Different isoforms of chitinases and β-1,3-glucanases of tobacco (Nicotiana tabacum cv Samsun NN) were tested for their antifungal activities. The class I, vacuolar chitinase and β-1,3-glucanase isoforms were the most active against Fusarium solani germlings, resulting in lysis of the hyphal tips and in growth inhibition. In addition, we observed that the class I chitinase and β-1,3-glucanase acted synergistically. The class II isoforms of the two hydrolases exhibited no antifungal activity. However, the class II chitinases showed limited growth inhibitory activity in combination with higher amounts of class I β-1,3-glucanase. The class II β-1,3-glucanases showed no inhibitory activity in any combination. In transgenic tobacco plants producing modified forms of either a class I chitinase or a class I β-1,3-glucanase, or both, these proteins were targeted extracellularly. Both modified proteins lack their C-terminal propeptide, which functions as a vacuolar targeting signal. Extracellular targeting had no effect on the specific activities of the chitinase and β-1,3-glucanase enzymes. Furthermore, the extracellular washing fluid (EF) from leaves of transgenic plants expressing either of the secreted class I enzymes exhibited antifungal activity on F. solani in vitro comparable to that of the purified vacuolar class I proteins. Mixing EF fractions from these plants revealed synergism in inhibitory activity against F. solani; the mixed fractions exhibited inhibitory activity similar to that of EF from plants expressing both secreted enzymes.

Plant-pathogen interactions leading to a hypersensitive response result in the induction of resistance both locally around the sites of infection and systemically in noninfected parts of the plant. Resistance is induced against a broad range of pathogens, irrespective of which pathogen triggered the hypersensitive response. For example, inoculation of tobacco (Nicotiana tabacum cv Samsun NN) with TMV leads to a systemic induction of resistance against the fungi Phytophthora parasitica var nicotianae and Peronospora tabacina (McIntyre et al., 1981).

Induced resistance in tobacco is accompanied by the induced synthesis of PR proteins, including Chi and Glu (for reviews, see Bol et al., 1990; Bowles, 1990; Linthorst, 1991). In tobacco, three classes of Chi have been identified based on the structural analysis of their genes (Shinshi et al., 1990). Class I contains vacuolar isoforms with an N-terminal domain homologous to hevein. The Chi-II, known in the literature as PR-3a and PR-3b (formerly PR-P and PR-Q), are very homologous to Chi-I, but lack the hevein domain (Linthorst et al., 1990b; Payne et al., 1990a) and are located extracellularly. Very recently, a third class of tobacco Chi genes was described (Lawton et al., 1992) that encodes proteins homologous to Chi in cucumber and Arabidopsis (Meilhac et al., 1989; Samac et al., 1990) but that are not related to class I or II isoforms. Based on their primary structures, three major classes of Glu have been identified (Payne et al., 1990b; Ward et al., 1991a). Class I contains basic, vacuolar isoforms (Glu-I), whereas class II consists of acidic, extracellular isoforms. In the literature, the latter proteins are also known as the PR proteins PR-2a, PR-2b, and PR-2c (formerly PR-2, PR-N, and PR-O). Glu-I and Glu-II are serologically related and show an identity in primary structure of approximately 50% (Linthorst et al., 1990a). To date only one class III enzyme has been identified, an acidic extracellular protein showing 54 to 59% identity with the class II isoforms (Payne et al., 1990b).

The major components of the cell walls of many fungi are the polysaccharides chitin and β-1,3-glucan, substrates for Chi and Glu, respectively (Wessels and Sietsma, 1981). In vitro, growth of a number of fungi is inhibited by a Chi-I from bean (Schlumbaum et al., 1986) and combinations of Chi-I and Glu-I from pea (Mauch et al., 1988). These observations, together with the notion of an apparent lack of chitin in plants, and the concomitant induction of Chi and Glu and fungal resistance have led to speculations about a role for these hydrolytic enzymes in systemically induced resistance (Mauch and Staehelin, 1989).

Here we report the antifungal activity of the Chi and Glu from tobacco. To this end, the various Chi and Glu were purified to homogeneity and tested, either alone or in combination, for their ability to inhibit in vitro growth of Fusarium solani. In addition, tobacco was transformed with modified gene constructs of the Chi-I and Glu-I and constructs containing both modified genes, resulting in extracellular targeting of these enzymes. It appeared that these proteins retained their enzymic activity and that EF harvested from these transgenic plants caused lysis and growth inhibition of F. solani germlings in vitro in the same manner as was observed with the purified proteins.

Abbreviations: Chi, chitinase(s); CTPP, carboxyl-terminal propeptide; EF, extracellular washing fluid; Glu, β-1,3-glucanase(s); I(II, III), class I (II, III); PR, pathogenesis-related; TMV, tobacco mosaic virus.

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MATERIALS AND METHODS

Biological Materials

Tobacco plants (Nicotiana tabacum cv Samsun NN) were grown in pots in a greenhouse according to standard methods. For induction of PR proteins, leaves of 5- to 6-week-old plants were inoculated with TMV as described previously (Woloshuk et al., 1991). Leaves were harvested and stored at −80°C 7 d after inoculation.

Fusarium solani was kindly provided by Dr. P.J.G.M. de Wit, Department of Plant Pathology at the University of Wageningen, The Netherlands. The fungus was maintained on potato dextrose agar at 25°C in the light. Spores were harvested from 3- to 6-week-old plates by flooding the plate with water. The spore concentration was adjusted to 10,000 spores/mL.

Protein Purification

Proteins were extracted and purified by a modification of the method described by Woloshuk et al. (1991). TMV-infected tobacco leaves (400 g) were homogenized at 4°C in a Waring blender with 500 mL of 0.5 M sodium acetate, pH 5.2, 0.1% (v/v) β-mercaptoethanol, and active charcoal (1 g per 100 g leaves). The homogenate was filtered through four layers of cheesecloth and centrifuged at 3000g for 15 min. The supernatant was centrifuged for 50 min at 20,000g. The final supernatant was passed over a Sephadex G-25 (medium coarse; Pharmacia) column (12 × 60 cm) equilibrated in 40 mM sodium acetate, pH 5.2. The eluted protein solution was incubated overnight on ice before centrifuging for 50 min at 20,000g. The resulting supernatant was loaded onto an S-Sepharose (Fast Flow; Pharmacia) column (5 × 5 cm), equilibrated in 40 mM sodium acetate, pH 5.2, and the adsorbed proteins were eluted with 500 mL of a linear gradient from 0 to 0.4 M NaCl in buffer. Fractions exhibiting Chi activity were pooled and concentrated by ultrafiltration through YM10 membranes (Amicon). The same was done for the fractions containing glucanase activity.

The Chi pool was brought to 20 mM sodium bicarbonate and further dialyzed against 20 mM sodium bicarbonate, pH 8.3. Chi-I were allowed to adsorb for 1 h to a matrix of 50 mL of regenerated chitin (Molano et al., 1977) equilibrated in 20 mM sodium bicarbonate, pH 8.3. The chitin matrix was then filtered, and a column was poured and washed with 100 mL of 20 mM sodium bicarbonate and 100 mL of 20 mM sodium acetate, pH 5.2. Bound proteins were eluted with 20 mM acetic acid, pH 3.5. The eluate was further purified by a Superdex-75 gel-filtration column (HR 10/30; Pharmacia). Gel filtration was carried out in 50 mM potassium phosphate buffer, pH 7.0, containing 0.2 M NaCl at a flow rate of 0.5 mL min⁻¹. The fractions showing Chi activity were pooled and dialyzed to 20 mM sodium acetate, pH 5.2, and applied to a Mono S column (HR 5/5; Pharmacia), equilibrated to the same buffer. Bound proteins were eluted from the Mono S column by a linear gradient (20 mL) of 30 to 60 mM NaCl in the above buffer (1 mL min⁻¹). Fractions were separated on 12.5% SDS gels, and the 32- and 34-kD Chi were pooled accordingly.

The pool exhibiting Glu activity was loaded onto a gel-filtration column as above. Glu activity eluted at an apparent molecular mass of 5 to 10 kD.

Acidic proteins running through the S-Sepharose column were dialyzed to 20 mM Tris-HCl, pH 8.0, and loaded onto a Q-Sepharose (Fast Flow; Pharmacia) column (5 × 5 cm) equilibrated in 20 mM Tris-HCl buffer, pH 8.0. Bound proteins were eluted with 500 mL of a 0 to 0.3 M NaCl gradient in 20 mM Tris-HCl, pH 8.0. Fractions were analyzed by 10% native polyacrylamide gels. Pools of the class II proteins were prepared based on the electrophoretic pattern, except PR-2c, which was pooled based on Glu activity measurements. In some instances Mono Q (FPLC) ion exchange chromatography was performed to enhance the separation of PR-3a and PR-3b. All of the class II proteins were further purified by gel-filtration chromatography as described above.

Protein Analysis

Protein concentrations were determined by the method of Bradford (1976) using BSA as the standard. Electrophoretic analysis was performed by SDS-PAGE on 12.5% polyacrylamide gels according to the method of Laemmli (1970). In the case of native gels, SDS was omitted.

Chi and Glu activity measurements were performed using [3H]chitin and laminarin as substrates, respectively (Molano et al., 1977; Kauffmann et al., 1987). Chi activity was expressed in cpm μg⁻¹ of protein. The activity of Glu was expressed in nkat mg⁻¹ of protein, for which a nkat was the number of nmol of Glc released per s.

Transformation and Analysis of Transgenic Plants

Transgenic tobacco plants were obtained as described by Melchers et al. (1993). Isolation of EF was done as described by de Wit and Spikman (1982). Extracellularly targeted enzymes were purified from the EF as described for the proteins isolated from TMV-inoculated leaf material (see “Protein Purification”). Specific activities of Glu and Chi were determined as above.

In Vitro Antifungal Assay

The protocol described by Woloshuk et al. (1991) was modified as follows. The assay was performed in a 24-well microtiter dish (Greiner). Potato dextrose agar (250 μL) was pipetted into each well. Five hundred F. solani spores in 50 μL were added per well. The spores were pregerminated for 6 to 7 h at 25°C. Protein samples were dialyzed for 2 to 4 h against 50 mM potassium phosphate buffer, pH 6, at 4°C and subsequently filter sterilized through 0.22-μm filters. Protein concentrations were determined and adjusted appropriately. One hundred microliters of protein sample was pipetted into each well, resulting in a total volume of 150 μL. Denaturation of enzymes was done by boiling the samples for 10 min. Two hours after the initiation of incubation, the fungus was monitored microscopically for possible effects of the added proteins. After 3 d fungal growth was stopped by staining the mycelium with lactophenol cotton blue. Subsequently, the plates were destained with water.
RESULTS

Purification of Chi and Glu

The 32- and 34-kD Chi-I, the Chi-II PR-3a and PR-3b, the 33-kD Glu-I, and the Glu-II PR-2a, PR-2b, and PR-2c were purified to homogeneity from TMV-infected Samsun NN tobacco plants. The identity of the class II proteins was confirmed by their mobility on a native polyacrylamide gel (Fig. 1A). In the outer lanes proteins from the EF of TMV-infected leaves were loaded and used as markers. The purity of the proteins was checked by SDS-PAGE (Fig. 1B). The specific activity of each of the Chi after purification was determined on tritiated chitin. For the determination of the specific activity of Glu, laminarin was used as a substrate. The results are summarized in Table I. As reported by Legrand et al. (1987), the specific activity of the Chi-I (10,000-11,000 cpm Mg⁻¹ for the 32-kD Chi-I and 17,000-19,000 cpm Mg⁻¹ for the 34-kD Chi-I) was 10 to 15 times higher than that of the Chi-II (1,000-1,100 cpm Mg⁻¹). In agreement with Kauffmann et al. (1987), the specific activities of the Glu-I and the extracellular PR-2c were comparable (300-500 nkat mg⁻¹). The specific activities of PR-2a and PR-2b were 50- to 70-fold lower (5-10 nkat mg⁻¹).

Table I. Specific activities of purified Chi and Glu of tobacco

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi-I (32 kD)</td>
<td>10,000-11,000 cpm µg⁻¹</td>
</tr>
<tr>
<td>Chi-I (34 kD)</td>
<td>17,000-19,000 cpm µg⁻¹</td>
</tr>
<tr>
<td>Chi-II (PR-3a)</td>
<td>1,000-1,100 cpm µg⁻¹</td>
</tr>
<tr>
<td>Chi-II (PR-3b)</td>
<td>1,000-1,100 cpm µg⁻¹</td>
</tr>
<tr>
<td>Glu-I</td>
<td>400-500 nkat mg⁻¹</td>
</tr>
<tr>
<td>Glu-II (PR-2a)</td>
<td>5-10 nkat mg⁻¹</td>
</tr>
<tr>
<td>Glu-II (PR-2b)</td>
<td>5-10 nkat mg⁻¹</td>
</tr>
<tr>
<td>Glu-II (PR-2c)</td>
<td>400-500 nkat mg⁻¹</td>
</tr>
</tbody>
</table>

a Chi and Glu activities were determined as described in "Materials and Methods." Ranges from three independent experiments are listed.

In Vitro Antifungal Effect of Purified Enzymes

A microtiter dish assay was used to determine the effects of the purified proteins on the growth in vitro of F. solani. Proteins were added, either alone or in combination, to a suspension with pregerminated spores on a layer of potato dextrose agar in wells of a microtiter dish. After an incubation period of 2 h, the germling suspensions were microscopically monitored for lysis. Up to 5 µg per well for Chi-I, 50 µg for PR-3a, and 30 µg for PR-3b was tested for the Chi and up to 10 µg for each of the Glu. Of the Chi, only the Chi-I were capable of causing lysis of F. solani germlings. Application of 5 µg of Chi-I per well showed strong lysis activity (50% for 5 µg of 32-kD Chi-I; 80% for 5 µg of 34-kD Chi-I). Smaller amounts of these proteins showed intermediate levels of lysis. The Chi-II PR-3a and PR-3b exhibited no lysis activity at all, even when up to 50 and 30 µg per well, respectively, was added. Testing of the Glu revealed that only the Glu-I isoform displayed lysis activity. Complete lysis was observed with 1 µg per well, but with 0.5 µg of Glu-I no lysis was observed. The Glu-II showed no lysis activity even at the highest concentration of 10 µg per well.

Three days after addition of the proteins, the growing mycelium was stained with lactophenol cotton blue. Subsequently, the amount of mycelium was taken as a measure of growth. Typical results are shown in the left panel of Figure 2A for the Chi and in the right panel for the Glu. Intermediate growth inhibition of the fungus was observed upon incubation of the fungus with 5 µg per well of Chi-I. Chi-II had no effect on growth even at the highest concentration of 50 µg per well. Addition of 0.5 µg of Glu-I showed intermediate growth inhibition, and with 1 µg of Glu-I per well no fungal growth was observed. The Glu-II PR-2c (Fig. 2A), PR-2a, or PR-2b (data not shown) had no effect on the growth of F. solani, even at the highest concentrations tested (10 µg). As a control, enzymes were denatured by boiling for 10 min. By using this treatment, the antifungal activities were completely abolished.

Synergism between Chi-I and Glu-I

Mauch and coworkers (1988) have shown that combinations of Chi and Glu are capable of synergistically inhibiting fungal growth in vitro. Here, we tested which specific combinations of enzymes exhibited synergistic antifungal activity.

Figure 1. Native (A) and SDS-polyacrylamide (B) gel electrophoresis of proteins purified from TMV-infected Samsun NN tobacco. Molecular masses are indicated in kD.
Figure 2. Effect of purified Chi and Glu (A) and combinations thereof (B) on in vitro growth of *F. solani*. Amounts of protein (in μg) per well are indicated. Chi-I, 32-kD Chi-I; Chi-II, PR-3a; Glu-II, PR-2c; C, highest amount of protein after boiling for 10 min.

In Figure 2B, results are shown of combinations of specific Chi and Glu. As Chi-I, the 32-kD isoform was used. Neither 0.1 nor 0.5 μg of Chi-I and Chi-II showed any effect if applied separately (Fig. 2A). The same was true for 0.1 or 0.5 μg per well of Glu-II. No effect or an intermediate effect was observed with 0.1 or 0.5 μg Glu-I, respectively. However, the combination of 0.1 μg of Chi-I with 0.1 μg of Glu-I caused complete tip lysis of germlings of *F. solani* 2 h after addition of the enzymes and complete growth inhibition after 3 d. The Glu-II, PR-2a, PR-2b (data not shown), and PR-2c (Fig. 2B) were not able to substitute for Glu-I. Especially with PR-

2c this is striking because the specific activity of this enzyme on laminarin is comparable to that of Glu-I (Table I). Mixing Chi-II with 0.1 μg of Glu-I showed no effect. However, an enhanced growth inhibition was observed in combination with 0.5 μg of Glu-I, as compared to 0.5 μg of Glu-I alone. There was no difference in level of growth inhibition whether 1 or 5 μg of the Chi-II PR-3a was added to 0.5 μg of Glu-I. The effect was significantly less than with Chi-I, and no lysis was observed. The Chi-II PR-3b exhibited an effect that was similar but slightly more pronounced than that of PR-3a: only in combination with Glu-I was growth inhibition observed without lysis activity, and the antifungal effect was far less than that of the combination of Chi-I and Glu-I.

**Antifungal Effect of Extracellularly Targeted Chi-I and Glu-I**

Chi-I and Glu-I are synthesized as preproproteins from which an amino-terminal signal peptide and a CTPP are cleaved off during posttranslational processing (Shinshi et al., 1988; Neuhaus et al., 1991). To study the role of the CTPP in the vacuolar targeting of the enzymes, transgenic plants have been created that constitutively express either a chimeric, modified Chi-I gene or a chimeric, modified Glu-I gene, or both. The modifications involved the introduction of a stop codon in the 3′-terminal coding regions of the genes, thereby specifically excluding the CTPP-encoding sequence from the open reading frame (Fig. 3). In these transgenic plants, the transgene products are targeted extracellularly (Neuhaus et al., 1991; Melchers et al., 1993). Purification of these proteins from the EF of the transgenic plants showed that the specific activity of the extracellularly targeted Chi-I and of the Glu-I was not affected (data not shown).

To determine the antifungal activity of the secreted proteins, in vitro assays were done on *F. solani* germlings using EF from the transgenic plants. As a control, EF was harvested from nontransformed, regenerated tobacco plants. Five mi-
showed clear inhibition of fungal growth. The denatured EF effect of the double construct could be mimicked by mixing denatured EF, EF (from a pMOG 556 plant) boiled for 10 min. to have a 50- to 70-fold lower specific activity, despite their laminarin the specific activities of the 33-kD Glu-I and the higher than those of the class II PR-3a and PR-3b proteins fractions had no effect on the fungus.

Chi-I or a GIu-I transgenic plant had a minor inhibitory plants with an equal amount of EF from either a retargeted Glu-I; C (control), regenerated nontransformed tobacco plant; total of 5 μg of protein was added per well. pMOG 189, Extracellularly targeted Chi-I; pMOG 549, extracellularly targeted Glu-I; pMOG 556, extracellularly targeted Chi-I and extracellularly targeted Glu-I; C (control), regenerated nontransformed tobacco plant; denatured EF, EF (from a pMOG 556 plant) boiled for 10 min.

DISCUSSION

To assess the in vitro antifungal activity of various Chi and Glu of tobacco, the class I and class II isoforms of these enzymes were purified to homogeneity. In accordance with Legrand et al. (1987), the specific activities of the 32- and 34-kD Chi-I were found to be approximately 10- to 15-fold higher than those of the class II PR-3a and PR-3b proteins when tested on tritiated chitin as substrate. When tested on laminarin the specific activities of the 33-kD Glu-I and the class II PR-2c protein were found to be very similar. In contrast, the class II enzymes PR-2a and PR-2b were shown to have a 50- to 70-fold lower specific activity, despite their >90% identity in primary structure with PR-2c (Linthorst et al., 1990a; Ward et al., 1991a). Similar results have been reported by Kauffmann et al. (1987).

To determine the effects of the purified proteins on the growth in vitro of F. solani, a microtiter dish assay was used. Both Glu-I and Chi-I exhibited high antifungal activities. Combinations of Glu-I and Chi-I revealed that these two purified enzymes acted synergistically to inhibit fungal growth. The Chi-II PR-3a and PR-3b are not inhibitory by themselves, even when amounts of protein up to 50 μg were added to the wells; in combination with Glu-I, synergism was observed, although to a much lesser extent than between Chi-I and Glu-I and without lysis activity. In contrast to Glu-I, none of the three Glu-II showed any antifungal activity, either alone or in combination with Chi. In view of the specific activity of the Glu-II PR-2c and its approximately 50% identity in primary structure with Glu-I, this result is unexpected. Apparently, hydrolytic activities as determined on the artificial substrate laminarin are not inhibitory per se for fungal growth in vitro. Clearly, the classification of enzymes based solely on their ability to hydrolyze a certain substrate is an oversimplification. Here we demonstrated that there is a clear distinction between enzymic and antifungal activities of the different Chi and Glu. This finding contributes to our understanding of why so many different kinds of hydrolytic proteins are produced in plants.

A hypersensitive reaction of plants to pathogenic infections leads to the induction of Chi and Glu activities, both locally and systemically. At the same time, resistance is induced, directed toward a broad range of pathogens, including fungi. The observation that Chi and Glu exhibit antifungal activities in vitro has led to speculations regarding a direct antifungal role of these hydrolytic enzymes in systemically induced fungal resistance (Boiler, 1988; Mauch and Staehelin, 1989).

Recently, it was shown that in TMV-infected tobacco the messengers for Glu-II and Chi-II are induced both locally around the site of infection and systemically in the noninfected parts of the plants. In contrast, the class I isoforms of both enzymes are induced to a high level locally, but they are not induced systemically (Brederode et al., 1991; Ward et al., 1991b). Here, we have shown that only the locally induced, class I hydrolytic enzymes exhibit antifungal activity in vitro. These observations together make it very unlikely that either class I or class II Chi and Glu fulfill a direct antifungal role in systemically induced fungal resistance. This does not rule out the possibility that the enzymes have an indirect role in plant defense or that they act synergistically with other types of antifungal proteins. They might be involved in the generation of signal molecules, which in turn trigger the defense system. Indeed, Keen and Yoshikawa (1983) have shown that Glu from soybean are capable of releasing elicitor-active carbohydrates from fungal cell walls.

In addition to their possible role in defense, Glu and Chi may have functions in a number of developmental processes in plants as well. Glu's are very likely involved in cell wall metabolism and cell expansion in seedlings (Wong and MacLachlan, 1980) and in microsporogenesis (Worall et al., 1992). Recently, a Chi was shown to have the capability to rescue a temperature-sensitive carrot somatic embryo mutant, suggesting that the enzyme might be involved in somatic embryogenesis (De Jong et al., 1992). In this context it is
noteworthy that during the early period of imbibition of barley seeds, two embryo-associated Chi are selectively released (Sweg et al., 1992) and that in Saccharomyces cerevisiae a Chi is required for cell separation during growth (Kuranda and Robbins, 1991).

Transgenic plants have been made that constitutively express a modified Chi-I, a Glu-I, or both. In these plants the modified gene products were targeted extracellularly. The specific activities of the targeted Chi and Glu were not decreased as determined by incubation on their respective artificial substrates, tritiated chitin and laminarin (our unpublished data).

The EF from transgenic plants expressing either the modified Chi-I or the modified Glu-I gene exhibited intermediate inhibitory activity on F. solani. The amount of Glu and Chi in the various EF fractions was calculated, and it appeared that the same amount of EF enzyme was needed to demonstrate antifungal activity as in the assays using the purified proteins. Clearly, the extracellularly targeted class I proteins have retained their antifungal activity. Mixed EFs of the plants containing the single constructs showed synergism similar to that observed with the purified proteins. Almost complete lysis of the germinating tips and growth inhibition of the fungus were demonstrated. In agreement herewith was the drastic antifungal effect found in the EF harvested from transgenic plants containing the construct of both modified genes. It can be concluded that targeting of these enzymes can be done successfully without any significant loss of antifungal activity.

Recently, Broglie and coworkers (1991) demonstrated increased resistance to Rhizoctonia solani in transgenic tobacco plants constitutively expressing a bean Chi-I. Many fungi penetrate their host through the stamens and never actually enter the plant cell but reside in the extracellular space. Future experiments will show whether targeting of Chi-I and Glu-I to the extracellular space is an effective way of increasing fungal resistance in plants.

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