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Virus and Fungal Resistance: From Laboratory to Field

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Virus and fungal resistance: from laboratory to field

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SUMMARY

Virus and fungal resistance traits are important targets in the genetic engineering of agricultural and horticultural crops. We have engineered resistance against potato virus X in important commercial potato cultivars. Four years of field trials with resistant potatoes have demonstrated the commercial feasibility of improving potato cultivars by selectively adding new traits while preserving intrinsic properties. In our pursuit for a broad resistance against fungi we have focused on the exploitation of genes encoding antifungal proteins. We present results demonstrating the antifungal effect of some of these proteins *in vitro*, as well as the synergy between specific chitinases and β-1,3-glucanases. We also report high level resistance against *Fusarium oxysporum* in transgenic tomato plants expressing a specific combination of genes encoding these enzymes.

1. INTRODUCTION

As a result of highly sophisticated breeding processes and mechanisation in agriculture, yields and quality of crops have increased dramatically over the past decades. However, the improved crops are still threatened by many diseases and pests. The high incidence of diseases and pests on crop plants as compared to wild plant species is most likely due to continuous growing of monocultures in combination with a selection in the breeding process for characteristics such as yield and quality rather than for disease and pest resistance. To control diseases and pests, industry has developed many chemical protectants against fungi, nematodes and insects. In contrast, chemical control of viruses and bacteria has, as yet, hardly been possible. Both because of the environmentally undesirable characteristics of many of the agrochemicals used for the first group of pathogens as well as the extremely laborious control strategies, or even the absence of adequate strategies for the second group of pathogens, resistance has become a strong focus of many plant breeding programs.

Since genetic engineering technology for plants became available in the early 1980s, it has been identified as a technology that could provide new strategies for controlling plant diseases. It was envisioned that some of these strategies might result in resistance traits that would last longer than the classical single-gene-resistance traits. In 1987 we embarked on a program aimed at the engineering of crop plants with resistance against viruses and fungi. For both types of pathogens various approaches to obtain resistance have been described. Most virus resistance strategies investigated to date involve the expression in transgenic plants of sequences derived from the genome of the virus against which resistance is desired, or of virus satellite sequences (Hull & Davies 1992). Where success has been reported, resistance was brought about by either the primary transcript of the transgene or by its processing or translation product. In all cases the observed resistance appeared to be specific. Recently we engineered two important commercial potato cultivars for resistance against potato virus X (Hoekema *et al.* 1989). To this end we used the most effective approach to date, i.e. the constitutive expression in transgenic plants of the viral gene encoding the coat protein (reviewed by Reimann-Philip & Beachy 1993; Gonsalves & Slightom 1993). After the successful introduction of resistance, transgenic potato plants were tested in the field for a number of consecutive years to address the question of the preservation of cultivar characteristics (Jongedijk *et al.* 1992).

One of the proposed strategies for broad-spectrum fungal resistance in transgenic plants involves the expression of genes encoding antifungal proteins (Lamb *et al.* 1992; Cornelissen & Melchers 1993). In a search for such proteins we have studied the phenomenon of induced resistance in plants in more detail. This form of resistance is triggered by a variety of elicitors and is directed to a whole range of pathogens (Sequeira 1983). Concomitantly with resistance the synthesis is induced of a large number of different proteins (reviewed by Bol *et al.* 1990; Bowles 1990; Linthorst 1991). These include various chitinases and β-1,3-glucanases, some of which exert antifungal activity *in vitro* against a broad spectrum of fungi (Schlumbaum *et al.* 1986; Mauch *et al.* 1988; Sela-Buurlage *et al.* 1993). We have isolated the genes for

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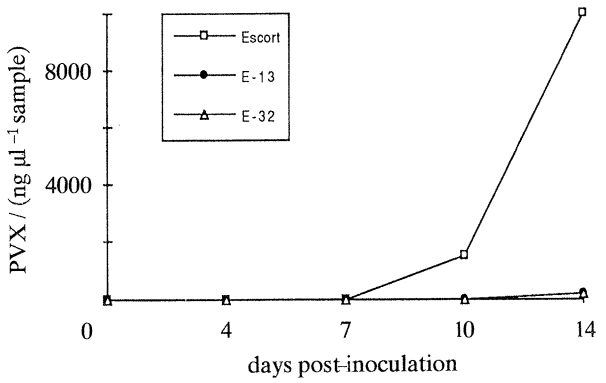

Figure 1. Accumulation of potato virus X in regenerants of potato variety Escort and two transgenic plant lines. Plantlets were inoculated with 1 µg ml^{-1} PVX. At various days post-inoculation the amount of virus was monitored by ELISA (Hoekema *et al.* 1989).

the antifungal hydrolases of tobacco and have shown recently that the simultaneous expression of these chitinase and β-1,3-glucanase genes in transgenic tomato plants results in a high level of resistance against the commercially important fungus *Fusarium oxysporum* (Melchers *et al.* 1993*a*).

2. PVX RESISTANCE AND PRESERVATION OF CULTIVAR PROPERTIES OF TRANSGENIC POTATO

(*a*) *Development of resistance against potato virus X*

To engineer resistance against potato virus X (PVX), a cDNA fragment containing the sequence encoding the viral coat protein (CP) was cloned between the cauliflower mosaic virus (CaMV) 35*S* promotor and the nopaline synthase transcription termination signal. The chimeric PVX CP gene was introduced into the commercial cultivars Bintje and Escort using *Agrobacterium* transformation technology. The resulting transgenic plants were tested for expression of the CP gene and for protection against virus infection under laboratory conditions (Hoekema *et al.* 1989). Transgenic Bintje and Escort clones showing high levels of PVX CP were selected, propagated in tissue culture, grown in pots and challenge inoculated with PVX (strain X^3; 1 µg ml^{-1}). As exemplified in figure 1, a considerable delay in PVX accumulation was observed in transgenic plants as compared to non-transgenic control plants (Hoekema *et al.* 1989). Tuber-grown plants displayed similar levels of protection against infection with PVX (Van den Elzen *et al.* 1989).

To establish the agronomic value of the reduced accumulation of PVX observed in our transgenic Bintje and Escort clones, we compared the frequencies of secondarily infected sprouts obtained from tubers of field grown transgenic plants, their non-transgenic counterparts and standard cultivars with known, but different levels of PVX resistance (Parlevliet *et al.* 1993). PVX-infected Bintje plants were planted in the field, evenly spaced throughout a randomized complete block with four plants per plot and four replicates. 'Natural' field infection was mimicked by dragging a harrow through the entire experimental field as in the current procedures in official Variety List research. Tubers were harvested from individual plants, soaked in a gibberellic acid solution to break dormancy and planted in trays. The emerging sprouts were separately analysed for PVX infection and the frequencies were calculated. Among progeny from transgenic clones expressing between 0.1% and 0.5% of PVX CP, relative to total soluble protein, a decrease in PVX incidence ranging from 56.6% (MGB-66) to 100% (MGE-32, 44) was observed (Jongedijk *et al.* 1992). These clones could be assigned Variety List ratings ranging from 7–8 up to *R* (field immunity), which was substantially better than the official Variety List score of 5 for untransformed Bintje and Escort after comparison with the standard cultivars (table 1). Simultaneous analyses of leaf and tuber samples collected from virus-free plants revealed a stable expression of the PVX coat protein gene throughout the growing season (Jongedijk *et al.* 1992).

To ascertain whether or not the observed PVX resistance might be overcome under extreme infection pressure, we performed a second experiment in which all experimental plots were rigorously inoculated using a dehaulming device, adjusted in such a way that the upper 25–30 cm of plants were severely bruised and partly stripped of their leaves. The overkill in PVX indeed resulted in much higher levels of PVX contamination among clonal progeny and worse discrimination between clones in the low-medium resistance group (Parlevliet *et al.* 1993). Some transgenic clones that ranked as moderately to highly resistant under normal conditions now proved to be largely susceptible. Despite the overall increase in PVX incidence, substantial and statistically significant levels of PVX resistance were, however, maintained in the transgenic clones MGB-66, MGE-13, MGE-32 and MGE-44. Under conditions of extreme PVX infection pressure, these latter clones could still be assigned Variety List ratings of 8–9 (table 1).

(*b*) *Preservation of cultivar identity*

Upon genetic modification, intrinsic cultivar characteristics may change due to somaclonal variation induced in the tissue-culture phase, insertion mutagenesis or possibly by effects of the transgene on the pattern of endogenous gene expression. To address the question to what extent the PVX resistant Bintje and Escort clones had maintained their intrinsic cultivar properties, we studied the karyotypes, morphological features and field performance of 39 independent Bintje and 22 independent Escort clones. Karyotyping was done using root tips collected from axenically grown plantlets. In total, 97% of all clones were observed to contain the normal tetraploid ($2n=4x=48$) chromosome number. One phenotypically normal aneuploid ($2n=47$) and one phenotypically aberrant polyploid ($2n\approx96$) Bintje clone (Hoekema *et al.* 1989) were of a deviant type. Subsequently, all transgenic clones and control Bintje and Escort plants were evaluated for 50 UPOV morphological features (21 plant, 10 flower, 7 tuber and 12 light sprout characteristics). These are

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Table 1. *Incidence of PVX-infected plants among clonal progeny from transgenic Bintje (MGB) and Escort (MGE) clones and untransformed standard cultivars following mechanical PVX infection using a harrow (moderate infection pressure) or a dehaulming device (severe infection presure)*

(Data derived from Jongedijk *et al.* (1992) and Huisman *et al.* (1992).)

	moderate infection pressure			severe infection pressure			PVX CP expression[c]	
cultivar	mean[a]	σ	variety list score[b]	mean[a]	σ	variety list score[b]	0.1–0.2	0.2–0.3
Bintje	38.5 ab	6.3	5	96.9 a	3.6	5		
Escort	36.5 abc	21.5	5	100.0 a	0.0	5		
Amazone	18.8 bcdef	8.0	7	93.8 ab	5.1	7		
MGB-66	16.7 cdef	15.2	7 8	82.4 bcd	9.1	7 8		+
Spunta	14.5 def	18.5	8	84.4 bc	13.0	8		
MGE-08	12.5 def	14.4	8 9	100.0 a	0.0	5		+
MGB-44	10.4 def	12.5	8 9	98.4 a	3.1	5		+
MGB-18	8.3 def	9.6	8 9	100.0 a	0.0	5		+
MGB-28	6.3 def	12.5	8 9	98.4 a	3.1	5		+
MGB-13	6.3 def	8.0	8 9	98.4 a	3.1	5	+	
MGE-21	4.2 ef	4.8	8 9	81.3 cd	13.5	$\geqslant 8$		+
MGE-13	2.1 ef	4.2	8 9	71.9 d	10.8	>8		+
Elles	2.1 ef	4.2	9	0.0 f	0.0	9		
MGE-44	0.0 f	0.0	R	76.0 cd	10.0	>8	+	
MGE-32	0.0 f	0.0	R	59.4 e	16.5	>8		+
Bildtstar	0.0 f	0.0	R	0.0 f	0.0	R		

[a] Different letters denote a significant difference (Duncan's multiple range test, $\alpha = 0.05$).
[b] PVX (strain X^3) resistance scores according to the Dutch Variety List: 5 9 = very susceptiblevery resistant; R = resistant in the field (immunity).
[c] Percentage of total soluble protein, data from growthroom experiments according to Hoekema *et al.* (1989).

currently used in official cultivar registration procedures (Anonymous 1986). The plants were also tested for yield and grading (<35 mm, 35–45 mm, 45–55 mm and >55 mm). These analyses were carried out in field experiments using uniformly graded, pre-germinated tuber material. The flower and light sprout characteristics (figure 2) were evaluated in a growth room and in a dimly illuminated lightproof cabinet, respectively. To allow statistical testing for the presence of differences between transgenic clones and control cultivars, all analyses were performed in randomized complete block experiments with five plants per plot and two replicates.

Typical changes in plant and tuber morphology as observed in some transgenic plants included a relatively compact plant appearance with small leaflets and higher frequency of secondary leaflets and relatively pronounced eyes and low yield of tubers. However, with respect to UPOV morphology, 41% of the transgenic Bintje clones and 82% of the transgenic Escort clones proved to be true to type. When total tuber yield and grading were also taken into account only 18% of the Bintje clones and 82% of the Escort clones were concluded to be true to type (figure 3; Jongedijk *et al.* 1992).

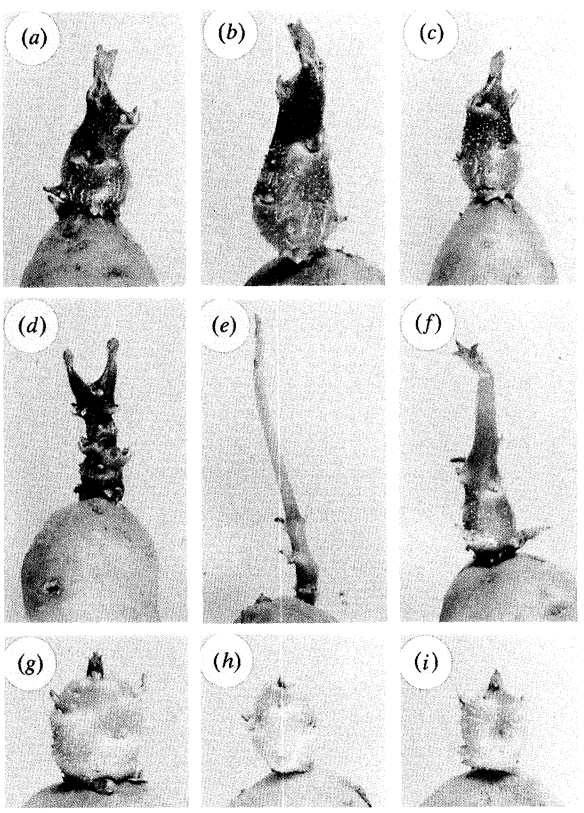

Figure 2. Typical light sprout morphology among transgenic Bintje (*a–f*) and Escort (*g–i*) clones. (*a,g*) Untransformed Bintje and Escort. (*b,c,h,i*) True-to-type transgenic Bintje and Escort. (*d–f*) Deviant transgenic Bintje. Note the cylindrical/narrow shape (*d,e*), extreme root tip protrusion and dark purple coloration (*d*) and branching (*f*). (Published with permission of *Bio Technology*.)

3. IDENTIFICATION OF ANTIFUNGAL PROTEINS AND USE OF THEIR GENES FOR THE DEVELOPMENT OF FUNGUS RESISTANT CROPS

(*a*) *Induced resistance and proteins in plants*

One of the strategies we are exploring to engineer durable resistance against fungi in plants involves the

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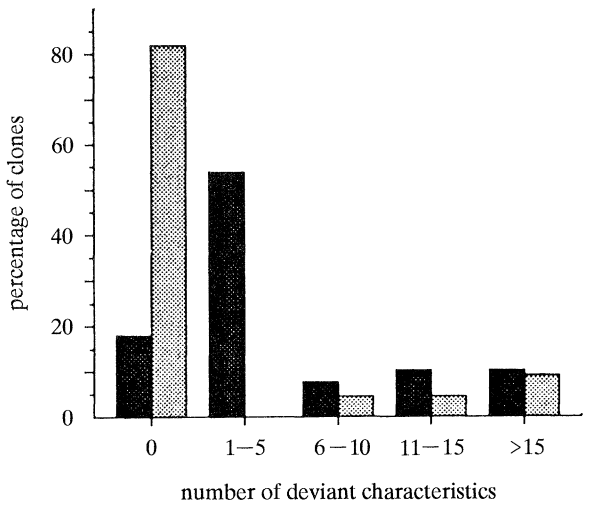

Figure 3. Percentages of true-to-type and deviant transgenic Bintje clones (filled bars, $n = 39$) and Escort clones (shaded bars, $n = 22$) as established by field evaluation of 50 UPOV morphological characteristics plus total yield and grading. (Published with permission of *Bio Technology*.)

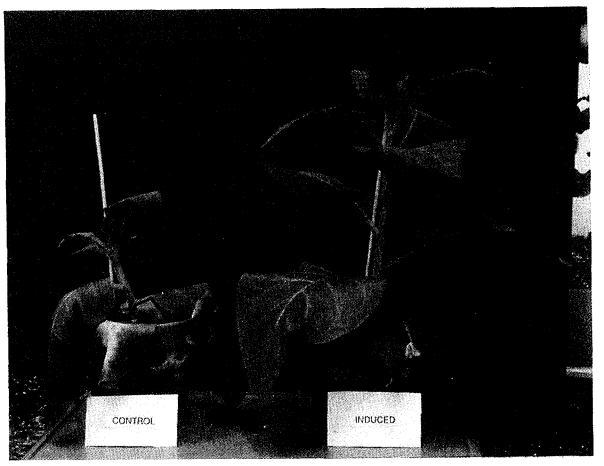

Figure 4. Induced resistance in *Nicotianae tabacum* cv Samsun NN against the soil phytopathogen *Phytophthora nicotianae*. A lower leaf of tobacco was injected with either water (left) or a cell wall extract of fungal hyphae (right), one week prior to the inoculation with *P. nicotianae*.

expression of genes encoding proteins able to inhibit fungal growth *in vitro*. As a potential source of such genes one could use the pool of plant genes of which the expression is induced concomitantly with the systemic induction of resistance against pathogens. Various biotic and abiotic elicitors are known to induce such a resistance, which is directed against a broad range of pathogens, irrespective the elicitor (Sequeira 1983). For example, as shown in figure 4, inoculation of leaves of Samsun NN tobacco with a cell wall extract of fungal hyphae leads to a systemic induction of resistance against pathogens including the fungal root pathogen *Phytophthora nicotianae* (McIntyre & Dodds 1979).

Concomitant with the appearance of resistance, the synthesis of a large number of proteins is induced (reviewed by Bowles 1990; Bol *et al.* 1990; Linthorst 1991). These include chitinases and β-1,3-glucanases, hydrolases of the sugar polymers chitin and β-1,3-glucan, respectively. These polymers are major cell wall components of many fungi (Wessels & Sietsma 1981). In hypersensitive tobacco at least three classes of chitinases are induced upon infection with TMV. The class I proteins occur in the vacuoles of the plant cell and possess an amino-terminal domain homologous to hevein (cysteine-rich domain) and a highly conserved catalytic domain (Shinshi *et al.* 1987). The class II, extracellular chitinases are immunologically and structurally related to class I chitinases, but lack the hevein domain (Linthorst *et al.* 1990*a*; Payne *et al.* 1990*a*). Class III chitinases of tobacco (Lawton *et al.* 1992) are not related to the class I or II enzymes but are homologous to the acidic chitinases of cucumber and *Arabidopsis* (Métraux *et al.* 1989; Samac *et al.* 1990). Two major classes (I and II) of β-1,3-glucanases have been identified in tobacco (Shinshi *et al.* 1988; Van den Bulcke *et al.* 1989; Linthorst *et al.* 1990*b*; Ward *et al.* 1991). The class I enzymes accumulate in the vacuole, whereas those of class II are found extracellularly. Proteins from the two classes are both structurally and immunologically related. To date, one class III enzyme has been identified, an acidic protein that is located extracellularly (Payne *et al.* 1990*b*).

Besides chitinases and β-1,3-glucanases, the set of TMV-inducible proteins of tobacco includes two classes of thaumatin-like proteins as well. The class I thaumatin-like proteins, initially defined as osmotins, were found to accumulate under salt-stress conditions in vacuoles of cells in suspension cultures (Singh *et al.* 1987). The class II thaumatin-like proteins were identified as polypeptides accumulating in the extracellular spaces of tobacco leaves after inoculation with TMV (Cornelissen *et al.* 1986). Both classes of proteins are related both structurally and immunologically.

(*b*) *Identification of antifungal activity*

In vitro growth of a number of fungi is inhibited by a class I chitinase from bean (Schlumbaum *et al.* 1986) and combinations of class I chitinases and class I β-1,3-glucanases from pea (Mauch *et al.* 1988). Recent experiments in our laboratory have demonstrated the *in vitro* antifungal activity on *Fusarium solani* of class I chitinases and β-1,3-glucanases purified from tobacco (Sela-Buurlage *et al.* 1993). Moreover, it was shown that these two hydrolases act synergistically: amounts of purified class I chitinase or β-1,3-glucanase by themselves insufficient to show any activity on fungal growth *in vitro* were found to be very effective inhibitors when applied in combination (figure 5). Surprisingly, in contrast to the class I proteins, the class II chitinases and β-1,3-glucanase exhibited no growth inhibiting effect at all, even when the amounts of enzymic activity were comparable to the activity present in the assay with class I enzymes (Sela-Buurlage *et al.* 1993). A combination of class II chitinases and class I β-1,3-glucanases displayed a weak synergistic effect on fungal growth, and only at high concentrations of class II chitinase. No effect could be observed in combinations of a class II β-1,3-

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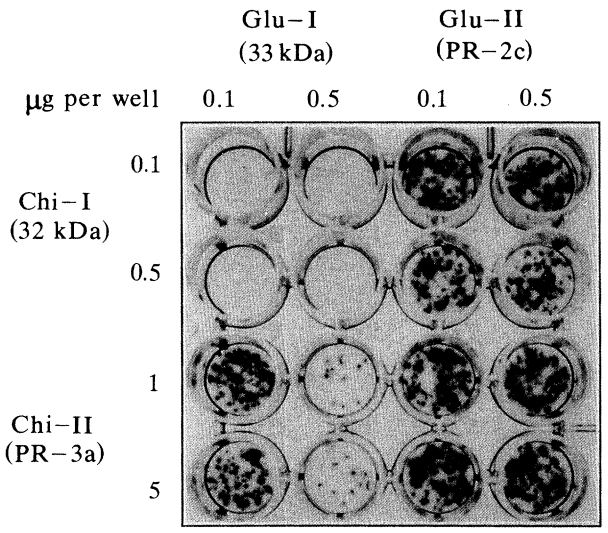

Figure 5. Synergistic activity of class I chitinase and class I β-1,3-glucanase inhibits growth of *Fusarium solani in vitro*. (Published with permission of *Pl. Physiol.*)

glucanase with either class I or II chitinases or a class I β-1,3-glucanase.

Cell walls of most fungi in the taxonomic class Oomycetes have a β-1,3-glucan component but contain no detectable amounts of chitin. *In vitro*, Oomycetes were shown to be insensitive to a mixture of chitinase and β-1,3-glucanase (Mauch *et al.* 1988). Because resistance against Oomycetes is inducible in plants including tobacco (see figure 4), factors other than chitinase and β-1,3-glucanase are probably involved in induced resistance against this class of fungal pathogens. Recently, we identified in TMV-infected tobacco a protein with an inhibitory effect on *Phytophthora infestans* (Woloshuk *et al.*,1991). Further characterization revealed that this 24 kDa anti-*Phytophthora* protein (AP24) was identical to osmotin, a class I thaumatin-like protein. Surprisingly, the purified class II thaumatin-like protein did not inhibit the growth of *P. infestans in vitro*.

(*c*) *Fungus-resistant plants: synergy of class I chitinases and β-1,3-glucanases in planta*

To test the hypothesis that constitutive expression in transgenic plants of genes encoding antifungal proteins, a class I chitinase gene and a class I β-1,3-glucanase, both driven by the cauliflower mosaic virus (CaMV) 35*S* promoter, were separately introduced into tomato cv. Moneymaker using *Agrobacterium*-mediated transformation (Van Roekel *et al.* 1993). Transgenic S1-progeny plants, constitutively expressing either the chitinase or the β-1,3-glucanase gene to relatively high levels, were challenge-inoculated with *Fusarium oxysporum* f.sp. *lycopersici* race 1. This soil-borne fungus infects the vascular tissue after penetration of roots through wound sites. None of the plants was found to be less sensitive to the fungal pathogen as compared to control plants (Melchers *et al.* 1993*a*). This is in contrast to the observation by Broglie and coworkers (1991) who observed resistance against the root pathogen *Rhizoctonia solani* in tobacco plants expressing a class I chitinase gene from bean. Next a four-gene construct encoding both class I and II chitinases and β-1,3-glucanases under control of the CaMV 35*S* promoter, was introduced into tomato. Transgenic S1-progeny plants were assayed for resistance to *F. oxysporum*. Disease severity varied among the different transgenic lines containing the four-gene construct. Four transgenic lines showed significantly increased resistance, while four other lines were essentially as sensitive as control plants to *Fusarium* infection (table 2). Transgenic tomato line MM-539-61 showed only a low disease severity and recovered from *Fusarium* infection by the time wild-type tomato plants had died (figure 6). We observed a significant correlation between expression levels of some of the transgenes and disease severity scored in the fungal assay. Concurrent expression of the class I chitinase (1.5–4%) and class I β-1,3-glucanase genes (0.1–2%) resulted in a significant enhanced disease resistance, while similar expression levels of either the class I chitinase or class I β-1,3-glucanase gene alone did not. As the expression level of the class II chitinase gene was low, the possible contribution of this protein in disease resistance remains unclear. The expression of the class II β-1,3-glucanase gene did not correlate with observed increased resistance to *Fusarium* in the lines tested.

In summary, tomato plants constitutively expressing both tobacco class I chitinases and class I β-1,3-glucanases exhibit substantially enhanced resistance against *F. oxysporum* f.sp. *lycopersici*, whereas tomato plants expressing either gene alone were not significantly protected (Melchers *et al.* 1993). These *in planta* results are consistent with the finding that class I chitinase and β-1,3-glucanase synergistically inhibit the growth of *Fusarium in vitro* (Mauch *et al.* 1988; Sela-Buurlage *et al.* 1993). Our work confirms and extends the observation that the overexpression of antifungal proteins is a feasible approach for enhancement of fungal resistance in agronomically important crop plants. Moreover, we provide evidence that the combination of specific genes, which encode enzymes with synergistic antifungal activities, yields a substantially better disease resistance in plants compared to those containing only a single transgene. Although the protection resulted in partial resistance rather than immunity, it is expected that under natural infection conditions in the field or greenhouse the tomato plants will benefit.

4. CONCLUSIONS

Our results on the engineering of commercial crops for resistance against either PVX or *Fusarium* has demonstrated unequivocally the potential of plant genetic engineering technology. With potato we have been able to obtain levels of PVX resistance in the field that could hardly be discriminated from the absolute resistance conferred by classical vertical resistance genes, such as in the variety Bildstar. In addition, we have shown that the potato varieties Bintje and Escort, which were used in this study, can be

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Table 2. *Disease severity of tomato S1-lines transgenic for a four-gene chitinase and β-1,3-glucanase construct inoculated with F. oxysporum f.sp. lycopersici race 1*

(Mean chitinase and β-1,3-glucanase protein expression levels and the number of T-DNA loci present, are shown for each plant line.)

	construct		disease severity[b]		expression levels			
cultivars[a]	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	—	—	0.00 a	0.00	0.0	0.0	0.0	0.0
Dombito	—	—	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-539-31	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-539-19	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-539-02	Chi-I,II/Glu-I,II	1	5.63 efg	0.67	0.5	0.0	1.0	3.0
MM-402-09	—	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

[a] Moneyemaker (MM) is a susceptible cultivar to *F. oxysporum* f.sp. *lycopersicie* race 1.

[b] Disease severity scored 30 days post-inoculation. Plants were cut longitudinal at the stem base and scored using the following severity scale: 0, no symptoms; 1, slight browning of vascular tissue, no wilting of leaves; 3, browning of vascular tissue and wilting of leaves; 5, browning of vascular tissue, wilting of leaves and some chlorosis; 7, severe wilting and chlorosis of leaves; 9, necrosis, dead plant. For each transgenic line mean levels of disease severity per plot (seven plants) were calculated and compared by one-way ANOVAR according to Duncan's multiple range procedure.

engineered with preservation of their intrinsic properties, even though a selection process is required. This conclusion is especially important for asexually propagated crops like potato. The heterozygosity typically found in potato cultivars does not allow the selective addition of desirable traits by classical breeding, while preserving the quality traits associated with specific varieties. These quality traits are often very important, because processing industries have designed their manufacturing procedures for use on specific varieties. For that reason varieties like Bintje and Russet Burbank have been grown since the beginning of this century, despite having some major limitations in agronomic performance. Some of the major problems are related to susceptibility to viruses and, most importantly, fungi and nematodes.

In our search for broad spectrum durable resistance genes against fungal infection, we have investigated the possibility of exploiting genes activated during the induced resistance reaction. We started an extensive screening program aimed at the isolation tobacco proteins with antifungal activity *in vitro* against a few selected fungi. Several isolated proteins appeared to be active against a subset of these fungi. For instance, class I chitinases and β-1,3-glucanases are active against fungi like *Fusarium*, *Alternaria* and *Cercospora*, but not *Phytophthora*. The protein AP24 however is active against the latter fungus but not against *Fusarium* (unpublished result from our laboratory). We speculate that the difference in spectrum of activity is related to the differences in, and availability of biochemical substrates for these proteins in the different fungi. For instance, Oomycetes such as *Phytophthora* do not have detectable amounts of chitin in their cell wall, whereas cell walls of other fungi contain considerable amounts of this polymer (Wessels & Sietsma 1981). The class I proteins tested so far appear to have an unexplained substantially higher antifungal activity *in vitro* than their class II counterparts. Because several fungi penetrate plant tissue through the intercellular space we have recently retargeted some of these intracellular proteins to the intercellular space and demonstrated that these proteins retained their antifungal activity (Melchers *et al.* 1993*b*). We are presently testing the degree of extra protection these retargeted proteins give against some specific fungi in transgenic plants as compared to plants constitutively producing the intracellular forms.

Figure 6. Resistance of tomato plants transgenic for a four-gene chitinase and β-1,3-glucanase construct to *Fusarium oxysporum*. Comparison of Moneymaker control plant and transgenic line MM-539-61, 40 days post-inoculation with *Fusarium oxysporum* f.sp. *lycopersici* race 1.

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In view of the commercial application of these genes some of the most spectacular results were observed upon infection of transgenic tomato plants expressing different combinations of chitinases and β-1,3-glucanases with the commercially important fungus *Fusarium oxysporum*. Broglie *et al.* (1991) and Logemann *et al.* (1993) reported a low level of tolerance against the fungi *Rhizoctonia solani*. This was observed in transgenic tobacco plants expressing a class I chitinase from bean and a bacterial chitinase, respectively. In our studies, transgenic tomato plants expressing only a class I chitinase (from tobacco) did not show any tolerance to *F. oxysporum*. Both the class I chitinase and class I β-1,3-glucanase are apparently necessary to obtain the maximum levels of resistance. Indeed these proteins also appear to act synergistically *in vivo*. Our results to date represent the first example of a specific trait conferred by engineering a combination of two genes into plants. They also increase the confidence with which we are testing a variety of combinations of these and other antifungal proteins in tomato and other crop species against a range of fungi.

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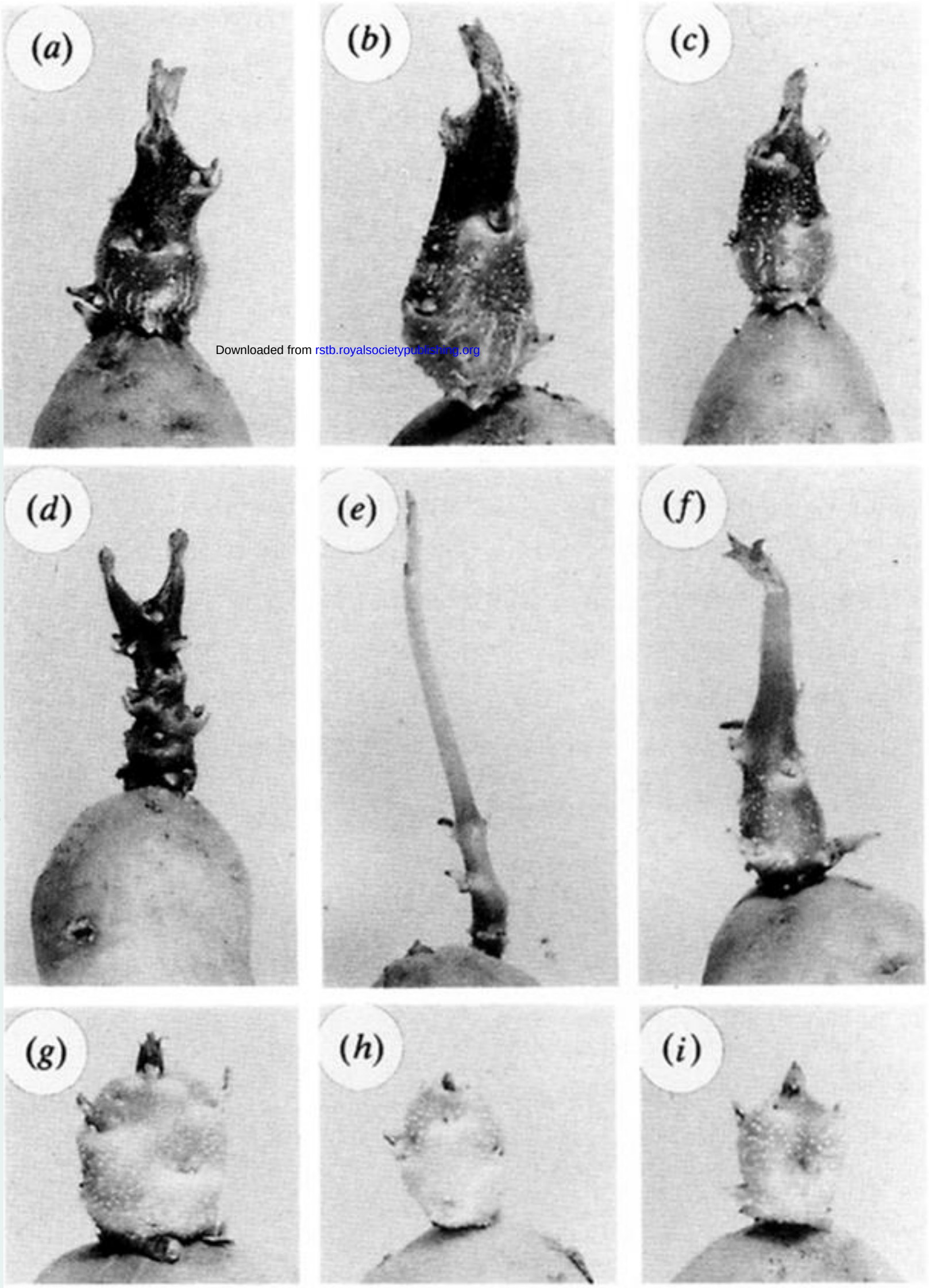

igure 2. Typical light sprout morphology among transge-
ic Bintje (*a–f*) and Escort (*g–i*) clones. (*a,g*) Untrans-
rmed Bintje and Escort. (*b,c,h,i*) True-to-type transgenic
intje and Escort. (*d–f*) Deviant transgenic Bintje. Note the
lindrical/narrow shape (*d,e*), extreme root tip protrusion
nd dark purple coloration (*d*) and branching (*f*). (Pub-
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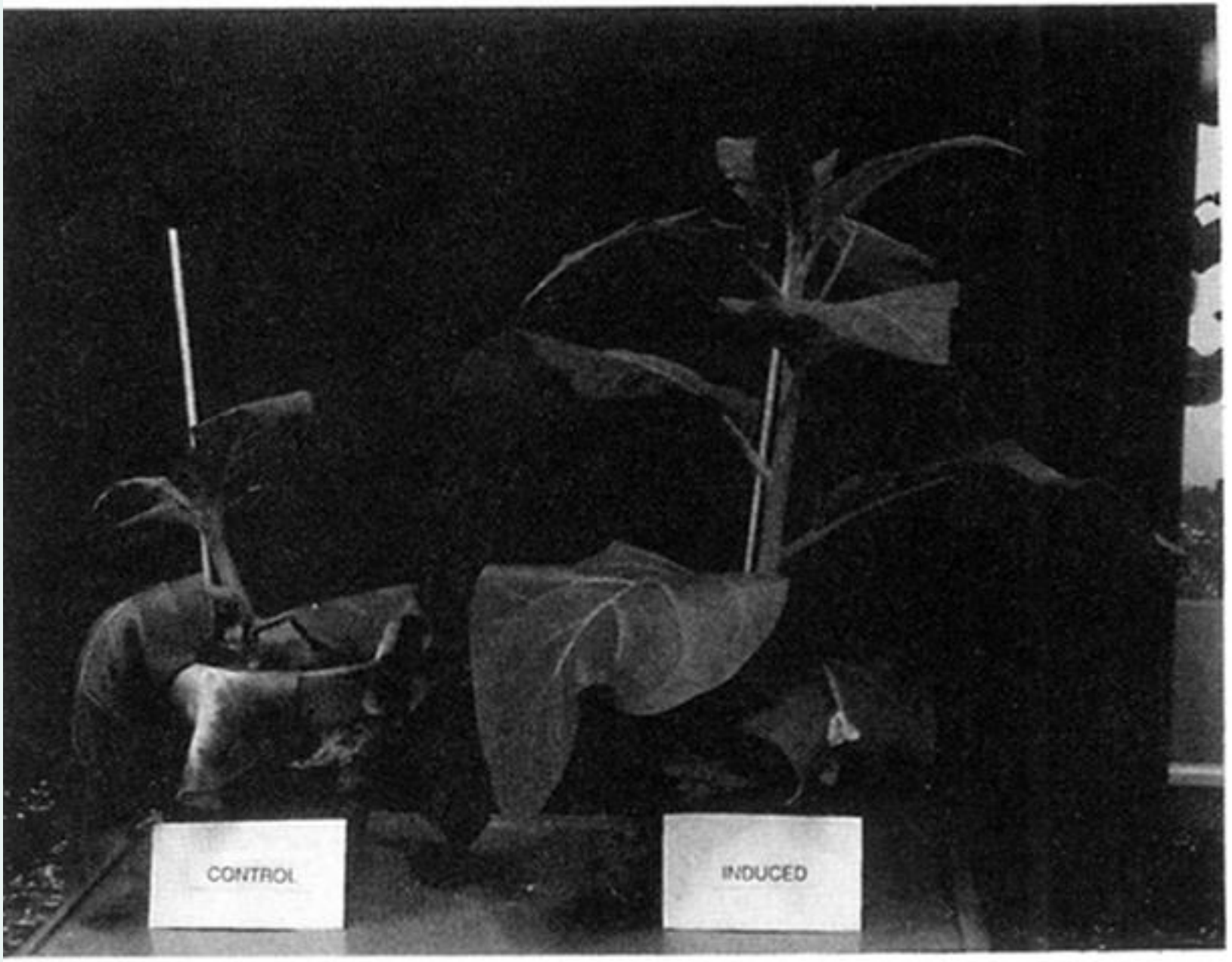
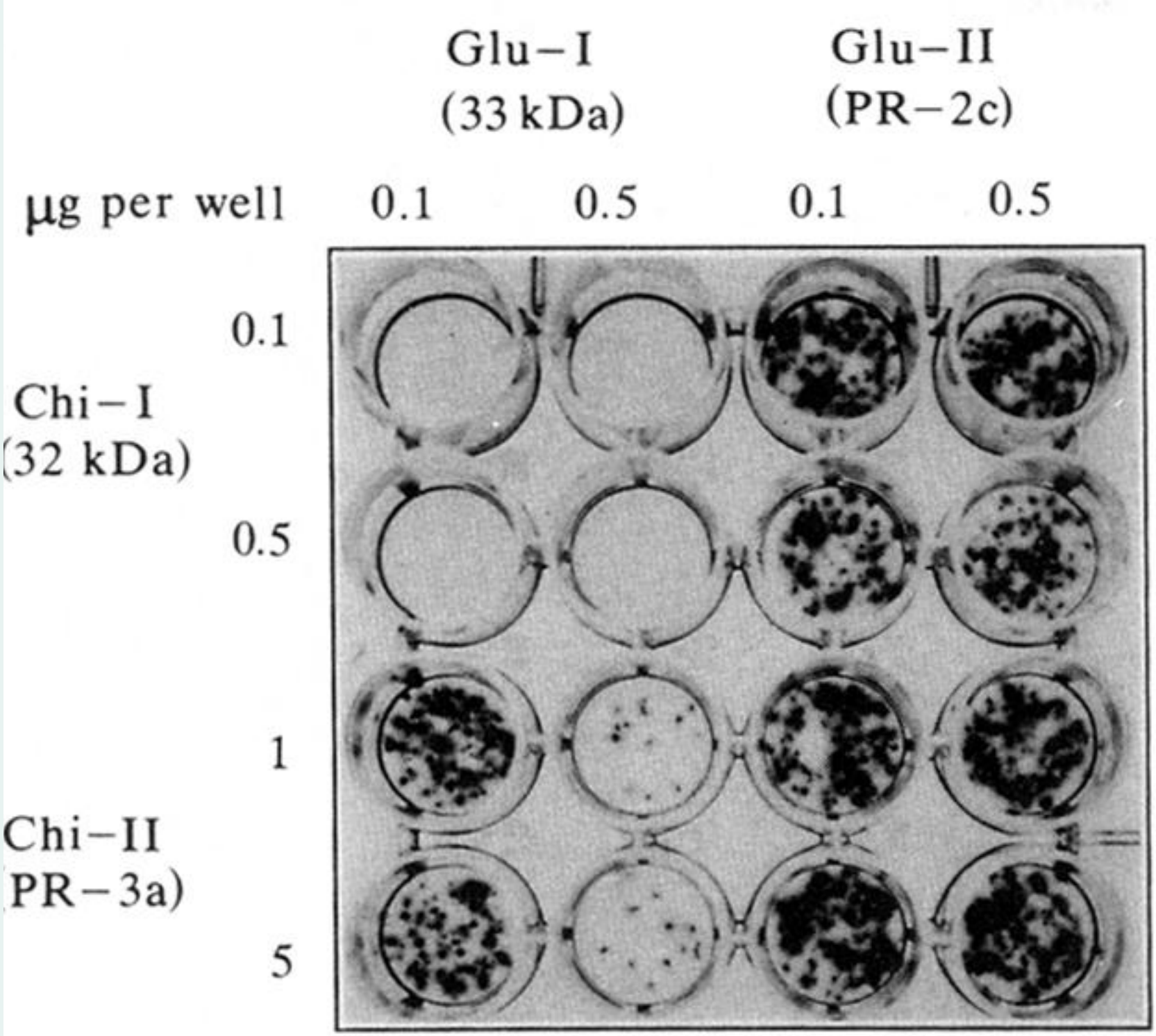


gure 4. Induced resistance in *Nicotianae tabacum* cv Samsun N against the soil phytopathogen *Phytophthora nicotianae*. A wer leaf of tobacco was injected with either water (left) or cell wall extract of fungal hyphae (right), one week prior the inoculation with *P. nicotianae*.



igure 5. Synergistic activity of class I chitinase and class I
-1,3-glucanase inhibits growth of *Fusarium solani in vitro*.
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gure 6. Resistance of tomato plants transgenic for a four-
ne chitinase and β-1,3-glucanase construct to *Fusarium*
sporum. Comparison of Moneymaker control plant and
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