombito') and in non-inoculated 'Moneymaker'. Similarly, the disease severity observed in the susceptible control cultivars ('Planet' and 'Moneymaker') did not significantly differ from that observed in the empty vector control (MM-402-09). This indicates that expression of the *nptII* gene (kanamycin resistance) dit not affect *Fusarium* resistance. Seven transgenic lines proved significantly more resistant whilst five were essentially as susceptible as control plants to *Fusarium* infection (Table 1). The reduction in disease severity in the resistant lines ranged from 36.3% (MM-539-16) up to 57.8% (MM-539-61) and was confirmed (50.9% up to 84.4% reduction) in a second experiment (data not shown). The transgenic lines MM-539-61 and MM-539-18 largely recovered from *Fusarium* infection by the time wild-type 'Moneymaker' had died (Fig. 3). In contrast, transgenic tomato lines constitutively accumulating similar levels of either the class I chitinase or the class I β-1,3-glucanase showed no increased resistance to *Fusarium* infection (Table 2). In fact, the only line with resistance significantly higher than that of wild-type 'Moneymaker' (MM-412-04) combined constitutive expression of the tobacco class I β-1,3-glucanase and induced expression of an endogenous tomato chitinase (Table 2), but did not differ significantly in disease response from the fully susceptible empty vector control plants (MM-402-09).

Correlation of gene expression and resistance

To establish to what extent expression of the class I and class II chitinase and β-1,3-glucanase genes contributed to resistance in the four-gene transgenic lines, mean expression levels in S1 lines were determined (Table

1) and correlated with the observed disease severity ratings. To avoid bias resulting from induction of chitinases and β-1,3-glucanases upon fungal infection (Joosten & de Wit, 1989; Tuzun et al., 1989), protein samples were collected directly prior to infection with *Fusarium*.

Correlation analyses indicated that higher expression of both the class I chitinase and the class I β-1,3-glucanase gene coincided with lower disease severity (Pearson correlation coefficients r(Chi-I) = - 0.77 [$P < 0.01$] and r(Glu-I) = - 0.54 [$P < 0.05$), respectively), while class II β-1,3-glucanase expression levels did not (Pearson correlation coefficient r(Glu-II) = - 0.22 [$P > 0.05$]). Correlation of the class II chitinase expression with disease severity could not be calculated, since its expression was confirmed to be either very low or lacking. Apparently expression of the class II β-1,3-glucanase gene does not play a pivotal role in enhancing *Fusarium* resistance. Whether expression of the class II chitinase can contribute to enhanced *Fusarium* resistance, obviously remains to be established.

Discussion and conclusions

The overall data presented here confirm and extend the observation that over-expression of antifungal proteins is a feasible approach for enhancing fungal resistance in economically-important crop plants. Enhanced levels of *Fusarium* resistance achieved in transgenic tomato lines accumulating multiple isoforms of chitinases and β-1,3-glucanases, were shown to result largely from the simultaneous expression of a class I chitinase and a class I β-1,3-glucanase gene because (i) higher expression of the class I chitinase and the class I β-1,3-glucanase gene correlated with lower disease severity, whereas expression of the class II β-1,3-glucanase gene did not, and (ii) simultaneous expression of the class I chitinase (1.5–4.0%) and β-1,3-glucanase (0.1–2.0%) did result in significantly enhanced resistance, while similar expression levels of either the class I chitinase or the class I β-1,3-glucanase alone, did not. These observations are consistent with previous data showing that only the class I chitinases and class I β-1,3-glucanases synergistically inhibit the growth of *Fusarium in vitro* (Mauch et al., 1988; Sela-Buurlage et al., 1993) and, as outlined in our earlier review paper (Van den Elzen et al., 1993), provide the first experimental evidence for the hypothesis that simultaneous expression of genes encoding antifungal proteins with synergistic activities *in vitro*, results in substantially higher levels of fungal resistance *in planta* than observed with expression of the individual antifungal genes alone.

Although the simultaneous expression of the class I chitinase and β-1,3-glucanase genes clearly resulted in partial resistance rather than immunity to infection with *Fusarium*, the observed delay in symptom development and apparent tolerance of infection in the best lines (MM-539-18 and MM-539-61), are expected to provide sufficient protection for survival of tomato plants following (early) natural *Fusarium* infection under field or glasshouse conditions. Experiments aimed at establishing the actual commercial value of *Fusarium* resistance in our best transgenic lines as well as resistance screens using a range of different fungal diseases are currently underway. If required, overall levels of partial resistance might be further enhanced by simultaneous expression of chitinases and β-1,3-glucanases with even stronger antifungal properties, by specifically expressing high levels of these hydrolytic enzymes in those tissues and cellular compartments that are predominantly invaded by the fungi of interest and/or by adding a variety of other genes encoding proteins with known antifungal activities.

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Note

Recently, a similar synergistic interaction between antifungal proteins has been reported by Zhu et al. (1994). They co-expressed a basic chitinase gene from rice and an acidic glucanase gene from alfalfa in transgenic tobacco plants and observed that 'the combination of the two transgenes gave substantially greater protection against the fungal pathogen *Cercospora nicotianae* than either transgene alone.'

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