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Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco

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Summary

cDNAs encoding three proteins from barley (*Hordeum vulgare*), a class-II chitinase (CHI), a class-II β-1,3-glucanase (GLU) and a Type-I ribosome-inactivating protein (RIP) were expressed in tobacco plants under the control of the CaMV 35S-promoter. High-level expression of the transferred genes was detected in the transgenic plants by Northern and Western blot analysis. The leader peptides in CHI and GLU led to accumulation of these proteins in the intercellular space of tobacco leaves. RIP, which is naturally deposited in the cytosol of barley endosperm cells, was expressed either in its original cytosolic form or fused to a plant secretion peptide (spRIP). Fungal infection assays revealed that expression of the individual genes in each case resulted in an increased protection against the soilborne fungal pathogen *Rhizoctonia solani*, which infects a range of plant species including tobacco. To create a situation similar to 'multi-gene' tolerance, which traditional breeding experience has shown to provide crops with a longer-lasting protection, several of these antifungal genes were combined and protection against fungal attack resulting from their co-expression *in planta* was evaluated. Transgenic tobacco lines were generated with tandemly arranged genes coding for RIP and CHI as well as GLU and CHI. The performance of tobacco plants co-expressing the barley transgenes *GLU/CHI* or *CHI/RIP* in a *Rhizoctonia solani* infection assay revealed significantly enhanced protection against fungal attack when compared with the protection levels obtained with corresponding isogenic lines expressing a single barley transgene to a similar level. The data indicate synergistic protective interaction of the co-expressed antifungal proteins *in vivo*.

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Introduction

Plant protection is a major challenge to agriculture worldwide. Significant yield losses due to fungal attack limits crop productivity and can be very severe in local epidemic infections. The control of fungal disease in modern agriculture is best achieved by 'integrated pest managment', based in part on plant cultivars with resistance to specific fungal races (Wenzel, 1985). Alternative strategies are being tested which aim to develop plants with a broad range resistance to fungal pathogens by genetic engineering (for reviews see Cornelissen and Melchers, 1993; Strittmatter and Wegener, 1993).

Monocot and dicot plants naturally respond to fungal attack by a complex network of defence mechanisms (Dixon and Harrison, 1990). These include the synthesis of polymers forming physical barriers (cutin, lignin, callose), of antimicrobial metabolites (phytoalexins), and of pathogenesis-related proteins (PR proteins). Hydrolytic enzymes, such as β-1,3-glucanases and chitinases which degrade fungal cell wall structural polysaccharides are well-known PR proteins (Joosten and de Wit, 1989; Kombrink *et al.*, 1988; Legrand *et al.*, 1987). Three classes of plant β-1,3-endoglucanases and five classes of plant endochitinases have been described (Collinge *et al.*, 1993; Melchers *et al.*, 1994; Ward *et al.*, 1991). Several of these enzymes have been shown to inhibit fungal growth *in vitro*, and their respective genes are therefore candidates as antifungal genes (Leah *et al.*, 1991; Mauch *et al.*, 1988; Schlumbaum *et al.*, 1986; Sela-Buurlage *et al.*, 1993). Indeed, recent studies have shown that ectopic expression of chitinases and β-1,3-glucanases in transgenic plants can mediate increased protection against phytopathogenic fungi (Broglie *et al.*, 1991; Van der Elzen *et al.*, 1993; Vierheilig *et al.*, 1993; Yoshikawa *et al.*, 1993; Zhu *et al.*, 1994).

Genes encoding ribosome inactivating proteins (RIPs) are also candidates as defence transgenes. RIPs possess 28S rRNA *N*-glycosidase activity which, depending upon their specificity, leads to the inactivation of conspecific and/or foreign ribosomes (Endo *et al.*, 1988; Stirpe *et al.*,

1992). Cytosolic Type I RIPs from the starchy endosperm of cereals are not significantly active on plant ribosomes *in vitro* (Taylor *et al.*, 1994), but readily modify foreign ribosomes, including those of fungi. Thus, Logemann *et al.* (1992) have shown that expression of a barley endosperm RIP under the control of an inducible promoter resulted in an increased protection of transgenic tobacco plants against *Rhizoctonia solani* without influencing plant growth.

A striking feature of plant response to pathogens is the coordinative nature of the complex network of defence. For hydrolytic enzymes such as chitinases or β-1,3-glucanases synergistic antifungal properties, leading to enhanced mycelial cell wall destructions, were demonstrated by combining these proteins in *in vitro* assays (Leah *et al.*, 1991; Mauch *et al.*, 1988). *In vitro* synergistically enhanced antifungal activity of other antifungal proteins such as the barley endosperm RIP combined with the barley class II chitinase or class II β-1,3-glucanase was also observed (Leah *et al.*, 1991). First evidence for enhanced protection *in planta* by co-expression of a rice basic chitinase and an acidic β-1,3-glucanase derived from alfalfa was recently provided by Zhu *et al.* (1994).

To evaluate the potential and limitations of a 'multi-transgene' tolerance strategy we combined different genes coding for hydrolytic enzymes as well as for a ribosome-inactivating protein and tested for tolerance of the transgenic plants to phytopathogenic fungi. Here we compare the tolerance of transgenic tobacco plants expressing cDNAs encoding a basic Class II chitinase (CHI) or basic Class II β-1,3-glucanase (GLU) or a Type I RIP from barley under the control of the CaMV 35S promoter with the tolerance of isogenic tobacco plants harbouring various combinations of these genes. Statistically meaningful semiquantitative infection assays with *R. solani* of 21 isogenic transgenic tobacco lines showed that plants expressing a combination of these defence transgenes exhibited increased levels of protection against infection by this fungus. The observed protection levels correlated well with the levels of the different proteins synthesized in the transgenic plants. Not only did a combination of the barley antifungal transgenes coding for the hydrolytic enzymes chitinase and β-1,3-glucanase result in a significantly increased protection but so also did a combination of chitinase and RIP.

Results

Fungal infection assay with Rhizoctonia solani

Our initial work with the *Rhizoctonia solani*/tobacco system indicated that an evaluation of fungal resistance based on a two class-scale (tallying rotted/dead plants versus surviving plants; Broglie *et al.*, 1991) did not properly reflect the growth of seedlings in *R. solani*-inoculated soil under our growth facilities. As shown in Figure 1, such plants exhibited a range of reactions in growth reduction and changes in morphology varying from virtually normal (uninfected) to severely macerated or dead. A correlation between the observed disease symptoms and the level of *R. solani* infection could be demonstrated by microscopy and re-isolation of the fungus from infected tissue (not shown). During the course of our experiments it then turned out that the variation in seedling growth caused by *R. solani* was best reflected by a five-class disease-severity-scale as defined below:

DI-class 0 = no disease symptoms—same size as uninfected controls;
DI-class 1 = no/few symptoms—growth reduction less than 25%;
DI-class 2 = clear symptoms, stem rot—growth reduction approximately 50%;
DI-class 3 = severe symptoms, stem rot, leaf curling—growth reduction greater than 75%;
DI-class 4 = macerated/rotted or dead.

Similar observations while evaluating *R. solani* disease severity in a range of plant species have been made by a number of phytopathologists during recent decades (Beagle-Ristaino and Papavizas, 1985; Boosalis, 1950; Elad *et al.*, 1981; Papavizas and Davey, 1960; Sneh *et al.*, 1966). We therefore used, in accordance with Boosalis (1950) as well as Sneh *et al.* (1966), the *DI*-classes defined above to calculate an average disease index (*DI*) for each group of 20 tested seedlings planted in random block design. The level of fungal inoculum which produced a half maximum *DI* of approximately 2.0 with wild-type control seedlings was initially established and subsequently used in all assays. This half maximum *DI* reference value reflected an intermediate response of control lines in the infection assays such that all five disease index classes could be represented. To better illustrate differences in the behaviour of the wild-type plants, reductions in *DI* were calculated using the following equation:

$$DI\text{-reduction (\%)} = ((\text{observed } DI - \text{reference } DI)/\text{reference } DI \text{ value}) \times 100.$$

Thus, an infected wild-type line exhibiting $DI = 2.0$ would have a *DI* reduction of 0%, while a transgenic line with $DI = 1.5$ would exhibit a *DI* reduction of 25%.

Experimental data were statistically analysed by means of the non-parametric Mann–Whitney–Wilcoxon-test (U-test). Additionally the *t*-test was used to prove the statistical significance of the differences in the mean *DI*-reductions. Mean *DI*-reductions were calculated for each plant line taking all repetitions of the respective infection assays into account (see Experimental procedures).

Kinetics and experimental variation of the *DI* were further

Figure 1. Appearance of wild-type seedlings after 14 days of growth in soil inoculated with *R. solani* exhibiting the five disease index (*DI*) symptom classes. See Results/Experimental procedures for details. The *DI* values range from none detectable (class 0) to completely macerated (class 4).

checked in repeated paired experiments for each plant line. Time course changes in *DI* and differences in *DI* of wild-type seedlings (SR1), and of seedlings transformed with an empty vector (pBIN19; trSR1) or with a CaMV 35S–*GUS* vector (pROK2275; 35S–*GUS*) were examined. Figure 2(a) clearly shows the time course of *R. solani* infection in which infection symptoms became visible 4–5 days after transplantation to inoculated soil (Figure 2b). The *DI* reached a maximum around 14 days after transplantation and remained constant thereafter (Figure 2a). Furthermore, *DI* did not differ significantly (±5%) between SR1, trSR1 or 35S–*GUS* seedlings (Figure 2b).

Protection against R. Solani *infection of transgenic plants expressing RIP*

RIP expression levels were analysed by RNA and protein extracts from tobacco plants that had been shown by DNA Southern blotting to carry intact copies of the chimeric 35S–*RIP* construct (L23; Figure 3). Figure 4(a) illustrates the immunodetection of cytosolic RIP in total protein extracts of these transgenic lines. The RIP level in the highest expressing line (L23-5) was calculated to be 0.15% of total leaf protein. The levels of RIP protein in different transgenic lines (Figure 4a) correlated well with levels of RIP RNA detected by Northern blotting (not shown). Segregation analysis of the transgenic plant lines indicated that the integrated T-DNAs behave as a single mendelian trait in the case of plant lines L23-4, -5 and -6, whereas in line L23-3 two genetic loci are present (not shown). This was in agreement with copy number estimation by Southern blotting which revealed a single T-DNA integration event in the case of lines 23-4 and -5, as well as two copies in the case of line L23-3. In the case of line L23-6, a complex banding pattern was observed indicating at least three intact copies in tandem array (not shown).

R_1 seedlings of selfed 35S–*RIP* transformants L23-3, 4, 5, and 6 were selected on resistance to kanamycin and tested for their performance in the *R. solani* infection assay. Lines L23-3, 4 and 5 showed *DI* reductions of 26–38% while line L23-6 exhibited a significantly lower *DI* reduction of about 15% (Figure 4b). These *DI* reductions correlated well with the relative levels of immunodetectable RIP in the different lines (Figure 4a and b, Figure 9). However, at higher amounts of RIP a saturation effect could be observed (Figure 9).

Plants transformed with the modified *RIP*-gene construct (35S–*spRIP*; pGJ-28; Figure 3), which produced a secreted RIP form, were also screened by DNA, RNA and protein analysis. Single copies of *spRIP*-gene were detected in the genome of the transgenic lines 4, 5, 6 and 7 by Southern blotting (not shown), which was in agreement with segregation analysis of these plant lines. In accordance with Northern blot analysis (not shown) RIP could be immunodetected in intercellular washing fluids of lines pGJ28-6 and 7, but not in fluids from lines pGJ28-4 and 5 (Figure 5a). The nature of multiple bands reacting with the RIP antiserum detected in the high expressing lines pGJ28-6 and 7 is not known.

R_1 seedlings from the 35S–*spRIP* transformants were also selected on kanamycin and tested for their performance in the *R. solani* infection assay (Figure 5b). *DI* reductions for lines pGJ28-4 and 5, which did not accumulate immunodetectable RIP, did not differ significantly from controls. In contrast, lines pGJ28-6 and 7, which accumulated RIP to 2.1% and 1.8% of IWF, respectively, (Figure

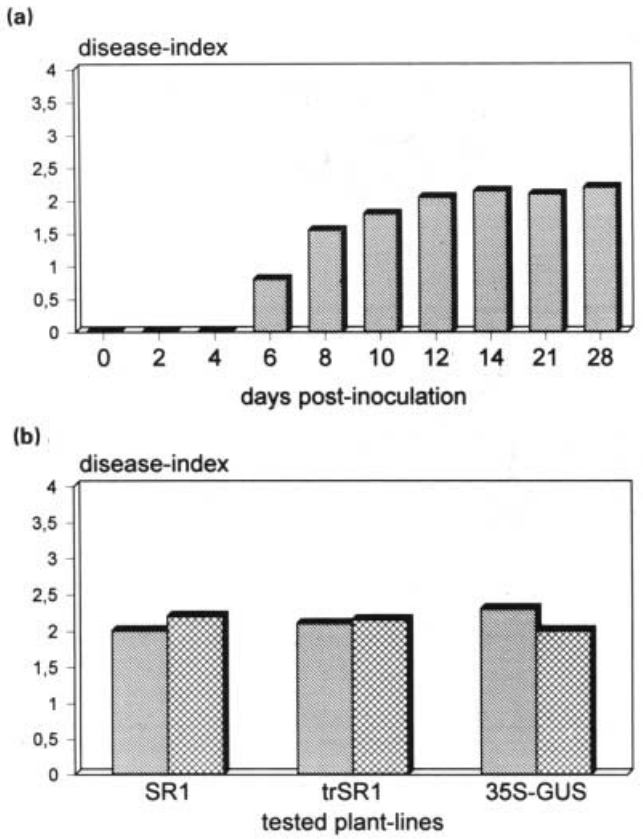

Figure 2. Kinetics and variation of the disease index.
(a) Changes in *DI* values of wild-type plants during 28 days of growth in soil inoculated with *R. solani*. Symptoms were first observed after 4 days and remained constant after 14 days. *DI* values were evaluated at the time points indicated.
(b) Variations in *DI* values estimated in two independent infection assays (indicated by the different shading of the bars) of wild-type plants (SR1), and plants transformed with the pBIN19 vector (trSR1) or a reporter gene (35S–*GUS*). The *DI* values were estimated 14 days after transplanting to infested soil. The differences between the respective *DI* values were within the range of ±5% and proved not to be statistically significant as judged by the Mann–Whitney–Wilcoxon-test (U-test).

5a), exhibited significant *DI* reductions (pGJ28-6, 51%; pGJ28-7, 48%).

These results indicate that accumulation of RIP intra- or intercellularly increased the protection of transgenic tobacco plants against infection by *R. solani*. The growth rates and morphologies of the transgenic plants which accumulate RIP or spRIP furthermore showed that intra- or intercellular expression of the Type I barley RIP was not deleterious to the growth of tobacco (data not shown).

Protection of transgenic plants expressing CHI *against* R. solani *infection*

CHI expression levels were again analysed in RNA and protein extracts of tobacco plants shown by Southern blot hybridization (data not shown) to carry intact copies of the 35S–*CHI* construct (pGJ24; Figure 1). Transgenic lines L24-2, -3 and -4 turned out to contain single copies of the *CHI*-transgene as determined by Southern blotting which all behaved as a single mendelian trait (not shown). Figure 6(a) shows the immunodetection of CHI in total protein from intercellular washing fluids (IWFs) of these transgenic lines. Comparable levels of RNA were seen in Northern blots of these lines (not shown). The highest level of CHI (4.1% of total IWF protein) was detected in line pGJ24-2.

Kanamycin-resistant R_1 seedlings of selfed 35S–*CHI* transformants pGJ24-2, 3 and 4 were tested for their performance in the *R. solani* infection assay. *DI* reductions for lines pGJ24-2 and 4 were approximately 35%. As shown in Figure 6(a) and (b), the *DI* reductions of the three lines tested did not correlate closely with their levels of immunodetectable CHI. For example, line pGJ24-2 accumulated more than two times the CHI level (4.1%) of line pGJ24-4 (2.1%), and five times the CHI level of line pGJ24-3 (0.7%), but exhibited only a slightly greater %*DI* reduction.

When grown in non-inoculated soil, no changes in growth rate or morphology were detected in lines expressing *CHI* as compared with control plants. This indicates that high-level, ectopic expression of the barley Class II chitinase was not deleterious to the growth of tobacco plants.

Protection against R. solani *infection of transgenic plants expressing* GLU

The *GLU* expression levels and growth of plants transformed with the 35S–*GLU* construct (L16; Figure 1) were analysed by the same assays as described above (Figure 7a and b). RNA and protein levels correlated well (not shown). In the case of line L16-7, Southern blot analysis revealed the presence of one intact copy of the *GLU*-transgene, whereas lines L16-2 and -5 were shown to carry two copies and line L16-6 three copies. Segregation analysis was in agreement with copy number estimation by Southern blotting (not shown). Protein analysis did detect low-level GLU accumulation in IWFs of line 7 (0.1%) and showed that GLU levels in lines 2, 5 and 6 were 6%, 9.8%, and 16.2% of total IWF protein, respectively (Figure 7a). These levels of GLU accumulation correlated well with the *DI* reductions of these lines (Figures 7b and 9) when analysed in the *R. solani* infection assay (L16-7, 0%; L16-2, 20%; L16-5, 28%; L16-6, 53%).

No changes in growth rates or morphology were detected in plants of line L16-6 as compared with control plants.

Protection against R. solani *infection of transgenic plants co-expressing* GLU/CHI *or* CHI/RIP

Expression levels of the respective genes were analysed by Northern and Western blot analysis of tobacco plants

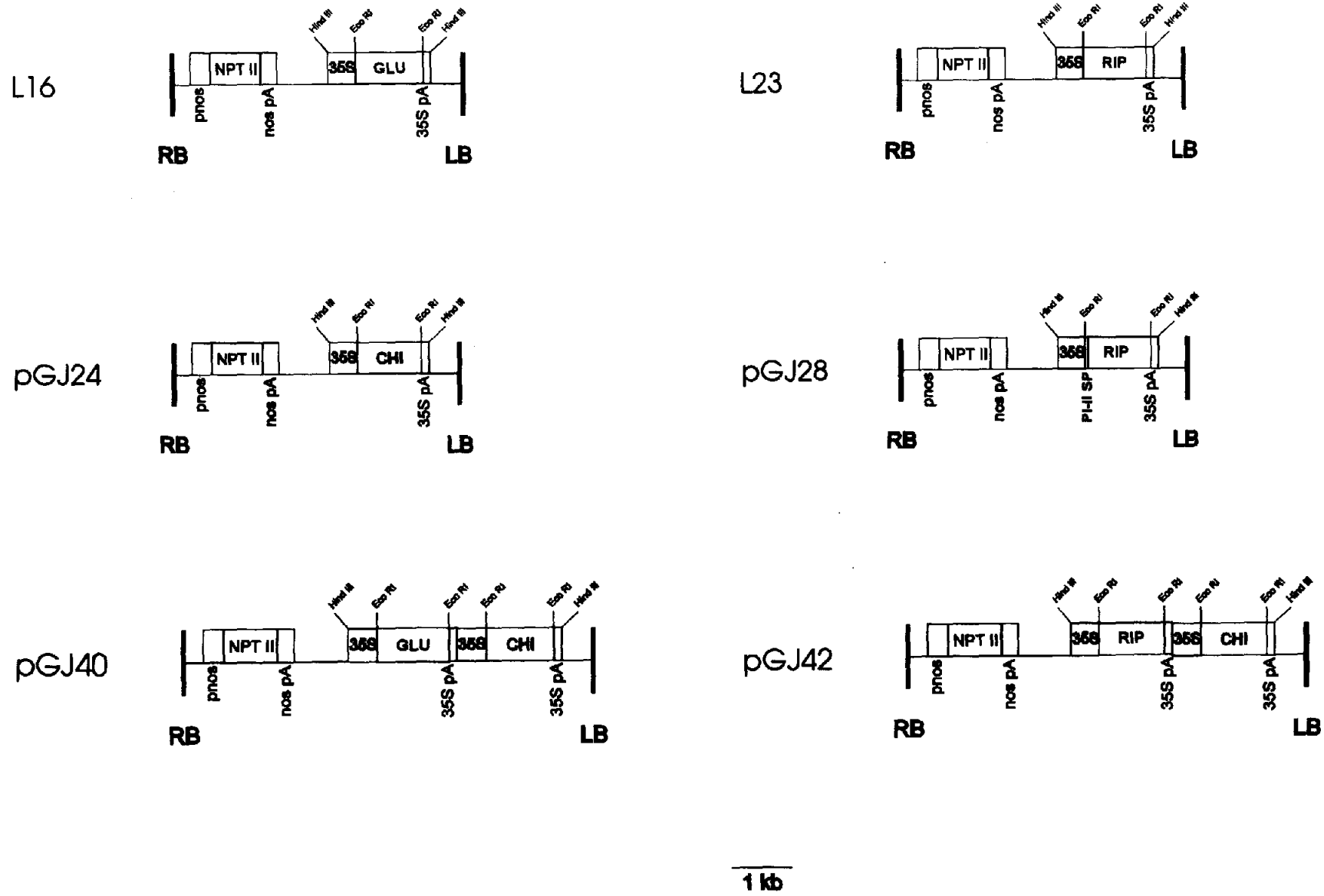

Figure 3. T-DNA regions of the different binary vectors (L16, L23, pGJ24, pGJ28, pGJ40 and pGJ42) for *Agrobacterium*-mediated transformation of tobacco. RB, right border; LB, left border.

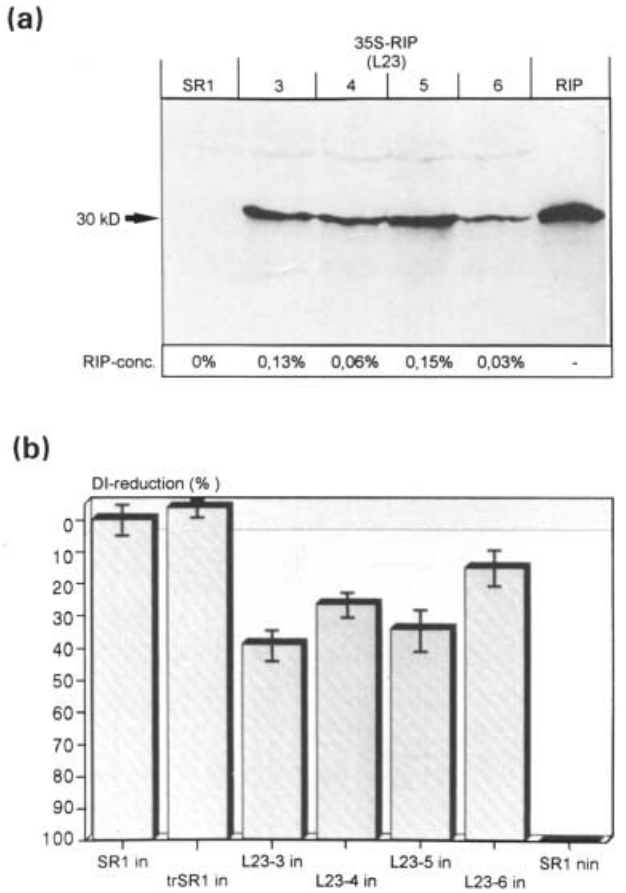

Figure 4. Fungal protection of *RIP* transgenic tobacco.
(a) Western blot analysis of plants transformed with the 35S–*RIP* construct (L23). Total leaf protein (300 μg lane^{-1}) of wild-type (SR1) and 35S–*RIP* transgenic lines (L23-3 to 6) were analysed by Western blot using RIP-specific antibodies. The *RIP* lane contained purified RIP (0.4 μg) and served as a positive control. The RIP protein content of the different samples is indicated below the corresponding lanes and is defined as % of total protein.
(b) Fungal infection assays of plants transformed with the 35S–*RIP* construct (L23). Mean values of % *DI*-reduction estimated in five independent infection assays for control and for 35S–*RIP* transgenic lines are given. nin, non-inoculated; in, inoculated soil. Statistical significance ($P = 0.05$) was proved for each assay with the Mann–Whitney–Wilcoxon-test (U-test). Standard deviations of the calculated mean values are given for each bar. The grey strip at the top of the diagram indicates non-significant *DI*-reductions.

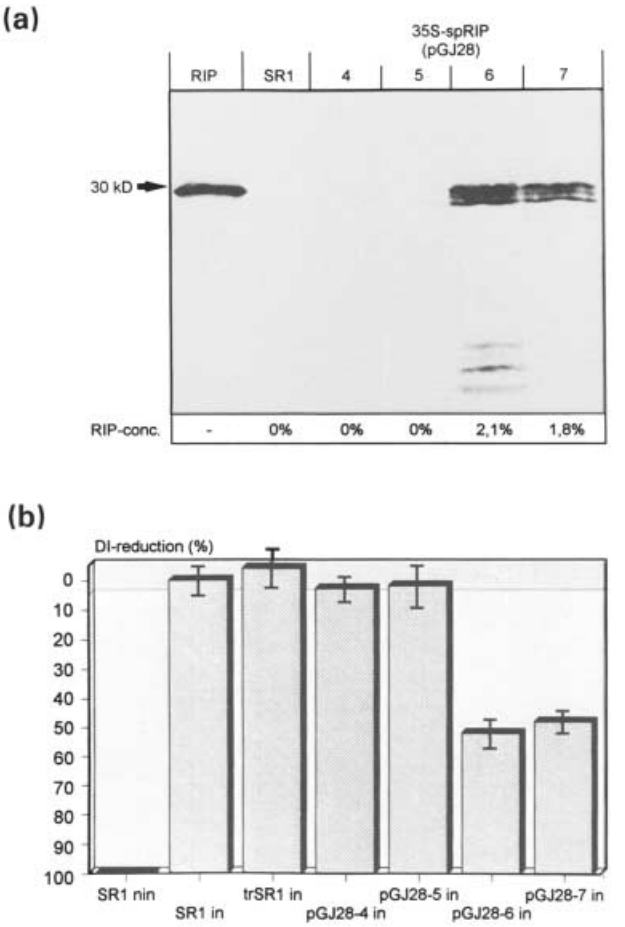

Figure 5. Fungal protection of *spRIP* transgenic tobacco.
(a) Western blot analysis of plants transformed with the 35S–*spRIP* construct (pGJ28). Leaf ICF protein (30 μg lane^{-1}) of wild-type (SR1) and 35S–*spRIP* transgenic lines (pGJ28-4 to 7) were analysed by Western blot using RIP-specific antibodies. The *RIP* lane contained purified RIP (0.4 μg) and served as a positive control. The RIP protein content (% of total IWF protein) of the different samples is indicated below the corresponding lanes.
(b) Fungal infection assays of plants transformed with the 35S–*spRIP* construct (pG28). Means of % *DI*-reduction estimated in four independent infection assays for control and for 35S–*spRIP* transgenic lines are given. Abbreviations are as shown in Figure 4. Statistical significance ($P = 0.05$) was proved for each assay with the Mann–Whitney–Wilcoxon-test (U-test). Standard deviations of the calculated mean values are given for each bar. The grey strip at the top of the diagram indicates non-significant *DI*-reductions.

shown by Southern blot hybridization to carry intact copies of either the 35S–*GLU*/35S–*CHI* (pGJ40; Figure 1) or the 35S–*RIP*/35S–*CHI* tandem gene constructs (pGJ42; Figure 1). Each of the transgenic lines pGJ40-1, -2, -3 and pGJ42-1, -2, -3 were shown to carry a single insertion of the corresponding T-DNA as judged by Southern blotting and segregation analysis (not shown). Immunodetection of the different proteins in intercellular washing fluids (GLU and CHI) or total protein extracts (RIP) of different transgenic lines can be seen in Figure 8(a). For lines pGJ40-3 and pGJ42-1 (Figure 8a) protein expression was sometimes below the detection level in this Western blot but was demonstrated in another set of experiments (data not shown). Amounts of the respective proteins, based on at least three independent experiments, are listed in Table 1. In all cases protein expression levels were fully consistent with the amount of specific RNA detected in these lines (not shown).

R_1-seedlings of selfed 35S–*GLU*/35S–*CHI* transformants pGJ42-1, 2 and 3 and from selfed 35S–*RIP*/35S–*CHI* transformants pGJ42-1, 2, and 3 were again selected for their resistance to kanamycin and tested for their performance in the *R. solani* infection assay. *DI* reductions for lines pGJ40-1, 2 and 3 were 49%, 60% and 25%, respectively, whereas lines pGJ42-1, 2 and 3 gave *DI* reduction values of 26%, 55% and 50%, respectively (Figure 8b).

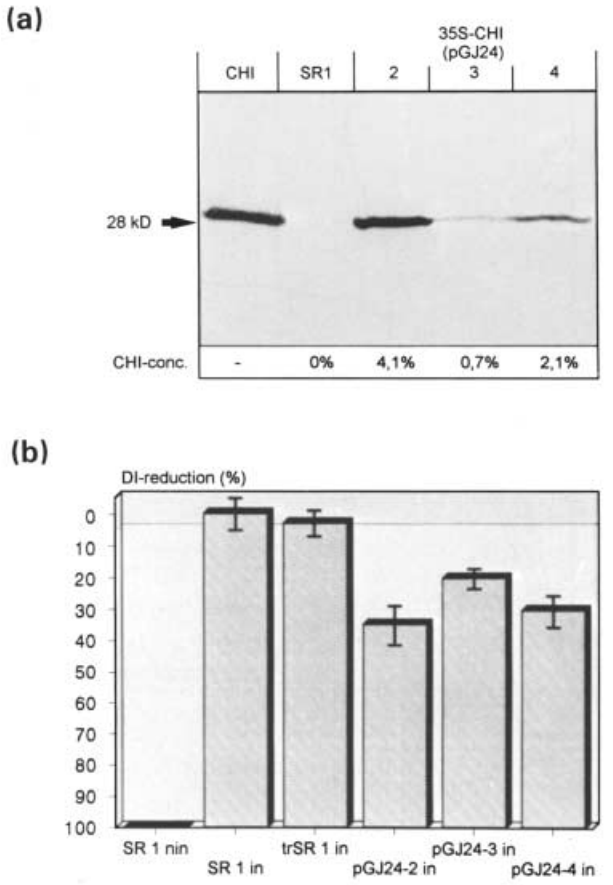

Figure 6. Fungal protection of *CHI* transgenic tobacco.
(a) Western blot analysis of plants transformed with the 35S–*CHI* construct (pGJ24). Leaf ICF protein (30 μg lane^{-1}) of wild-type (SR1) and 35S–*CHI* transgenic lines (pGJ24-2, 3 to 4) were analysed by Western blot using CHI-specific antibodies. The *CHI* lane contained purified CHI (0.4 μg) and served as a positive control. The CHI protein content, defined as % of total IWF protein, of the different samples is indicated below the corresponding lanes.
(b) Fungal infection assays of plants transformed with the 35S–*CHI* construct (pGJ24). % *DI*-reductions (mean values) estimated in three independent infection assays for control and for 35S–*CHI* transgenic lines are given. Abbreviations are as shown in Figure 4. Statistical significance ($P = 0.05$) was proved for each assay with the Mann–Whitney–Wilcoxon-test (U-test). Standard deviations of the calculated mean values are given for each bar. The grey strip at the top of the diagram indicates non-significant *DI*-reductions.

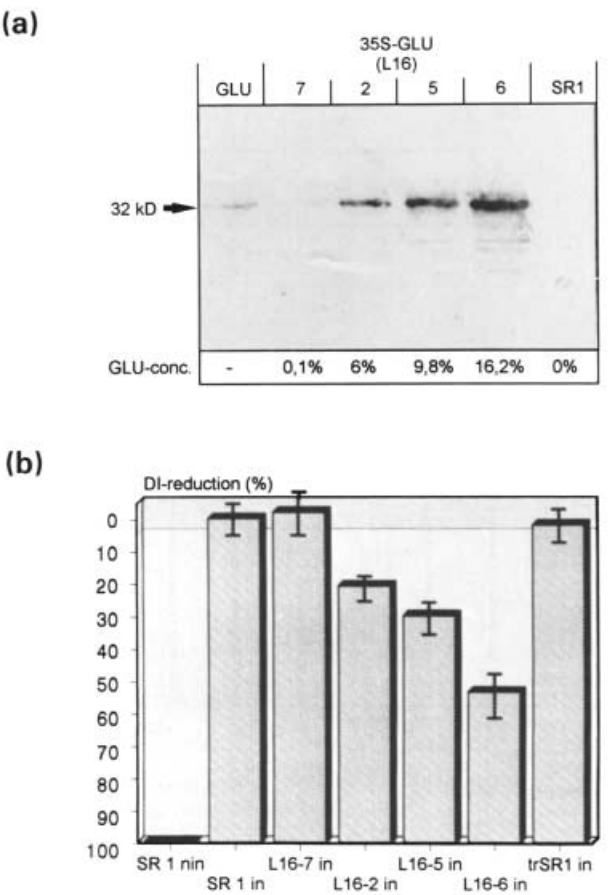

Figure 7. Fungal protection of *GLU* transgenic tobacco.
(a) Western blot analysis of plants transformed with the 35S–*GLU* construct (L16). Leaf ICF protein (10 μg lane^{-1}) of wild-type (SR1) and 35S–*GLU* transgenic lines (L16-2, 5 to 7) were analysed by Western blot using GLU-specific antibodies. The *GLU* lane contained purified GLU (0.4 μg) and served as a positive control. The GLU protein content (% of total IWF protein) of the different samples is indicated below the corresponding lanes.
(b) Fungal infection assays of plants transformed with the 35S–*GLU* construct (L16). Mean values of % *DI*-reductions estimated in six independent infection assays for control and for 35S–*GLU* transgenic lines are given. Abbreviations are as shown in Figure 4. Statistical significance ($P = 0.05$) was proved for each assay with the Mann–Whitney–Wilcoxon-test (U-test). Standard deviations of the calculated mean values are given for each bar. The grey strip at the top of the diagram indicates non-significant *DI*-reductions.

In order to judge whether resistance of the different plant lines is due to additive or synergistic protective interaction of the co-expressed antifungal proteins, expected *DI* reductions were calculated based on infection data obtained with isogenic transgenic lines containing only a single antifungal transgene. For this purpose dose–response curves (% *DI* reduction versus protein amount) were plotted for the *RIP, CHI* and *GLU* lines (L23; pGJ28; pGJ24; L16) (Figure 9) and used to determine the % *DI* reduction which could be expected for the observed expression levels of the individual proteins in the co-expressing plant lines (Table 1). In all cases experimentally observed % *DI* reductions were at least 1.5–2-fold higher than the calculated additive effect of the proteins (Table 1), clearly indicating the synergistic protective interaction of the co-expressed antifungal proteins. Perhaps the most striking results were obtained with the plant lines pGJ40-3 and pGJ42-1. In these lines, even in the case of additive protective effects no significant protection was expected, based on data with isogenic lines containing only one of these barley antifungal transgenes. However, *DI* reductions of 25 and 26% were observed in the infection assays (Table 1). Figure 10 shows a typical infection assay with the *CHI/RIP* line pGJ42-2 and clearly demonstrates the protective potential of expression of the barley antifungal transgenes.

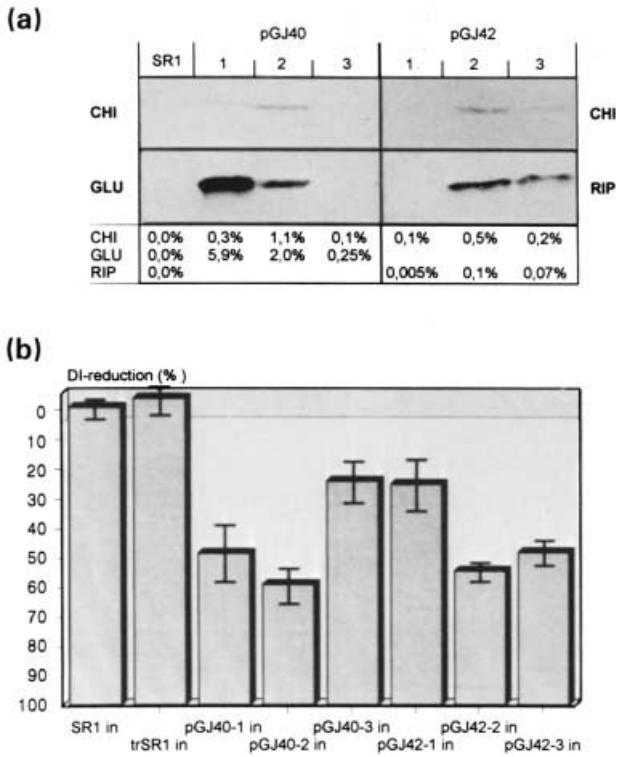

Figure 8. Fungal protection of *GLU/CHI* and *RIP/CHI* transgenic tobacco.
(a) Western blot analysis of plants transformed with the either 35S–*GLU*/35S–*CHI* construct (pGJ40) or the 35S–*RIP*/35S–*CHI* construct (pGJ24). Leaf ICF protein (30 μg lane^{-1}) of wild-type (SR1) and 35S–*GLU*/35S–*CHI* transgenic lines (pGJ40-1,2,3) and 35S–*RIP*/35S–*CHI* transgenic lines (pGJ42-1,2,3) were analysed by Western blot using specific antibodies. RIP protein was detected in total protein extracts (300 μg lane^{-1}) of the transgenic plants pGJ42-1, 2 and 3 by specific antibodies. The amounts of GLU, CHI and RIP protein in the different samples were calculated with purified protein as a standard as already described. Values of GLU (% of total IWF protein), CHI (% of total IWF protein) and RIP (% of total protein) are indicated below the corresponding lanes.
(b) Fungal infection assays of plants transformed with the 35S–*GLU*/35S–*CHI* construct (pGJ40) and 35S–*RIP*/35S–*CHI* construct (pGJ42). Mean values of % *DI*-reductions estimated in five independent infection assays are shown. Abbreviations are as shown in Figure 4. Statistical significance ($P = 0.05$) was proved for each assay with the Mann–Whitney–Wilcoxon-test (U-test). Standard deviations of the calculated mean values are given for each bar. The grey strip at the top of the diagram indicates non-significant *DI*-reductions.

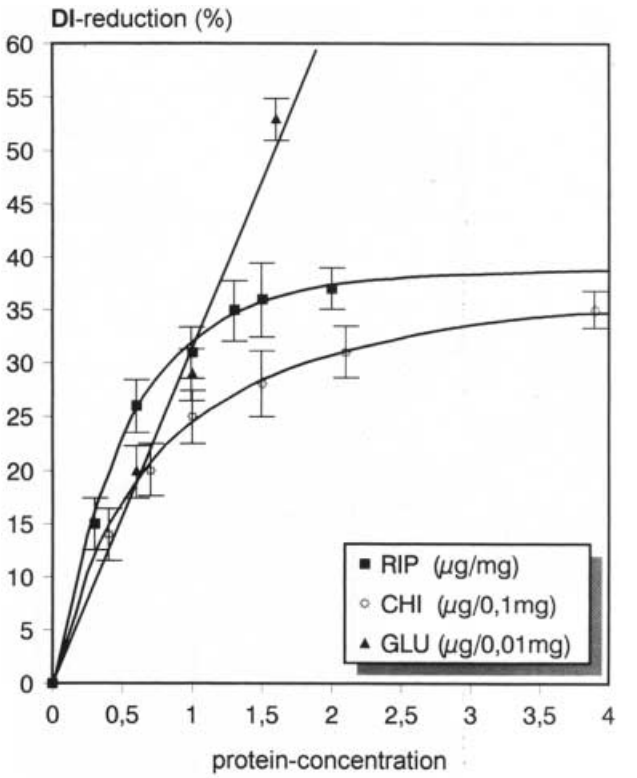

Figure 9. Dose–response curves for the antifungal proteins RIP/CHI/GLU. *DI*-reductions measured for individual transgenics expressing RIP, CHI or GLU were plotted against the respective concentration of the antifungal protein immunodetected in these plants. A strong linear correlation is obvious for GLU proteins, whereas CHI and RIP show some saturation effects at higher protein amounts. Variations of the *DI*-reduction measured in three to six experiments are indicated by the bar.

Discussion

The phytopathogenic fungus *R. solani* is the causative agent of root rot, stem canker and damping off diseases in a broad range of crops (Anderson, 1982; Kataria and Verma, 1992; Ogoshi, 1987; Sneh *et al.*, 1966; Uchimiya *et al.*, 1993). Damping off and seedling rot caused by *R. solani* are serious diseases of rapeseed and canola, for example, in Canada (Kataria and Verma, 1992). In some years and locations 80–100% of the plants are infected, resulting in partial or nearly complete loss of plant stands and an annual yield loss of 30% (Davidson, 1977; Sippel *et al.*, 1985). Earlier studies have shown that the fungus also infects tobacco (Broglie *et al.*, 1991; Logemann *et al.*, 1992; Roby *et al.*, 1990) and that the *R. solani*/tobacco system is therefore suitable for studies aiming to develop transgenic plants expressing foreign or modified proteins with antifungal properties. A statistically meaningful semiquantitative infection assay was used to compare the protective effects of RIP, CHI and GLU *in planta* against *R. solani* attack either expressed singly or in combination. In all cases cDNAs of these barley antifungal proteins were expressed under the control and the CaMV 35S promoter with the expectation that this would contribute to immediate protective effects during fungal attack. The performance of CaMV 35S–*CHI*, -*GLU*, and -*RIP* transgenic tobacco lines in a *R. solani* infection assay revealed that % *DI* reductions were significant for plant lines expressing these barley transgenes individually to relatively high levels (38% for *RIP*; 51% for *spRIP*; 35% for *CHI* and 53% for *GLU*). Co-expression of different PR proteins *in planta* normally occurs in response to invading fungal pathogens. By analogy combinatorial ectopic expression of different antifungal proteins might further increase protection. Indeed, the co-expression of either *GLU/CHI* (pGJ40-series) or *CHI/RIP* (pGJ42-series) resulted in a significantly enhanced

Table 1. Comparison of the expected and actually measured *DI*-reductions for the *GLU/CHI* and *RIP/CHI* expressing transgenic lines

Plant	Expression level		*DI*-reduction (expected)[b]			*DI*-reduction (measured)
	GLU[a]	*CHI*[a]	GLU	CHI	Addition	
pGJ40-1	5.9	0.3	20%	10%	30%	49%
pGJ40-2	2.0	1.1	5%	20%	25%	60%
pGJ40-3	0.25	0.1	0%	0%	0%	25%
Plant	Expression level		*DI*-reduction (expected)[b]			*DI*-reduction (measured)
	RIP[c]	*CHI*[a]	RIP	CHI	Addition	
pGJ42-1	0.005	0.1	0%	0%	0%	26%
pGJ42-2	0.10	0.5	26%	14%	40%	55%
pGJ42-3	0.07	0.2	22%	7%	29%	50%

Values for the expected *DI*-reductions were read from the established dose–response curves shown in Figure 9.
[a]% of IWF protein.
[b]Based on data obtained with transgenic plants carrying the respective single-gene constructs.
[c]% of total leaf protein.

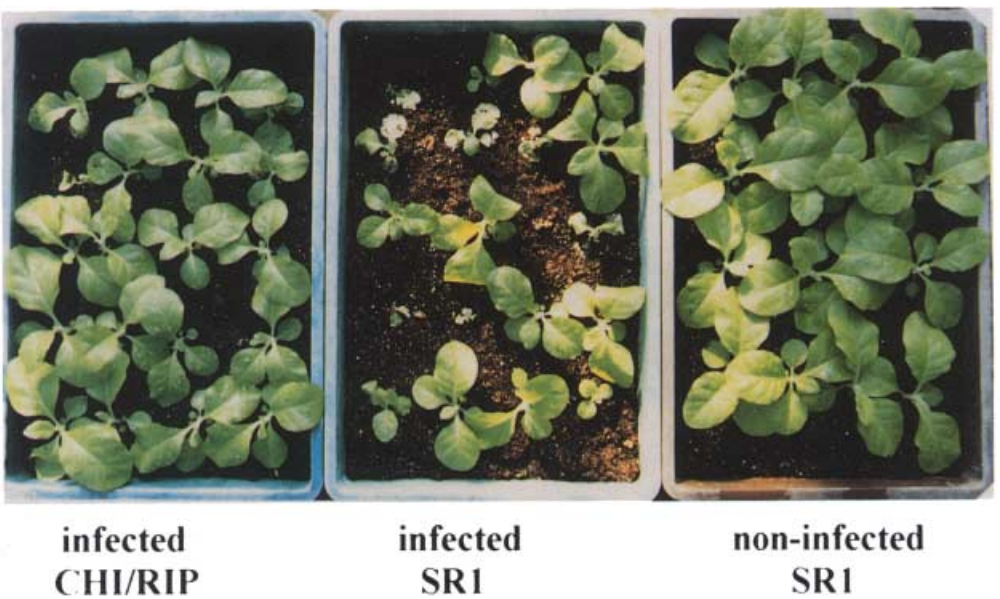

Figure 10. Appearance of control lines and of a transgenic line co-expressing high levels of *RIP* and *CHI* (pGJ42-2, Figure 7) after 18 days of growth in a typical *R. solani* infection assay.

protection against fungal attack (Figures 8 and 10; Table 1). Preliminary infection assays with other phytopathogenic fungi such as *Alternaria alternata* and *Botryis cinerea* also revealed significantly enhanced protection against fungal attack of *GLU/CHI* or *CHI/RIP* transgenic tobacco lines (Gieffers *et al.*, unpublished). These results indicate that the combinatorial expression of different antifungal proteins can lead to improved protection against a broad range of phytopathogenic fungi.

Although endogenous substrates for chitinases have not been identified in plants yet, substrates for β-1,3-glucanases are known for a number of plants tissues, and several studies have indicated that these enzymes can play a role during early embryogenesis or flower development (de Jong *et al.*, 1992; Neale *et al.*, 1990), as well as seed germination and microsporogenesis (Vögeli-Lange *et al.*, 1994; Worral *et al.*, 1992). In this respect it is interesting that the barley β-1,3-glucanase (GLU) and chitinase (CHI) used in this study were found not to cause any deleterious effects on the growth and development of transgenic tobacco. This indicates that non-defensive metabolic or developmental processes in which β-1,3-glucanase and chitinase may play a role are not affected by constitutive expression of these barley enzymes in tobacco.

In the case of another antifungal protein, the barley endosperm Type I RIP, earlier work showed that local expression of this RIP, driven by a wound-inducible potato promoter, increased the protection of tobacco plants against *R. solani* (Logemann *et al.*, 1992). This prompted us to examine whether tobacco plants could accumulate high levels of RIP either in the cytosol or in intercellular spaces, by engineering the addition of a signal peptide sequence to the original cDNA (spRIP). Transgenic plants in which up to 2.05% of leaf IWF protein was spRIP were obtained and found not be affected in their growth and development (pGJ28-6). This would appear to confirm earlier *in vitro* studies showing that Type I endosperm RIPs do not act on plant ribosomes (Leah *et al.*, 1991; Stirpe *et al.*, 1992; Taylor *et al.*, 1994).

Defence proteins have been the focus of numerous studies aiming to develop genetically engineered fungal resistance. Broglie *et al.* (1991) first showed that constitutive, ectopic expression of one of these defence transgenes, a bean class I chitinase, increased the protection against *R. solani* infection in tobacco and canola. Detailed cytological investigations of bean chitinase transgenic plants revealed that fungal invasion was primarily restricted to the cortex of these transgenic plants, and that the hyphae of invading fungi exhibited severe morphological alterations (Benhamou *et al.*, 1993a, 1993b). This effect was most pronounced in hyphal tip cells, presumably because the chitin-containing primary cell wall is most exposed in the hyphal tips and otherwise covered by glucans (Arlorio *et al.*, 1992; Benhamou *et al.*, 1993a). In tobacco transgenic lines expressing barley chitinase (pGJ24-series, Figure 5) limited accessibility of the pathogen to chitin could explain the fact that protection does not increase in plants which accumulate the enzyme to levels greater than about 1% of IWF protein (line pGJ24-4, see Figures 6 and 9). As is obvious from Figure 9 this saturation effect can also be observed for the RIP protein which might be due to limited uptake across fungal membranes preferentially at the hyphal tip.

From this point of view the combined action of chitinases and glucanases as well as chitinases and RIP was expected to lead to enhanced destruction of hyphal cell walls and therefore improved resistance against fungal attack. In the case of barley antifungal hydrolases, earlier *in vitro* observations of Leah *et al.* (1991) clearly demonstrated enhanced growth inhibition of different fungi by a combination of different hydrolases. Synergistically enhanced protection against fungal attack by co-expression of chitinase and glucanase *in planta*, was recently demonstrated by Zhu *et al.* (1994). These data are in accord with our independent data showing that barley chitinase and β-1,3-glucanase also act synergistically (pGJ40-series, Table 1).

When barley RIP, which normally accumulates only in the endosperm of barley seeds, is expressed in tobacco roots and leaves along with a barley chitinase, interesting antifungal effects were observed. Our data with transgenic tobacco plants co-expressing *CHI/RIP* (pGJ24-series, Table 1) clearly reveal that this strategy of producing different antifungal proteins in the same plant is not limited to the combination of different hydrolytic enzymes attacking fungal cell walls. It is possible that the hydrolytic activity of chitinase or β-1,3-glucanase could result in an increased uptake of RIP into fungal cells and therefore drastically enhance the inhibition of the growth of invading fungi. This is further supported by preliminary data with transgenic plants co-expressing a bacterial exo-chitinase gene (*CHI A*) from *Serratia marcescens* (Ordentlich *et al.*, 1988) together with the barley *RIP*, which also revealed synergistically improved protection against *R. solani* attack (Jach *et al.*, unpublished). This clearly indicates that chitinases of completely different origin can increase the antifungal activity of RIP. Another candidate as a defence transgene is the antifungal peptide (AFP) from the mould *Aspergillus giganteus* (Nakaya *et al.*, 1990). Interestingly, preliminary data with this antifungal peptide (AFP) revealed protection against fungal attack *in planta* as well as dramatically enhanced inhibition of fungal growth *in vitro* upon combination with RIP (Görnhardt *et al.*, 1994), suggesting further interesting combinations of antifungal transgenes.

Historically, early strategies for breeding of fungal resistance were based on single genes that were inherited in a mendelian fashion. However, it soon became obvious that this 'single gene' strategy favoured the rapid co-evolution of resistance-breaking pathogen strains. Consequently, more recent breeding programmes aim to select for more durable resistance using 'multigene' combinations (Vanderplank, 1978; Wenzel *et al.*, 1985). Likewise, breeding for fungal resistance in transgenic plants with 'multigene' combinations may make good sense since it is likely that this 'multigene' resistance strategy could result in a reduction of the probability of the emergence of resistance-breaking strains of pythopathogenic fungi. A number of different combinations of antifungal genes that could be co-expressed in transgenic plants await evaluation. They may represent a powerful line of defence against different invading fungi and could give rise to cultivars with a broad and durable field resistance.

Experimental procedures

Construction and transfer of chimeric genes to tobacco

Full-length *CHI*, *GLU* and *RIP* cDNAs were subcloned into the *Eco*RI site of pRT101 (Töpfer *et al.*, 1987) giving rise to the plasmids L16, L23 and pGJ23. In the resulting chimeric genes the CaMV 35S promoter (−420/+9) was fused to the different cDNAs and a 200 bp fragment containing the 35S polyadenylation signal (Töpfer *et al.*, 1987). These chimeric genes were subcloned into the

*Hin*dIII site of the binary vector pBin19 for transfer to tobacco (Bevan, 1984).

For the construction of spRIP, coding for RIP combined with a signal peptide, *RIP* cDNA was subcloned into the *Eco*RI site of the plasmid pRT104 (Töpfer *et al.*, 1987). The resulting plasmid (pGJ5) was digested with *Nco*I and religated to produce plasmid pGJ6 which lacks 60 bp from the 5′ untranslated region of the *RIP* cDNA. A 680 bp fragment of plasmid pMA1017 containing the CaMV 35S-promoter and the sequence encoding the first 32 amino acids of the signal peptide of the potato proteinase-inhibitor-II gene (Sanchez-Serrano, 1986; Sonnewald, unpublished) was then PCR amplified using the following primers:

Primer-1: 5′-CAGGAGCCATGGTGCAAGCCTTCGCATCAA-3′;
Primer-2: 5′-GAATTCCCATGGAGTCAAAGATTC-3′.

This product, which contained an inframe ATG (*Nco*I site) at the 3′ end of the signal peptide, was subcloned into the *Nco*I site of pGJ6 to produce plasmid pGJ26. The second copy of the 35S-promoter which was thus introduced was removed by digestion with *Xho*I and Asp718, followed by a Klenow fill-in reaction and ligation to produce plasmid pGJ27. Finally, the chimeric gene 35S–*spRIP* of pGJ27 was subcloned as a *Hin*dIII fragment into pBin19 (Bevan, 1984) to produce plasmid pGJ28.

To produce vectors containing combinations of these chimeric genes the 1.7 and 1.9 kb *Hin*dIII fragments of plasmids L16 (35S–*GLU*) and L23 (35S–*RIP*) were isolated and treated with Klenow-enzyme to fill up the recessed ends. The blunt-ended fragments were then inserted into the *Hin*cII site of plasmid pGJ23 (35S–*CHI*), which marks the 5′ end of the 35S-promoter. The resulting plasmids contained either the tandemly arranged chimeric genes 35S–*RIP*/35S–*CHI* (pGJ37) or 35S–*GLU*/35S–*CHI* (pGJ38). These gene combinations were moved as *Hin*dIII fragments to the binary vector pBin19 (Bevan, 1984) producing the vectors pGJ40 (GLU–CHI) and pGJ24 (RIP–CHI).

The resulting binary vectors (Figure 1: CHI, pGJ24; GLU, L16; RIP, L23; spRIP, pGJ28; GLU–CHI, pGJ40; RIP–CHI, pGJ42) were introduced into tobacco by *Agrobacterium*-mediated gene transfer according to Hoekema *et al.* (1983) and initially screened for resistance to kanamycin.

Southern and Northern blot analysis

DNA and RNA from putative tobacco transformants was screened by Southern and Northern blot analysis with the appropriate cDNA probes to identify plants carrying intact copies of the chimeric genes and assay RNA amounts. Genomic DNA from tobacco leaves was isolated according to Murray and Thompson (1980). Isolation of total RNA and separation of RNA on denaturing agarose gels was performed after Logemann *et al.* (1987). Transfer to Hybond-N membrane (Amersham) and hybridization to radioactive probes were done as described in standard protocols (Maniatis *et al.*, 1982).

Preparation of total proteins and of proteins in intercellular washing fluids from tobacco leaves

Leaf tissue was powdered in liquid nitrogen with mortar and pestle, then homogenized in ice-cold extraction-buffer (50 mM phosphate-buffer (pH 7.0), 5 mm β-mercaptoethanol, 5 mM EDTA) and centrifuged at 4°C for 10 min at 7800 ***g***. The supernatant was frozen at −20°C overnight, thawed on ice and centrifuged again. The resulting total leaf protein extract was transferred to a fresh tube and aliquots were used for SDS–PAGE and Western blotting.

Proteins in the intercellular washing fluids of tobacco leaf tissue (IWFs) were prepared as described by Parent and Asselin (1988) except that vacuum-infiltration was carried out for 5 min. Protein concentrations in total leaf extracts and IWFs were quantified according to Bradford (1976) using the Bio-Rad Protein dye reagent.

Western blot analysis

Proteins were separated by SDS–PAGE according to Laemmli (1970) and transferred to Immobilon filters (Millipore) with a Trans-Blot-SD unit (Bio-Rad). To reduce non-specific binding, filters were incubated for 30 min with 1% gelatin (Bio-Rad) in TBS and then incubated overnight in the same solution with specific antisera diluted 1:1000. The preparation and specificity of these antisera towards barley RIP, CHI and GLU have been described previously (Leah *et al.*, 1991; Mundy *et al.*, 1986). For immunodetection AP-conjugated second antibodies and BCIP and NBT as substrates (Sigma) were used. The amounts of the respective proteins (CHI, GLU and RIP) in different samples and control lanes containing purified proteins were estimated by scanning Western blot filters with a video image system (INTAS/Göttingen) and subsequent densitometric measurements of the signal strengths of the immunoreactive bands.

R. solani/*tobacco infection assays*

The fungus *R. solani* was propagated on solid potato-dextrose-medium (PDA, Difco) at 25°C in the dark with fresh inoculations every month. These inocula were regularly reisolated from infected plant tissue to maintain fungal virulence. Fungus for soil inoculation was cultivated in 400 ml liquid medium (potato-dextrose-broth (Difco)) for 6 days at 25°C at 100 r.p.m. Mycelia were then harvested by filtration on Whatman 3M-paper in a buchner-funnel, weighed, and homogenized in a Warring blender in PDB-media for 2–3 sec at low speed. Subsequently, fresh medium was added to give a 0.1g fresh-weight ml^{-1} mycelial suspension. Fifty to sixty millilitres of suspension were then thoroughly mixed with 2 l standard soil (ED73, not sterilized) filled into plastic trays (45×30×6 cm) and overlayed with 3 l non-inoculated soil.

Seeds from selfed tobacco lines were surface-sterilized and germinated on MS-medium supplemented with 30% sucrose (w/v) and with 100 μg ml^{-1} kanamycin in the case of seeds from transgenic lines. After about 3 weeks, 60 seedlings of each line at the four-leaf stage were transferred to the greenhouse and cultivated for 7–10 days in non-sterile standard soil (ED73) prior to infection-experiments. Twenty seedlings of the same size (1.5 cm total length, 0.1 cm stem diameter) of the transgenic test lines, as well as of wild-type (SR1) and vector-transformed (trSR1) lines, were then transplanted to control soil or to soil inoculated with *R. solani*. A random block inoculation and planting design were used to minimize variation in inocula strength and growth chamber micro-environments. Seedling growth in a chamber with constant environmental conditions (23°C; 75–80% relative humidity, daily irrigation with tap water) was monitored for 15–20 days. The severity of infection symptoms for plants grown in inoculated soil were estimated using a disease index scale (*DI*, see Results). In all cases, severity of the infection symptoms was rated by two people independently, one of whom was constantly without knowledge of the actual experimental design and code (blind design).

Statistical evaluation of the infection assays

In all cases statistical analysis was performed in two ways. First, the Mann–Whitney–Wilcoxon-test (U-test) was used to determine

significant differences of the distribution of the plants over the five disease classes since these experimental data already represent a ranking, which is usually the initial step of this test procedure. Within one experiment with 20 seedlings each this statistical method proved significance ($p = 0.05$) for distribution differences leading to an approximately 20% reduction of the corresponding calculated *DI* value (reduction compared with wild-type *DI*). With data collected from three or more replications of the experiment even less than 10% reduction of the *DI* could be demonstrated to be statistically significant. Secondly, *mean DI*-reductions were calculated for each plant line taking all repetitions of the respective infection assays into account. Significance of the differences between the *mean DI*-values of the tested transgenic plant lines and the wild-type controls were proved by means of the *t*-test ($p = 0.05$) following an analysis of variance (Sachs, 1972).

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