*In vitro*sensitivity and tolerance of *Fusarium solani* towards chitinases and ß-1,3-glucanases

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Voor Uri, Lior en Yarden

IN VITRO SENSITIVITY AND TOLERANCE OF *FUSARIUM SOLANI* TOWARDS CHITINASES AND ß-1,3-GLUCANASES

PROEFSCHRIFT

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door

Marianne Beatrix Sela-Buurlage
geboren op 7 maart 1962
te Amersfoort

Stellingen

1) Hoewel chitinases en ß-1,3-glucanases substraten in de schimmelcelwand kunnen afbreken is dat geen garantie voor een brede antischimmel aktiviteit van deze eiwitten.
Dit proefschrift.

2) De synergie tussen de enzymen Chi-I en Glu-I is zo groot dat alleen met behulp van transgene planten aangetoond kon worden dat deze twee eiwitten afzonderlijk zeer beperkte antischimmel aktiviteit bezitten.
Dit proefschrift.

3) Ofschoon de eiwitten Chi-I en CBP20 beiden zowel chitinase aktiviteit bezitten als een chitine bindend domein bevatten, vertonen zij toch synergie in antischimmel aktiviteit.
Dit proefschrift.

4) Hoewel de antischimmel aktiviteit van Chi-I wordt verhoogd door de aanwezigheid van een chitine-bindend domein in dit enzym, is in specifieke situaties dit domein verantwoordelijk voor verminderde gevoeligheid voor dit eiwit.
Dit proefschrift.

5) Het is onwaarschijnlijk dat pathogenese-gerelateerde eiwitten verantwoordelijk zijn voor systemisch geïnduceerde resistentie, daar die eiwitten met de hoogste antischimmel aktiviteit slechts locaal worden geïnduceerd.
Brederode et al., PMB 17: 1117-1125; Ward et al., Plant Cell 3: 1085-1094.
Dit proefschrift.

6) Het feit dat de term "pathogenese-gerelateerd" vaak tussen aanhalingstekens wordt geplaatst, geeft de twijfel aan over de rol van "pathogenese-gerelateerde" eiwitten bij pathogenese.
Legrand et al., (1987), PNAS 84: 6750-6754; Kauffmann et al., (1987), EMBO J. 6: 3209-3212; Kauffmann et al., (1990), PMB 14: 381-390; Stintzi et al., (1994) Biochimie 75: 687-706.

7) Het feit dat Oomyceten er uitzien als schimmels, groeien als schimmels en planten kunnen aantasten als schimmels, is onvoldoende deze in te delen in het schimmelrijk, daar DNA-analyse en promoter studies aangeven dat zij dichter bij alg, mens en *Arabidopsis thaliana* staan dan bij andere schimmels.
Coffey & Tyler, APS-meetings, 1994.

8) De snelheid waarmee de nummering van genconstructen oploopt, weerspiegelt de beschikbare onderzoeksgelden in een plantenbiotechnologisch bedrijf.

9) In allerlei organisaties kan het "Peterprincipe" worden waargenomen, waarbij werknemers in een hierarchie opklimmen tot een nivo van incompetentie en het werk in feite wordt verricht door mensen die nog net niet dat nivo bereikt hebben.
Peter & Hull.

10) Het feit dat een uur forensen in Nederland als onredelijk wordt beschouwd, maar in vele steden in de Verenigde Staten als gemiddeld wordt gezien, onderschrijft de bewering dat afstand een relatief begrip is.

Stellingen behorend bij het proefschrift: "*In vitro* sensitivity and tolerance of *Fusarium solani* towards chitinases and ß-1,3-glucanases"

Wageningen, 12 september 1996 Marianne B. Sela-Buurlage

Contents

List of Abbreviations

CBD	chitin binding domain
CBP20	20 kD chitin-binding protein
Chi-I	class I chitinase
Chi-I*	Chi-I targeted extracellularly in transgenic plants
CTPP	carboxy-terminal propeptide
EF	extracellular washing fluid
f. sp.	forma speciales
GI	growth inhibition
Glu-I	class I ß-1,3-glucanase
Glu-I*	Glu-I targeted extracellularly in transgenic plants
IF	intercellular washing fluid
kD	kilo Dalton
ODu	optical density units
PDA	potato dextrose agar
PR	pathogenesis-related
PVDF	polyvinylidene difluoride
sp	spores
spp	species
TMV	tobacco mosaic virus
win	wound-induced

AIM AND OUTLINE OF THIS THESIS

In agriculture, fungal diseases have always been one of the major problems. Many options exist to combat the pathogens responsible. Application of fungicides is for specific diseases a very effective means of control. However, new strains of fungal pathogens may emerge showing resistance to such compounds. Moreover, environmental and health concerns have made these chemicals less favourable. Crop rotation is a possibility to control disease, but is economically less attractive for farmers. Traditional plant breeding to obtain resistant cultivars fits best in a system of sustainable agriculture. However, this technology is very laborious and time consuming. Also, desired resistance traits might not be available within the species or even within related species. Since the development of technology for genetic engineering of plants, new strategies for introducing resistance in plants to fungal pathogens have emerged.

In the first chapter of this thesis, a review is presented on the various strategies that are used or could possibly be used in the future to genetically engineer fungal resistance. One of the strategies followed at MOGEN involves overexpression of one or more antifungal proteins. The work, presented in this thesis, is part of this strategy. An *in vitro* assay had been established to assist in the isolation and identification of such antifungal proteins (Woloshuk *et al.*, 1991) and has played a pivotal role in the results described here. In search for such antifungal proteins, the phenomenon of induced resistance is exploited. *Nicotiana tabacum*, cv. Samsun NN, when inoculated with tobacco mosaic virus (TMV), acquires resistance to subsequent pathogen attack. Synthesis of a large number of pathogenesis-related (PR) proteins is induced (Linthorst, 1991).

In Chapter 2 results are described using protein extracts from tobacco leaves inoculated with TMV. These induced extracts were calibrated for the levels of known PR-proteins and tested *in vitro* on a variety of fungi. The majority of fungi were inhibited in growth by these extracts. Spores of all fungi were far more sensitive to induced protein extracts if pregerminated before addition of the extracts, when compared to assaying without pregermination.

The natural location of many antifungal tobacco PR-proteins, such as Chi-I, Glu-I and AP24, is the vacuole. However, since many pathogens reside in the intercellular spaces, overexpression of these proteins is expected not to yield the desired protective effect. Therefore, genes were modified in such a way that proteins, in stead of being targeted to the vacuole, were rerouted extracellularly. Results of these experiments are presented in Chapter 3.

In Chapters 4 and 5 several of the tobacco PR-proteins were purified and assayed for their *in vitro* antifungal effects. In Chapter 4, the proteins of group PR-2, ß-1,3-glucanases, and PR-3, chitinases, were assayed for their antifungal activity, either alone or in synergy. Apoplastic

5, the isolation, enzymatic activity and antifungal activity of the class I PR-4 CBP20, is described.

In Chapter 6, the proteins from transgenic plants described in Chapter 4, were reisolated in order to analyze whether extracellular targeting had affected antifungal activity.

As observed in Chapter 2, non pregerminated fungal spores were far less sensitive to induced protein extracts compared to germlings. In Chapters 7 and 8, the phenomenon of decreased sensitivity occurring during incubation with antifungal proteins is further investigated using *F. solani* f.sp. *phaseoli* as a model system. The effect of germination time before addition of proteins was studied. Results presented indicate that macroconidia adapt to the presence of specific chitinases only during the first three hours of germination. Concomitantly, as described in Chapter 8, specific protease(s) are released by the germinating spore capable of cleaving the chitin-binding domain from Chi-I and CBP20. The influence of this chitin-binding domain on the level of antifungal activity of Chi-I and CBP20 as well as its role on the adaptation phenomenon has been determined.

The overall results described in this thesis are summarized in Chapter 9. The use of an *in vitro* assay to assist in the isolation of antifungal proteins is addressed in detail. Since it was demonstrated that macroconidia can adapt to the presence of specific antifungal proteins, the relevance of this observation is discussed. Finally, the importance of *in vitro* identification of antifungal proteins in engineering fungal resistant plants is demonstrated.

Chapter 1

Host Plant Defense Mechanisms and their Exploitation in Engineering Fungal Resistant Plants

INTRODUCTION

In nature, plants coexist with numerous potential pathogens, such as viruses, bacteria and fungi. Most plants are non-hosts for most of these micro-organisms, of which many are not capable of entering the plant and establishing an interaction. In exceptional cases, a micro-organism is capable of penetrating and colonizing the host tissue. The resulting interaction can be of symbiotic nature, in which both partners benefit from the interaction. Examples include *Rhizobium* spp. able to fix nitrogen in the Leguminosae and mycorrhizal fungi which colonize the root tissue of many plant species. In the latter case, the extended root system is beneficial for retrieving more nutrients from the soil, especially under growth limiting conditions.

In stead of being beneficial, the interaction between a plant and a micro-organism can also be harmful to the plant. The micro-organism is then called a pathogen. When the interaction between the pathogen and the plant is compatible (the plant is susceptible and the pathogen is virulent), the pathogen is capable of spreading throughout the host tissue causing extensive damage and even death of the plant. However, in many cases the plant is able to resist entry by the pathogen and the interaction is termed incompatible (the plant is resistant and the pathogen avirulent).

In this review, various resistance mechanisms are discussed, which are now or maybe in the future exploited in engineering resistant plants. The final aim of this type of research is to generate transgenic plants resistant to various pathogens. The strategies to genetically engineer resistance into commercial crops might serve as alternatives to conventional resistance breeding and chemical control against pathogens. The former being time consuming and laborious and the latter coming under increasing scrutiny, since resistance in the pathogens against these chemicals occurs frequently. Additionally, environmental constraints on these compounds make them less favourable in practice.

In literature, often a distinction is made between two types of resistance. The first type is called passive or constitutive defense, which is present in plants even in the absence of a pathogen. The second type of resistance is called active, whereby the plant responds to pathogen attack with reinforcement of existing barriers or *de novo* synthesis of antimicrobial compounds. However, these two types of resistance mechanisms are often not so distinct, but rather overlap. Nevertheless, in this review we will follow this distinction. Firstly, pre-existing defense systems will be described, where the various components of the cell wall structure and composition will be dissected as well as toxic compounds and other 'resistance factors'

constitutively present inside the host cell. Secondly, the various steps in active defense systems will be discussed. Perception of either specific or aspecific elicitors of the pathogen, responses to these elicitors, transduction of signals to induce defense responses, both locally, such as the hypersensitive response (HR) or phytoalexin production, as well as distally, such as the phenomenon of systemic acquired resistance (SAR), will be discussed. Progress that has been made in exploitation of these various types of defense systems in genetic engineering will be discussed. Additionally, other strategies, focusing on the use of non-plant compounds will be incorporated into the various sections, in case their expected mode of action is similar to that involving plant factors.

1. CONSTITUTIVE DEFENSE SYSTEMS

1.1 Host surface

The first contact between a potential pathogen and a host occurs on the plant surface. The pathogen has various tools to facilitate successful contact and orientation followed by penetration. In this section some of these tools will be described as well as possibilities for genetic engineering to interfere with the pathogen in this initial process.

1.1.1 Cuticle

The outer layer of the above ground parts of the plants is protected by a waxy cuticle which is made up of cutin, an insoluble polyester of C_{16} and C_{18} fatty acid derivatives. The main function of this layer is to prevent these aerial parts from desiccation. However, an additional role of the cuticle layer might be a barrier against potential pathogens. Various fungi, are capable of penetrating the cuticle, either mechanically through force or by secreting enzymes capable of degrading the cuticle such as cutinases (Kolattukudy, 1985). For *Fusarium solani* f. sp. *pisi* it has been shown that pathogenicity on pea was significantly decreased upon disruption of the cutinase gene (Rogers *et al*, 1994). Moreover, transfer of this cutinase gene to *Mycosphaerella* spp., a papaya pathogen, only capable of infecting through wounds, allowed transformants to infect fruit through the intact cuticle (Dickman *et al.*, 1989).

Altering cuticle composition in transgenic plants might render specific cutinases ineffective, thus preventing penetration. More knowledge is needed both on specific target sites of the cutinase on its substrate as well as on key enzymes in the biosynthesis of cutin. Recently, it was shown by Gilbert and Dean (1995) that in germlings of *Magnaporthe grisea* appressoria are specifically induced by 1,16-hexadecanediol. The authors determined that both the length of the carbon chain as well as the number and position of hydroxyl groups were crucial. Theoretically, another option to obtain fungal resistant plants is interference with cutinase by overproduction of cutinase inhibitors in transgenic plants. However, such a strategy will be very complicated since these products needed to be targeted through the cuticle. Additionally, once these proteins would reach the surface of the host, they would be exposed to varying environmental conditions which could destabilize them. At present it remains doubtful whether genetic engineering will venture into this field.

1.1.2. Morphology

Most bacteria, viruses and many fungi cannot penetrate the cuticle. Their only means of successful entry into the host tissue is through wounds and stomates. In case a fungus enters its host through stomates, sensing mechanisms in the pathogen are necessary to locate these entry sites. Morphology of the plant surface as well as chemical stimuli are important for spore germination, directional fungal growth and penetration (for overview: see Buurlage, 1987). It has been demonstrated that poplar cultivars, resistant to *Melampsora larici-populina* possess less stomates than susceptible varieties. Additionally, specific ridge heights on the leaf surface are necessary for good directional growth of the fungus. They were shown to be less pronounced in resistant varieties (Mlodzianowski *et al.*, 1978). Similarly, *Uromyces phaseoli* needs a specific change in ridge height, either on artificial substrates or of the stomates of bean leaves, to produce appressoria (Hoch, 1987). Too low a height will not induce an appressorium, which is essential for successful penetration. Similarly, chemical stimuli such as glucose, saccharose and potassium salts are inducers of appressorium formation of rusts (Kaminskyi and Day, 1984; Staples *et al.*, 1983). Additionally it was shown that cAMP is capable of inducing appressoria in *Magnaporthe grisea* (Gilbert and Dean, 1995).

Once plant factors have been identified responsible for the recognition responses in the pathogen, such as oriented germ tube growth or appressorium formation, morphology of the outer parts of the plant could be altered such that the pathogen no longer recognizes the host and

therefore can not form infection structures ("evasion resistance"). At present it is not known which gene(s) code(s) such structural features in plants and subsequently their usefulness in genetic engineering remains limited.

1.2. Cell Wall

During penetration, the potential pathogen is confronted with the plant cell wall. The cell wall consists of a rigid crystalline matrix of cellulose, a polymer of crosslinked ß-1,4-glucans. These glucans allow attachment of various hemicelluloses such as glucuronoarabinoxylans or xyloglucan (Condit and Meagher, 1986). A plant cell wall also contains many structural proteins, which are rapidly immobilized after deposition through a still unclear mechanism. Examples are hydroxyproline-rich glycoproteins (HRGPs) or extensins (Fry *et al.*, 1986) which are found in phloem and cambium cells and proline- or glycine-rich proteins which are mainly present in xylem cells or lignifying cells. In many cell walls lignin is present, which is a polymer of various products derived from the phenylpropanoid pathway. Between cells the middle lamella is located, consisting of pectin and pectate. Pectins consist mainly of polygalacturonic acids and rhamnogalacturonans. They form the matrix into which cellulose microfibrils are embedded. Specific soluble HRGPs are also located in the middle lamella (Showalter, 1993).

The plant cell wall provides the cell its structural integrity and is involved in other functions such as water and metabolite flow, cell enlargement and cell development. Additionally, the plant cell wall plays a role in defense against pathogen attack. With the exception of pathogens that reside in the intercellular spaces of plant tissues, most pathogens have to penetrate the plant cell and are confronted with the cell wall barrier. Enzymes, capable of degrading specific cell wall components have been identified in a number of bacteria and fungi. Examples include endopolygalacturonases produced by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Benhamou *et al.*, 1990) and *Colletotrichum lindemuthianum* (De Lorenzo *et al.*, 1990), endopolygalacturonate lyases by *Erwinia carotovora* (Davis *et al.*, 1984), pectinases, pectate lyases, pectin methyl esterases, xylanases by *Trichoderma viride*, although not a plant pathogen, (Bailey *et al.*, 1990; Lotan and Fluhr, 1990), cellulases (McNeil *et al.*, 1984) and glucanases by *Xanthomonas campestris* (Gough *et al.*, 1988). Inhibitors of certain polygalacturonases have been identified. They will be discussed in section 2.2.1, since these inhibitory proteins also seem to play a role in the elicitation of defense systems.

No significant progress has been made on exploiting pre-existing defense systems in genetic engineering since they are difficult to manipulate. More knowledge is needed on genes involved in cuticle and cell wall synthesis. Possibilities to increase resistance to fungal attack might depend on modification of the chemical composition or structure of the cuticle or the cell wall, leading to decreased susceptibility to enzymatic degradation by pathogens.

1.3 Toxic Compounds

A last line of defense for the host lies in the release of toxic compounds upon penetration of the plant cell, either preformed or *de novo* synthesized upon attack. The latter will be discussed in section 2 of this review. Examples of the former include small, acid-soluble, basic antimicrobial peptides produced in high abundance by maize kernels (Duvick *et al.*, 1992) as well as small cyclic peptides, produced by blue-green algae, which showed marked *in vitro* antifungal activity (Frankmolle *et al.*, 1992). Other examples include the family of thionines (for reviews, see Bohlmann, 1994; Florack and Stiekema, 1994), small basic proteins, found in seeds of many crops, including *Raphanus sativus* and *Mirabilis japonica*. They were shown to be very inhibitory to fungal growth *in vitro* (Cammue *et al.*, 1992; Terras *et al.*, 1992).

Another family of toxic compounds are the saponins, triterpenoids that are found in many plant species. A well studied example is avenacin, a saponin formed by oat. Specific pathogens of oat such as *Gaeumannomyces graminis* f. sp *avenae* are capable of detoxifying this avenacin by an enzyme, avenacinase, whereas for example the wheat pathogen *G. graminis* f.sp. *tritici*, unable to attack oat, lacks this enzyme. A related enzyme, called tomatinase, was identified in tomato pathogens such as *Septoria lycopersici*, capable of detoxifying the saponin, tomatine, from tomato, but not from oat (Bowyer *et al.*, 1994). Once the biosynthetic pathway of these compounds is known, saponin structure can be altered in transgenic plants such that the fungal enzyme of the corresponding pathogen can no longer detoxify such a compound. Alternatively, heterologous saponins could be overexpressed, for example avenacin in transgenic tomato.

Many plant species produce so-called ribosome inactivating proteins (RIPs). They are very toxic due to the fact that protein synthesis is inhibited by modification of 28S rRNA such that elongation factor 2 binds inefficiently (Leah *et al.*, 1991, Logemann *et al.*, 1992, Stirpe *et al., 1992*). The RIPs do not inactivate endogenous ribosomes, but only foreign ones.

Overexpression of antifungal compounds probably represents the most obvious strategy of engineering fungal resistance in transgenic plants. Overexpression of antifungal proteins in

plants can easily be achieved. However, for production of non-proteinaceous compounds, detailed knowledge on the biosynthesis and possible key enzymes is crucial. So far, only successful examples of the first case have been reported. For example, tobacco plants, stably transformed with the gene encoding the *Rs*-AFP2 thionine showed enhanced resistance to *Alternaria longipes* and *Botrytis cinerea* (Terras *et al.*, 1994). Similarly, Logemann *et al.* (1992) have shown that overexpression of a barley RIP in transgenic tobacco led to enhanced resistance to *Rhizoctonia solani* .

1.4 Resistance Factors

An alternative approach which has been used, is to identify specific resistance factors which might eventually be incorporated into genetic engineering strategies. It is based on comparing a resistant host cultivar with a susceptible one. This strategy has been successfully applied in pea where a disease resistance response (DRR) gene has been cloned from a resistant variety (Chiang and Hadwiger, 1990). The function of the protein is unknown, but homologs have been identified in parsley (Somssich *et al.*, 1988), potato (Matton and Brisson, 1989), bean (Walter *et al.*, 1990) and birch pollen allergen (Breiteneder *et al.*, 1989). Recently, homology to RNAse was suggested (Moiseyev *et al.*, 1994). A second example consists of a 45 kD protein correlated with resistance in musk melon to *Pseudoperonospora cubensis* (Balass *et al.*, 1992). Conceivably, overexpression of such genes might enhance fungal resistance. However, since functions of these proteins are presently unknown their role in the actual defense is as yet uncertain.

An example of this approach showing that a resistance factor directly encodes a resistance function, has been described in maize. *Helminthosporium carbonum* produces a toxin, HC-toxin, that is crucial to the fungus for successful colonization of the host tissue (Yoder, 1980). In maize cultivars resistant to HC-toxin producing isolates, an enzyme is present, HC-toxin reductase, which is capable of degrading HC-toxin. The HC-toxin reductase protein as well as the coding gene *Hm1* have been isolated (Meeley and Walton, 1991; Johal and Briggs, 1992). Constitutive expression of such detoxifying enzymes might provide control to fungal attack. However, HC-reductase only detoxifies the toxin produced by race 1 of the fungus which limits its usefulness. Research is now directed towards isolation of additional detoxifying genes. Possibly co-expression of two or more of such genes might prove more effective.

Finally, another approach has been followed where fungal compounds, deleterious for the plant, are identified and subsequently inactivated. During infection, *Sclerotinia sclerotiorum*

produces high levels of oxalic acid, which are very harmful to host cells. An enzyme has been identified, oxalate oxidase, which degrades oxalic acid. Constitutive overexpression of this gene in transgenic tobacco and oilseed rape rendered these hosts less sensitive to the acid (Dumas *et al.*, 1994). In contrast, however, it has been shown that mutant strains of *Sclerotinia minor* which do not produce oxalic acid were not less virulent on either peanut or lettuce compared to oxalic acid producing strains (Li *et al.*, 1995b). These data suggest that oxalic acid is very likely not the sole pathogenicity factor.

It can be concluded that knowledge of the various constitutive, pre-existing defense systems present in plants, might generate successful strategies to obtain transgenic plants. Altering the structure of cells or organs of a plant are not feasible yet. More molecular and biochemical studies need to be performed to elucidate the nature and mode of action of genes involved in physical and chemical structure of the cell wall. Obviously, negative effects on plants can be anticipated if crucial parts of plants are altered.

2. INDUCED DEFENSE SYSTEMS

2.1 General

In this section, induced defense systems, as a second type of resistance mechanism, are reviewed. It is an active defense response, whereby the plant responds to pathogen attack with reinforcement of existing barriers or *de novo* synthesis of antimicrobial compounds. The various phases in this process will be discussed. Perception of either specific or aspecific elicitors of the pathogen, responses to these elicitors, transduction of signals to induce defense responses, both locally, such as the HR or phytoalexin production, as well as distally, such as the phenomenon of systemic acquired resistance (SAR), will be discussed. Furthermore, possibilities for exploitation of various aspects of induced resistance in genetic engineering will be discussed.

Upon challenge of the host by a pathogen, a set of defense responses is induced in the plant. Both in susceptible and resistant plant,s responses are observed, however timing, speed and intensity can vary and thus the efficacy of resisting attack. In the case of *Cladosporium fulvum* on tomato similar protein patterns were observed both in compatible and incompatible

interactions. In compatible interactions, however, the induction of proteins was delayed and sometimes at lower levels compared to the incompatible situation (De Wit *et al.*, 1986). Similarly in bean it was shown, that induction and response of expression of defense genes were not correlated and were induced via various mechanisms (Jacobek and Lindgren, 1993; Jacobek et al., 1993). Both avirulent bacterial pathogens (either living or heat killed) and non-pathogens were shown to activate bean defense genes. The hypersensitive response (HR) is only observed with a living avirulent bacterial pathogen, whereas this is suppressed by a virulent, metabolically active, bacterial pathogen.

As stated earlier, a distinction is often made between pre-existing and induced defense mechanisms. However, induced defense mechanisms often involve reinforcement of preexisting barriers in the presence of pathogens.

2.2 Elicitors

Generally, elicitors are defined as compounds capable of interacting with the host and inducing defense responses. Many elicitors have been identified. They can be divided into two major classes. One class is represented by aspecific elicitors, which are able to induce defense responses in more than one plant species. These elicitors are described in section 2.2.1. The second class, discussed in section 2.2.2., consists of specific elicitors, only formed by avirulent races of a pathogen.

2.2.1 Aspecific Elicitors

Aspecific elicitors can induce defense responses in various host and non-host species. They can either be produced by the pathogen or released from the host cell wall by fungal enzymes. Examples of the first group are harpins, hypersensitive response-inducing bacterial proteins (Wei *et al.*, 1992) and the tobacco mosaic virus coat protein, which can be regarded as a necrosis-inducing protein (Culver and Dawson, 1991). Elicitors released from the fungal cell wall have been identified including chitin fragments of specific sizes (Barber *et al.*, 1989; Barber and Ride, 1988; Felix *et al.*, 1993; Ride, 1975 ; Ride *et al.*, 1989) and chitosan fragments (El Ghaouth *et al.*, 1994; Kauss *et al.*, 1989; Young *et al.*, 1982). Acetyl residues appear to be necessary for their elicitor activity (Barber *et al.*, 1989). Interestingly, a chitin deacetylase has been identified from *Colletotrichum lagenarium.* This enzyme can inactivate the elicitor activity of chitin and chitosan, thus preventing induction of host defense responses.

(Siegrist and Kauss, 1990). Additionally, glucan fragments of *Phytophthora megasperma* f. sp. *glycinea* walls have been shown to possess elicitor activity (Kopp *et al.*, 1989).

From the latter fungus also a glycoprotein has been isolated capable of eliciting phytoalexin synthesis in parsley cell and protoplast suspensions (Parker *et al.*, 1991; Renelt *et al.*, 1993). Nearly all *Phytophthora* species produce an additional 10 kD protein which induces necrosis in tobacco leaves. These proteins have been termed 'elicitins" and are produced *in vitro* and *in vivo* (Huet and Pernollet, 1989; Huet *et al.*, 1992; Ricci *et al.*, 1989; Terce-Laforgue *et al.*, 1992). It is assumed that elicitins are primarily toxins that weaken the host enabling the pathogen to invade the plant tissue. The elicitor function leading to systemic acquired resistance is more rare but therefore these molecules could be considered avirulence factors (Huet *et al.*, 1995). Only *P. nicotianae*, a pathogen of tobacco, does not produce these elicitins. When elicitins are added at sublethal doses, they provoke rapid membrane depolarization and induce the biosynthesis of phytoalexins. Recently, also *Pythium vexans* has been shown to excrete such elicitins (Huet *et al.*, 1995). Possibly, expression of such elicitor proteins in transgenic plants under control of a pathogen inducible promoter, could provide resistance to fungal attack by inducing the plant's own defense systems.

Apart from elicitors of pathogenic origin, specific host cell wall fragments are capable of eliciting defense responses. Many oligosaccharides with elicitor activity, such as oligogalacturonides, can be released from the host cell wall by enzymes produced by the pathogen (Darvill & Albersheim, 1983; Keen & Yoshikawa, 1983; Mathieu *et al.*, 1990; Ryan, 1994). However, only oligogalacturonides of certain size, released from the bean cell wall by endopolygalacturonases from *Colletotrichum lindemuthianum* elicit a host defense response. The highest activity of elicitation is observed with fragments with a degree of polymerization between 9 and 16 (Ryan, 1994). Polygalacturonase inhibiting proteins (PGIPs) have been isolated from bean capable of inhibiting the degradation of the elicitors (Cervone *et al.*, 1989; De Lorenzo *et al.*, 1990). Similar proteins have also been isolated from pear. Transgenic tomato plants have been created overexpressing pear PGIP and reported to show enhanced resistance to *Botrytis cinerea* (Powell *et al.*, 1994).

Finally, application of chemicals, including polyacrylic acid, (acetyl)salicylic acid, isonicotinic acid derivatives, heavy metals and ethylene as well as stress conditions such as wounding, UV treatment or ethylene, elicit specific defense responses which will be discussed later (Kessmann *et al.*, 1995; Ryals *et al.*, 1992; Sequeira, 1983). Conceivably, genes necessary for recognition of such compounds can be isolated and overexpressed in transgenic plants. Upon treatment of plants with these chemicals, a resistant state is induced.

2.2.2 Specific Elicitors

Apart from aspecific elicitors also specific elicitors have been identified. They represent products of avirulence genes only formed avirulent races of a pathogen. Examples including bacterial and fungal elicitors are described (review by De Wit, 1995). Avirulence genes or avirulence gene products (elicitors) have been identified from both bacterial (*Pseudomonas syringae* spp. *syringae* (Innes *et al.*, 1993; Staskawicz *et al.*, 1984) and fungal pathogens (*Cladosporium fulvum* (De Wit *et al.*, 1988; Scholtens-Toma and De Wit, 1988), *Rhynchosporium cerealis* (Hermann *et al.*, 1994; Rohe *et al.*, 1995); *Magnaporthe grisea* (Valent, 1994). Even the coat protein of TMV can be regarded as a necrosis-inducing protein in specific cultivars of the host (Culver and Dawson, 1991). One of the best studied examples of elicitors and their role in induction of HR which will be discussed later, is the AVR 9 protein from *Cladosporium fulvum*, capable of inducing necrosis on leaves of tomato lines carrying the matching *Cf9* resistance gene (De Wit *et al.*, 1988; Scholtens-Toma and De Wit, 1988). The *Avr9* gene has been cloned (Van Kan *et al.*, 1991) and transfer of this gene to a virulent *C. fulvum* race lacking the gene rendered this transgenic race avirulent on tomato lines with the *Cf9* gene (Van den Ackerveken *et al.*, 1992).

A strategy for engineering resistance might be achieved by overexpression of an avirulence gene, by which the transgenic plant might be continuously in an induced state. Conceivably, given the fact that many interactions between elicitors and their receptors induce HR, such a transformation would not yield viable plants. Keen and coworkers (1994) have produced transgenic tobacco plants overexpressing the *AvrD* gene. These plants showed enhanced resistance not only towards the bacterial pathogen *Pseudomonas syringae* pv. *tabacina* from which the avirulence gene was originally isolated but also against *Pseudomonas syringae* pv. *solanacearum* and even fungal pathogens such as *Alternaria alternata* and *Phytophthora nicotianae* var. *nicotianae.*

Most recently, a gene was isolated from the rice blast fungus, *Magnaporthe grisea*, which determines host specificity (Sweigard *et al.*, 1995). The gene, *PWL2*, encodes a glycine-rich, 16 kD, hydrophillic protein with a putative secretion signal sequence. Isolation of the corresponding resistance gene from weeping lovegrass on which the fungus is avirulent, might be a very attractive source for engineering durable resistance.

2.3 Signal Perception and Transduction

Much research is focused on perception of pathogens by plants and transduction of signals

to trigger the onset of defense responses. It is generally assumed that host receptors exist, which are capable of recognizing elicitors. These receptors in turn activate a cascade of events eventually leading to a resistant state. In section 2.4.1 dealing with the HR, these putative receptors are described in more detail. In this section, early responses, both locally and systemically, involving perception and signal transduction will be discussed.

Often, a distinction is made between two types of signals. A first group of signals are transported over short distances and act as inducers of defense responses at the site of penetration of a pathogen. They will be described in section 2.3.1. A second group of signals is believed to be transported throughout the plant and is responsible for inducing systemic defense responses. They will be reviewed in the section 2.3.2.

2.3.1 Local Signalling

To understand the signalling pathway(s) necessary for induction of a rapid response at the site of attack, much effort has focused on the early steps in such (a) pathway(s). Precise mode of action of many elicitors is presently unknown, but it is generally assumed that elicitors specifically bind to receptors in the host thus activating a cascade of defense responses. In the interaction of oligogalacturonide elicitors with tomato and potato cells it was shown that a 34 kD membrane associated protein became phosphorylated (Farmer *et al.*, 1991). Similarly, inhibition of protein kinases inhibited elicitor induced activation of defense genes in tomato (Grosskopf *et al.*, 1990). Receptor protein kinases and calcium/calmodulin dependent protein kinases have been reported to occur in many plant species (Walker, 1993; Watillon *et al.*, 1993). A specific receptor exists in soybean cell membranes which binds a hepta-ß-glucoside elicitor (Schmidt and Ebel, 1987; Cosio *et al.*, 1990; Cheung and Hahn, 1991). The ability of this elicitor to bind to the membrane is correlated with its ability to elicit phytoalexin synthesis (Cheung and Hahn, 1991). Elicitins, mentioned earlier, cause necrosis at the site of application. High affinity binding sites for elicitins are thought to be present in the membrane fractions of tobacco cells (Blein *et al.*, 1991).

Transient depolarization of membranes (K^+ efflux, H^+ influx, Ca^{2+} influx and Cl^- efflux) is thought to be one of the earliest events leading to HR and to general activation of defense genes (Dixon and Lamb, 1990). It has been shown that tobacco cell suspension cultures are capable of reacting to harpins, elicitors produced by *Pseudomonas syringae* (Atkinson, 1994). Surprisingly, this non-host response was identical to the response observed in resistant soybean cultivar cell suspension cultures, where within minutes after treatment with the elicitors efflux

of K^+, alkalinization of the medium and influx of Ca^{2+} was observed. In tomato cell suspensions specific chitin fragments were demonstrated to elicit these responses too (Felix *et al.*, 1993). Also in parsley cell suspensions similar results were obtained after application of fungal elicitors (Nürnberger *et al.*, 1995). Research is in progress now to isolate receptors capable of recognizing such elicitors (Hahn, 1994; Baureithel and Boller, 1994)

Upon elicitor treatment or in response to avirulent pathogens, enzymes involved in peroxidation of lipids, such as lipoxygenase (LOX) and phospholipases were activated (for overview, see Beckman and Ingram, 1994). A scheme has been proposed by these authors for signal transduction leading towards both early senescence as well as HR: 1) Ca^{2+}/calmodulin-stimulated activation of phospholipases, 2) release of polyunsaturated fatty acids (PUFA) from membrane lipids, 3) stimulation of LOX by free PUFAs, 4) subsequent damage to membranes due to LOX-dependent and LOX-independent lipid peroxidation, leading ultimately to membrane leakiness, Ca^{2+} influx and phospholipase activation. LOX-genes have been cloned and are being overproduced in transgenic potato to study their effect on disease and stress response (Findantsef, 1994)

Another very early event observed is the so-called oxidative burst, whereby rapid and transient production of active oxygen species occurs, such as the superoxide anion radical (O_2^-), hydroxyl radical ($OH\cdot$) and hydrogen peroxide (H_2O_2). Transgenic potato plants overexpressing glucose oxidase accumulate hydrogen peroxide (Wu *et al.*, 1995). The plants exhibited enhanced resistance to not only *Erwinia carotovora* subsp. *carotovora* but also to the fungal pathogen *Phytophthora infestans*.

Jasmonic acid (JA) or its methyl ester (MJ) are also thought to be involved in induction of responses, since infection and wounding lead to accumulation of these compounds (Creelman *et al*, 1992; for review see Reinbothe *et al.*, 1994). In tomato, proteinase inhibitors are wound inducible and thought to play a role in defense against insects (Ryan 1990). It has been demonstrated that MJ is capable of inducing proteinase inhibitors. Similarly, thionines in barley leaves are induced by JA (Andresen *et al.*, 1992). Via the atmosphere, MJ could contribute to systemic induction of these proteins within the plant or even act as an interplant stress signal (Farmer and Ryan., 1990). Jasmonic acid also induces the proteinase inhibitor genes (Farmer et al., 1992) as well as octadecanoid precursors of jasmonic acid, which are released from membranes of wounded cells by specific lipases (Farmer and Ryan, 1992). Not much information is available on rate-limiting factors in the biosynthesis of JA and related compounds. However, newly synthesized protein(s) appear to be rate-limiting in the biosynthesis since cycloheximide affected the accumulation of JA in wounded potato tubers (Koda and Kikuta 1994).

Plants are thus able to recognize specific microbial components or elicitors released from the plant cell wall. This recognition is followed by a cascade of events, leading to the HR, synthesis of phytoalexins, increased lignification and synthesis of pathogenesis-related proteins. Insight into sensing mechanisms is crucial in the development of strategies exploiting these phenomena. Interference in the recognition and signal transduction processes might enhance resistance of transgenic plants. Conceivably a plant could be transformed in such a way that the detection system for the presence of pathogens is most optimal. However, as stated earlier, often the early defense responses are similar in susceptible and resistant or even non-host plants. Detailed knowledge on the timing and level of induction of genes in resistant and susceptible plants, is essential.

2.3.2 Systemic Signalling

In many cases, the host plant does not only resist attack locally around the site of penetration, but also at sites distal from the invading pathogen. Much attention has been directed towards the identification of the transported signal which is responsible for these systemic defense responses. From tomato, an 18-amino-acid polypeptide, systemin, has been isolated which is a very powerful systemic inducer of proteinase inhibitor genes (Pearce *et al.*, 1991; 1993). This peptide is derived from a large precursor protein which is extensively processed by an unknown protease, possibly as a result of the loss of compartmentalization of the cell caused by wounding. By suppressing systemin expression (via antisense RNA), no systemic induction of the proteinase inhibitor genes occurred upon wounding (McGurl *et al.*, 1992). These results point towards the involvement of systemin in systemic signal transduction in tomato. Elicitins, as mentioned earlier, can cause necrosis at the site of application, but also at remote sites by movement through the vascular system of tobacco leaves (Devergne *et al.*, 1992; Zanetti *et al.*, 1992). Concomitantly, resistance is induced systemically.

It has been shown that in tobacco synthesis of the class II PR-genes is induced not only locally around the site of infection but also systemically by application of salicylic acid (SA) (Ward *et al.*, 1991). Similarly, in *Arabidopsis thaliana* SA also induces PR-1, PR-2 and PR-5 type proteins (Uknes *et al.*, 1992). These data suggest that SA by itself might be a systemic signal or is involved in the generation or transmission of the systemic signal. The endogenous SA levels in tobacco plants resistant to tobacco mosaic virus (TMV) indeed increase 20-fold in virus-infected leaves and 5-fold in uninfected leaves of the same plant. This rise is not observed in tobacco lines susceptible to TMV (Malamy *et al.*, 1990). The increase in endogenous SA

precedes and is sufficient for the induction of PR-proteins (Malamy *et al.*, 1992; Yalpani *et al.*, 1991). Recently, a SA-binding protein (SABP) has been identified exhibiting catalase activity. SA specifically inhibited catalase activity and induced elevated levels of H_2O_2. The action of SA in systemic acquired resistance (SAR) is hypothesized to be mediated through elevated amounts of H_2O_2 (Chen *et al.*, 1993). Overexpression of the gene encoding SABP in transgenic tobacco led to increased resistance to TMV (Chen and Klessig, 1991). Whether this gene is capable of conferring fungal resistance needs to be determined. Bi *et al.* (1995) questioned this hypothesis since their results indicate that the 3-AT, an inhibitor of catalase, had no effect on SA induction of PR-genes. Therefore, if H_2O_2 plays a role downstream of SA or even can act as an SA-independent inducer, it is unlikely to arise from the inhibition of endogenous catalases. Accordingly, also Neuenschwander *et al.*. (1995) found no increase of H_2O_2 during the onset of SAR. Additional experiments also do not support a role of H_2O_2 in signalling, but possibly this compound is important in tissues undergoing a hypersensitive response. Supposedly, inhibition of catalase by SA might enhance the accumulation of reactive oxygen species in hypersensitively reacting cells.

Similar to tobacco, SAR in cucumber coincides with increased SA levels in the phloem sap (Metraux *et al.*, 1990). Rasmussen *et al.*, (1991) measured levels of SA in various tissues of cucumber after sequential removal of a leaf infiltrated with *Pseudomonas syringae*. Surprisingly, the inducing leaf could be removed 4 to 8 hours post inoculation, before significant SA accumulation, without preventing the systemic induction of either SA or SAR gene expression, suggesting the existence of a systemic signal other than SA. The essential role of SA in the induction of SAR in tobacco has been definitively proven by raising transgenic tobacco plants expressing a salicylate hydroxylase gene, *NahG*, from *Pseudomonas putida*. These transgenic plants accumulate little or no SA in response to tobacco mosaic virus infection and are consequently unable to develop SAR (Gaffney *et al.*, 1993). Proof that SA is not the translocated signal responsible for inducing SAR came from grafting experiments involving these transgenic tobacco plants, overexpressing the *NahG* gene. Transgenic rootstocks, although unable to accumulate SA, were fully capable of delivering a systemic signal to induce SAR in the graft, indicating that the translocating signal is not SA. However, reciprocal grafting experiments demonstrated that SA must be present in tissues distal from the infection site in order to allow systemic resistance to be induced (Vernooij *et al.*, 1994). A synthetic inducer of SAR, 2,6-dichloroisonicotinic acid (INA), was shown not to induce SA during the time required for SAR. Apparently, INA activates the signalling pathway either at the same site or downstream of SA accumulation (Vernooij *et al.*, 1995). This hypothesis was confirmed by the fact that in transgenic plants expressing *NahG* INA was fully capable of inducing SAR towards

Peronospora parasitica, although the lesion size was larger. An explanation for this observation might lie in the fact that SA has two roles, namely both in the HR and in the signalling pathway leading to SAR. Conrath *et al.* (1995) demonstrated that INA like SA, binds the SABP/catalase described above and inhibits its enzymatic activity. Dose-response curves were found to be similar. These results again indicate that INA and SA share the same mechanism of action, namely binding and inhibiting catalase, but do emphasize a role for reactive oxygen species in the induction of plant defense responses.

Bowling *et al.* (1994) identified a single recessive mutation, *cpr1*, in *Arabidopsis* which leads to elevated expression of PR-genes. It was shown that such a mutant plant contains higher levels of endogenous SA. Interestingly *cpr1* plants are resistant to both *Peronospora parasitica* and *Pseudomonas syringae* pv *maculicola*. This indicates that the *CPR1* gene product acts upstream of SA as a negative regulator of SAR and that the mutation constitutively activates SAR. In *Arabidopsis* another mutant has been identified, *nim1*, which is insensitive to chemical and biological inducers of SAR genes and resistance (Delaney *et al.*, 1995), although it is able to accumulate wild-type levels of endogenous SA, required for SAR. The authors suggest that the wild-type *NIM1* gene product functions in a pathway regulating SAR, at a position downstream of SA accumulation but upstream of SAR gene induction and expression of resistance. They propose therefore that there possibly are two separate SA-dependent pathways, whereby one is defined by the *nim1* mutation whereas the other is NIM1-independent and partially responsible for the NahG plants. Depletion of SA would block both pathways leading to hypersusceptibility. A third single recessive mutation has been identified in *Arabidopsis*, *npr1*, which abolishes the SAR induction by either SA, INA or avirulent pathogens (Cao *et al.*, 1994). Moreover, localized expression of PR-genes is disrupted and lesion formation by *Pseudomonas syringae* is less confined.

Some of the tobacco genes induced by TMV or SA can also be induced by ethephon (Ye *et al.*, 1992), which upon hydrolysis, yields the stress hormone ethylene (for overview: see Ecker, 1995). It has been suggested that ethylene acts as a signal in SAR (Raz and Fluhr, 1992). Since the key enzymes involved in ethylene synthesis, such as ACC-synthase and ethylene forming enzyme have been identified, their local overexpression at the site of penetration of a pathogen, might be an effective means of introducing resistance (Yang and Hoffmann, 1984). Studies are being performed in the group of Bol (Leiden University in the Netherlands), involving sense and antisense expression of either gene alone or simultaneously (Knoester *et al.*, 1995) to study the exact role of ethylene in SAR. Application of ethylene, rather than ethephon, has not been shown to increase disease resistance, although increased

synthesis of enzymes such as phenylalanine ammonia lyase, chalcone synthase as well as the vacuolar chitinase and ß-1,3-glucanase was induced (Ecker and Davis, 1987; Mauch *et al.*, 1992). Ethylene production by plants is increased under various stress conditions such as wounding and pathogen attack (Mauch *et al.*, 1984; Yang and Hoffman, 1984). Thus the role of stress ethylene in the induction of PR-proteins is not very clear and it is believed that it plays an indirect rather than a direct role (Boller, 1988). By using mutants of *Arabidopsis* non-responsive to ethylene, it has been shown that ethephon induction of PR-gene expression is a SA-dependent process. Caution must be taken since it was demonstrated that phosphoric acid released from ethephon could cause this effect rather than released ethylene (Lawton *et al.*, 1994). Transgenic *Arabidopsis* plants expressing *NahG* were unable to accumulate SAR mRNAs in response to ethephon, indicating that ethephon induces SAR gene expression through SA and that ethylene may play a role in SAR by enhancing sensitivity of tissue to the action of SA (Lawton *et al.*, 1994).

In conclusion, an important process in plant defense against fungal attack is "self-immunization" upon contact with a pathogen. Many of the genes induced during SAR are the same as those induced during local defense responses. Different signal molecules capable of mimicking the SAR response have been discovered, but the link between them is at present not fully understood. Application of this knowledge in engineering fungal resistant plants will largely depend on results coming from SABP expressing transgenic plants. An option for engineering of such transgenic plants might be achieved through overexpression of either a receptor or a 'master switch', the essential gene responsible for activation of the cascade of defense responses. An absolute requirement in interfering with signal transduction pathways leading to SAR is, that the plants to be transformed are capable of showing the SAR phenomenon. This type of resistance has been demonstrated in a number of species, but has been studied most extensively in tobacco and cucumber. Extensive genetic studies in for example *Arabidopsis* will demonstrate the feasibility of this approach.

2.4 Local Defense Responses Induced by Pathogens or Elicitors

One option by which a host might resist pathogen attack is the reinforcement of preexisting barriers in the presence of pathogens. For example in wheat, increased lignification has been observed around wound sites (Barber *et al.*, 1989; Barber and Ride, 1988; Ride, 1975; Ride *et al.*, 1989). Similarly, upon attempted penetration of the pathogen, papillae are

often produced locally. Papillae are cell wall thickenings, consisting generally of callose, a ß-1,3-glucan, but may contain other compounds such as lignin, cellulose, phenols or silicon (Kauss *et al.*, 1989; Kohle *et al.*, 1985; Paxton and Groth, 1994). Papillae might delay penetration and allow additional defense responses to occur (Aist, 1976). In the case of obligate parasites with limited food reserves, they might cause the fungus to starve. Bradley *et al.*, (1992) presented evidence that in bean and soybean preexisting hydroxyproline-rich structural proteins were insolubilized in the cell wall within 10 minutes after treatment with fungal elicitors. Cross-linking, mediated by H_2O_2, was proven to be responsible for the observed insolubilization.

As the pathways to synthesize many of these, constitutive/reinforced, cell wall components are being elucidated, the application of this knowledge might be useful in genetic engineering for resistant plants. However, success will depend largely on the absence of side effects which could be caused by stress or wounding, leading to unwanted expression of the transgene(s).

2.4.1 Hypersensitive Response (HR)

One of the most studied responses to pathogen attack is the HR. Upon pathogen attack, host plants respond with rapid collapse and desiccation of the host tissue locally at the site of penetration. This is called HR. Moreover, cells in the immediate vicinity of the dead cells react by activation of defense mechanisms, preventing further ingress of the pathogen. HR is invariably associated with resistance (Collinge and Slusarenko, 1987; Farmer and Ryan, 1991). This localized necrosis of plant tissue is believed to limit the multiplication and spread of the invading pathogen (Jacobek and Lindgren, 1993). In section 2.4.1.1 this naturally occurring phenomenon and its possible employment in genetic engineering for fungal resistance are discussed. Since death of the host cells at the attempted point of entry of the pathogen is sufficient to restrict the pathogen and activate defense systems, research has been directed towards engineering controlled host cell death. In the section 2.4.2.2 some examples of artificially engineered HR in transgenic plants to enhance disease resistance are described.

2.4.1.1 Naturally Occurring HR

HR mostly occurs in gene-for-gene relationships, initially described by Flor (1955)

for flax and *Melampsora lini* (Ellingboe, 1981, 1982). In this relationship, avirulence is caused by interaction between the product of the avirulence gene of a distinct physiologic race of a pathogen and the matching resistance gene product of the host. In many plant-pathogen systems such types of relationships are observed (for review see: Crute, 1985). Initially, all research effort was focused on the pathogen side of the interaction. Many avirulence genes have been identified from both bacterial and fungal pathogens and some fungal avirulence gene products (elicitors) have been isolated (see Section 2.2.2).

Much effort has been put into isolating and characterizing corresponding resistance genes, which were hypothesized to encode receptors for elicitors produced by the pathogen (for review see: Dangl, 1995). It is generally assumed that upon recognition of the elicitors by plant receptors, signalling pathways are activated and induce defense mechanisms both locally at the site of penetration and also systemically in non-infected parts of the host. Martin *et al.* (1993) were the first to identify an HR-type of resistance gene. The *Pto* gene, coding for resistance to *Pseudomonas syringae* pv. *tomato* with avirulence gene *avrPto*, resembles a serine-threonine protein kinase. Also, a myristylation site necessary for membrane anchoring has been identified.

More recently, the *RPS2* gene has been isolated from *Arabidopsis thaliana* conferring resistance to *Pseudomonas syringae* pv.*tomato* expressing avirulence gene *avrRpt2* (Bent *et al.*, 1994; Kunkel *et al.*, 1993). Various specific regions were identified in this resistance gene such as a region of leucine-rich repeats, suggested to be involved in protein-protein interactions and ligand binding. A second (N-terminal) region carries an additional potential protein-protein interaction domain, called a leucine zipper. Downstream of this region is a P-loop, involved in nucleotide triphosphate binding.

Most recently, resistance genes were identified conferring resistance to pathogens other than bacteria. The *N*-gene from tobacco conferring resistance to TMV was identified, sharing high homology to the *RPS2* gene from *A. thaliana*, however lacking the transmembrane segment (Whitham *et al.*, 1994). One of the best studied plant/fungus examples of 'gene-for-gene' incompatibility is the interaction between tomato and *Cladosporium fulvum*. The *Cf9* resistance gene has been isolated using transposon tagging. This gene encodes a membrane-anchored extracytoplasmic glycoprotein with 26 leucine-rich repeats (Jones *et al.*, 1994). Surprisingly, the recently identified *L6* gene from flax coding resistance to *Melampsora lini* was shown to be similar in structure to the *RPS2* gene and the *N*-gene (Lawrence *et al.*, 1994). Also the *Xa21* gene from rice encoding resistance to *Xanthomonas campestris* pv. *oryzae* (Wang *et al.*, 1995) shows a high degree of similarity. It has a signal peptide, leucine-rich

repeats, a transmembrane domain, as well as a serine/tyrosine kinase domain. It is striking that R-genes coding for resistance against bacteria, a virus and fungi, respectively, appear to share such a high degree of homology. This might indicate a common underlying mechanism which, once identified, could be used in genetic engineering. Theoretically, a synthetic gene could be engineered harboring the necessary regions to confer resistance to a wide variety of pathogens. Many speculations are made on the function of the proteins encoded by this genes, none of which have been isolated (yet). The general assumption is that the extracellular domain functions as a receptor which through the membrane-spanning domain activates the inner part of the protein or neighbour protein, which in turn triggers the signal transduction cascade, which has been described earlier.

This area of research is very exciting as the mechanism of action of the proteins encoded by these resistance genes will be unravelled. Dissection of the gene-for-gene interaction by comparing various resistance genes in a given host with various matching avirulence genes in the pathogen will be required to further reveal the mechanism underlying HR.

However, to complicate matters, it has been shown recently that the one-gene-for-one-gene interaction does not always occur. The *RPM1* gene in *Arabidopsis* confers specific resistance to two avirulence genes in *Pseudomonas syringae* pv. *tomato* (Bisgrove *et al.*, 1994; Dangl and Innes, 1995; Grant *et al.*, 1995). It has features as found in other R-genes, such as a leucine zipper, two motifs for a nuclear binding site and 14 leucine-rich repeats (Grant *et al.*, 1995). At present it is not known whether the RPM1 molecule has two *avr*-signal binding sites or whether it interacts in a pathway with signals transduced through one promiscuous receptor of *avr*-dependent signals. Even more striking was the isolation of a mutant of *Arabidopsis*, *ndr1-1*. Mutation in this locus rendered the plant susceptible to *Pseudomonas syringae* pv. *tomato* containing various cloned avirulence genes and even the fungal pathogen *Peronospora parasitica* (Century *et al.*, 1995). The authors demonstrated that this locus is not required for non-host resistance and they propose that this locus is important in a common signal transduction pathway, downstream of the initial recognition of the avirulence factor. It could well interact with such a receptor, for example *RPS2*, or alternatively it may play a role in the SA pathways. Transgenic plants unable to accumulate SA show a similar pattern of susceptibility as the *ndr1* mutants, however the difference in growth between a virulent and an avirulent strain as observed on the transgenic plants was not observed on mutant plants.

It remains unclear whether HR is caused by toxic effects or the activation of programmed cell-death. Mutants of *A. thaliana* have been identified, which in the absence of a pathogen, form necrotic lesions, mimicking the HR and are resistant to pathogen attack. They are the so-

called *acd* (accelerated cell death) mutants, identified by Greenberg *et al.* (1994) and the *lsd* (lesions simulating disease) mutants by Dietrich *et al.* (1994). It has been shown that a single gene is involved in a (the) pathway(s) that regulate HR. Possibly such a gene involved in signal transduction leading to HR could be exploited in engineering fungal resistance by placing it under control of a pathogen-inducible promoter. Upon activation of such a gene by a penetrating pathogen, HR would be induced.

2.4.1.2 Artificial HR

The most promising strategies at present in the area of engineering artificial HR in transgenic plants involve two-component systems. One strategy has been proposed by De Wit and coworkers (1992) for engineering fungal resistance using the gene-for-gene interaction described above. The model proposes production of transgenic plants simultaneously expressing both an avirulence gene placed under control of a pathogen-inducible promoter and a corresponding resistance gene constitutively expressed. Upon pathogen attack, HR will be induced locally around the site of penetration. More specifically the *Avr9* gene and the *Cf9* gene are employed. It has been shown that artificial cell death can be accomplished by using these genes. Tomato plants carrying the *Cf9* gene were crossed with transgenic *Cf0* tomato plants constitutively expressing the *avr9* gene. Of the progeny of such crosses 50% of the seedlings showed severe necrosis (Hammond-Kosack *et al.*, 1994). At Mogen, transgenic tomato plants could be raised of the *Cf0* cultivar overexpressing the *avr9* gene. However, if the same construct was transformed into plants carrying the *Cf9* gene only a very limited number of transgenic plants could be obtained having an extremely aberrant, necrotic, phenotype. These preliminary data indicate that artificial cell death can be engineered (Honee *et al.*, 1995).

Similarly, another two-component strategy is being exploited. A system is employed consisting of two genes from *Bacillus amyloliquefaciens*, called Barnase, an RNAse and a RNAse-neutralizing protein from the same bacterium called Barstar, which irreversibly binds to the RNAse (Mariani *et al.*, 1990). Potato plants have been transformed with a construct combining the pathogen-inducible *prp1-1* promoter (Martini *et al.*, 1993) with Barnase. To minimize the detrimental effects of background Barnase expression in non-infected tissue, the transgenic plants also expressed the specific inhibitor Barstar, under control of a constitutive promoter. In transgenic plants harboring this two-component system, the level of Barnase expression is expected to exceed the level of Barstar expression only in the vicinity of fungal infection sites. Initial results involving transgenic potato inoculated with *Phytophthora infestans*

were promising. For 12 out of 75 primary transformants the efficiency of fungal sporulation was reduced (Strittmatter *et al.* 1995).

Most recently, an intriguing strategy has been proposed by the group of Jones (pers. comm.), which involves insertion of a transposable element in the *Cf9* gene. After controlled transposition, local necrotic lesions will occur in transgenic plants expressing the *Cf9* gene in addition to the *Avr9* gene. These necrotic lesions will induce local and systemic defense responses in the transgenic plant. This type of resistance has been termed genetically engineered acquired resistance (GEAR).

2.4.2 Antifungal Proteins

Apart from HR, other defense systems are also induced at the site of penetration such as the production of antifungal proteins. In the section, 2.4.2.1, a brief overview will be presented of such antifungal proteins and the results obtained by overexpression of (combinations of) such proteins. Since overexpression of antifungal proteins from non-plant origin has been studied extensively as well, some of the most promising results are described in section 2.4.2.2.

2.4.2.1 Plant Antifungal Proteins

Pathogenesis-related proteins can be defined as plant proteins that are induced in pathological or related situations (Van Loon *et al.*, 1994). They are induced both locally at the site of infection and systemically throughout the whole plant. They are invariably induced after a biotic agent has caused necrosis (Linthorst, 1991). However, this is not an absolute requirement. In incompatible tomato-*Cladosporium fulvum* interactions necrosis is observed late in the infection process and it has been reported that induction of glucanases precedes the necrotic response (Ashfield *et al.*, 1994). These authors showed that the induction of tobacco ß-1,3-glucanase in *C. fulvum* infected tomato leaves depended on the specific *Cf* gene present. Similarly, in tobacco leaves infiltrated with *Pseudomonas syringae* pv. *tabaci* both Hrp$^-$ and wild-type strains induced accumulation of chitinase transcripts as well as PAL, CHS, CHI, even though no HR develops at the Hrp$^-$ mutant infection site (Jacobek and Lindgren, 1993). First described in hypersensitively reacting tobacco, PR proteins were initially defined as pathogen-inducible, acidic and relatively protease-resistant proteins and mainly occurring in the

extracellular spaces, the so called class II proteins (Parent and Asselin, 1984; Van Loon, 1985). However, more recently, serologically related, class I, isoforms have been identified intracellularly (for reviews see: Linthorst, 1991; Stintzi *et al.*, 1993). Overall, twelve different families of PR-proteins are recognized (Van Loon *et al.*, 1994). In tobacco, members of at least five groups of proteins are induced (Antoniw *et al.*, 1980; Van Loon, 1982; Van Loon *et al.*, 1987) and will be briefly discussed below since they were shown to possess antifungal activity. An summary is presented in Table I.

The PR-1 family comprises four members divided over two classes, being the class I basic PR-1 protein and three class II extracellular proteins, PR-1a, -1b and -1c. The function of these proteins is unknown, however the class I PR-1 protein from tomato, P14a, and from tobacco PR-1g were shown to possess growth inhibiting activity *in vitro* towards *Phytophthora infestans* (Nidermann *et al.*, 1995).

The PR-2 proteins are ß-1,3-glucanases (Kauffmann *et al.*, 1987; for review see: Simmons, 1994) and able to hydrolyze ß-1,3-glucan polymers, a cell wall constituent of many fungi (Wessels and Sietsma, 1981). Indeed, ß-1,3-glucanases were shown to possess antifungal activity in synergy with chitinases (Mauch *et al.*, 1988). The PR-2 proteins can be divided into three structural classes (Payne *et al.*, 1990). The class I enzymes are basic and located in the vacuole. The class II enzymes, PR-2a, -2b and -2c (formerly known as PR-2, -N and -O) are located extracellularly and are acidic in nature. The class III enzymes show a higher specific activity on laminarin, although the products are larger oligosaccharides than those produced by the class I and II enzymes. Structural homology is observed with a soybean elicitor-releasing ß-1,3-glucanase (Payne et *al.*, 1990; Takeuchi *et al.*, 1990). The class I PR-2 was shown to inhibit growth of *Fusarium solani* f.sp. *phaseoli in vitro* , both alone as well as in synergy with other PR-proteins, including the class I PR-3 and the class I PR-4 (Ponstein *et al.*, 1994; Sela-Buurlage *et al.*, 1993).

The proteins belonging to the PR-3 group are chitinases (Legrand *et al.*, 1987; for review see: Colling *et al.*, 1993; Graham and Sticklen, 1994). Chitin, a high molecular weight polymer of 1,4-N-acetyl-ß-D-glucosamine, is found in almost all fungal cell walls with the exception of the Oomycetes. The class I chitinases are basic in charge and located in the vacuole. They contain a N-terminal chitin binding (lectin-like) domain, a hinge region and a C-terminal catalytic domain, with a high percentage of identity with the class II chitinases. These class II enzymes are acidic in nature, located extracellularly and missing the N-terminal chitin binding domain. The specific enzymatic activity as determined on labelled colloidal chitin is 5-10 times lower than that of the class I enzymes. A class III chitinase has been identified in tobacco, which shares homology to chitinases identified in cucumber and in *Arabidopsis*, acidic in

TABLE 1. Overview of PR-proteins, induced in tobacco Samsun NN by inoculation with tobacco mosaic virus

	Intracellular (basic)		Extracellular (acidic)				
Group	Class I	Mol wt (kD)	Class II		Mol wt (kD)	Function	References
1	PR-1g	17	PR-1a		15.5	Transg. PR-1a tob/P. tabacina	1
			PR-1b		15.5	PR-1a, PR-1b: anti P.infestans	35
			PR-1c		15	PR-1g: anti P. infestans	27
			PR-1g		17		
2	Glu-I	33	PR-2a	PR-2	31	ß-1,3-glucanase	15
			PR-2b	PR-N	33	Glu-I antifungal alone and in synergy	32
			PR-2c	PR-O	35	with Chi-I, CBP20, Chi-V	32
				PR-Q'	35		
3	Chi-I	32	PR-3a	PR-P	27	chitinase	19
		34	PR-3b	PR-Q	28	Chi-I antifungal alone and in synergy	32
						with Glu-I, CBP20	32
4	CBP20	20	PR-4a	PR-r1,2	14.5/13	WIN-homologous	34
			PR-4b	PR-s1,2	14.5/13	CBP20 chitinase, antifungal	29
						CBP20 synergy w/ Chi-I, Glu-I	29
5	AP24	22.3	PR-5a	PR-R	24	AP24,osmotin, antifungal	43
			PR-5b	PR-S	24	Transg. AP24 potato/P.infestans	21
11	Chi-V	40				chitinase, antifungal alone and in	26
		42				synergy with Glu-I	

charge and located in the extracellular space (Lawton *et al.*, 1992; Metraux *et al.*, 1989; Samac *et al.*, 1990). Chitinases were shown to possess antifungal activity *in vitro*, alone and in synergy with ß-1,3-glucanases (Arlorio *et al.*, 1992; Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993). Similarly, microinjection of a bacterial chitinase into epidermal cells of barley could suppress disease establishment through digestion of the feeding structures, the haustoria, of the powdery mildew pathogen *Erysiphe graminis* f.sp. *hordei* (Toyoda *et al.*, 1991). As with the PR-1 and PR-2 groups, the class I enzymes of PR-3 were far more antifungal than the class II isoforms (Sela-Buurlage *et al.*, 1993).

The PR-4 proteins comprise an additional group of PR-proteins. Two proteins initially identified are located extracellularly and acidic in nature. The function of these proteins is unknown, but their primary structure is homologous to the C-terminal domain of wound induced proteins in potato tubers, WIN-1 and WIN-2 (Stanford *et al.*, 1989) and the C-terminus of hevein (Broekaert *et al.*, 1990). The WIN proteins and hevein all contain a chitin binding domain, similar to the class I chitinases in tobacco. The relationship between PR-4 and WIN-1/WIN-2/hevein, therefore is similar to that of class I and class II chitinases. Recently, in tobacco a class I PR-4 was identified, CBP20, with chitinase activity and *in vitro* antifungal activity towards *Fusarium solani* and *Alternaria radicina* alone and in synergy with either the class I PR-2 or the class I PR-3 (Ponstein *et al.*, 1994). The class II PR-4 protein from tobacco lacks antifungal activity *in vitro*.

PR-5 proteins can also be divided into two classes, namely a class I isoform, again basic in nature and located in the vacuole and a class II extracellular, acidic form. The class I PR-5 shows high homology to thaumatin, a sweet-tasting protein from *Thaumatococcus daniellii* (Cornelissen *et al.*, 1988; Edens *et al.*, 1982) and osmotins, which accumulate in response to high salt stress (Singh *et al.*, 1987). The function of the proteins is unknown, however, high homology was observed to an α-amylase/trypsin inhibitor from maize (Richardson *et al.*, 1987). The class I PR-5 from tobacco and tomato were found to possess antifungal activity *in vitro* towards the fungus *Phytophthora infestans*, by causing lysis of the sporangia (Woloshuk *et al.*, 1991), whereas the class II counterpart was not antifungal. Similarly activities were observed for thaumatin-like PR-5 proteins from maize, barley and tomato (Hejgaard *et al.*, 1991; Malehorn *et al.*, 1994; Vigers *et al.*, 1991; 1992).

Recently, another class of chitinases has been identified from tobacco, Chi-V. Significant homology with bacterial exochitinases was observed. However, Chi-V possesses endochitinase activity. It is induced by TMV, ethephon and UV treatment and is therefore termed a PR-protein (Melchers *et al.*, 1994) and initially placed in group PR-12 by the authors. (According to guidelines proposed for PR-protein nomenclature by van Loon *et al.* (1994) it should be placed

in group PR-11.)

Various antifungal PR-proteins have been identified so far and their corresponding genes have been cloned. An obvious strategy involves overexpression of (combinations) of antifungal PR-proteins. Transgenic plants have been created expressing one or more PR-proteins and have been assayed for enhanced fungal resistance. Transgenic tobacco plants constitutively expressing PR-1a showed enhanced resistance to *P. nicotianae* and *Peronospora tabacina* (Alexander *et al.*, 1993). Similarly, transgenic tobacco constitutively expressing a bean class I chitinase showed enhanced resistance to *Rhizoctonia solani* (Broglie *et al.*, 1991) although Neuhaus and coworkers (1991) could not increase resistance to *Cercospora nicotianae* in transgenic tobacco by constitutive overexpression of class I chitinases. Antisense expression of an *Arabidopsis* class I chitinase in *Arabidopsis* was used by Samac and Shah (1994) to study the role of chitinase in induced resistance. They found a slight, but not statistically significant increase in susceptibility in transgenic plants towards *Botrytis cinerea*, indicating the limited role chitinase exerts*in vivo*. It must be stated that antisense RNA was not totally effective in suppressing induced chitinase expression, since chitinase levels still increased in transgenic leaves to 47% of control leaves within 24 hours after inoculation. Constitutive co-expression of a chitinase and a ß-1,3-glucanase in transgenic plants seems far more promising. In transgenic tomato plants, simultaneous expression of the class I chitinases and class I ß-1,3-glucanase from tobacco increased resistance to *Fusarium oxysporum* f.sp. *lycopersici* r1 (Van den Elzen *et al.*, 1993). The same constructs were shown in carrot to enhance resistance in the field simultaneously towards *Alternaria dauci, Alternaria radicina* as well as *Cercospora carotae* (unpubl. results). In tobacco, overexpression of a rice basic chitinase and an alfalfa acidic ß-1,3-glucanases provided enhanced resistance to *Cercospora nicotianae* (Zhu *et al.*, 1994). Likewise, the co-expression of a barley class II chitinase and a barley class II ß-1,3-glucanase enhanced resistance in transgenic tobacco towards Rhizoctonia *solani* (Jach *et al.*, 1995). Synergy between barley RIP and chitinase and ß-1,3-glucanase has been demonstrated *in vitro* (Leah *et al.*, 1991). Transgenic tobacco plants simultaneously expressing RIP and either a barley class II chitinase or a barley class II ß-1,3-glucanase showed enhanced resistance towards *Rhizoctonia solani* (Jach *et al.*, 1995). Also the class I PR-5, osmotin, from tobacco could enhance resistance in transgenic potato (Liu, 1994).

2.4.2.2 Antifungal Proteins of Non-plant Origin

Apart from plant antifungal proteins, many non-plant antifungal proteins have been reported in literature. Overexpression of such proteins might be very successful in increasing resistance to pathogen attack. In the case of genetically engineering bacterial resistance, positive results have been achieved, such as overexpression of cecropinB in transgenic tobacco leading to increased resistance to *Pseudomonas syringae* (Norelli *et al.*, in press). Similarly ,a hen egg white lysozyme, active against grampositive bacteria, has been overproduced in *Brassica*, potato and tobacco and positive results were obtained (Trudel *et al.*, 1992).

An antifungal protein from *Aspergillus giganteus* was identified and the corresponding gene isolated (Nakaya *et al.*, 1990). Also, a gene was isolated from *Enterobacter cloacae* that is responsible for biological control of *Pythium ultimum* (Maloney and Nelson, 1994). Conceivably, overexpression of such proteins enhances resistance. Promising results have been obtained with a chitinase, ChiA, from *Serratia marcescens* overexpressed in transgenic tobacco. These plants were significantly better protected against *Rhizoctonia solani* than control plants (Jach *et al.*, 1992).

Identification of antibodies specifically raised against certain key factors of pathogens and subsequent overexpression of these specific (monoclonal) antibodies in transgenic plants, the so called plantibodies, is a non-conventional approach for obtaining resistance to pathogens. In tobacco, overexpression of monoclonal antibodies raised against a viral coat protein yielded effective protection against subsequent viral attack, indicating that the concept works (Benvenuto *et al.*, 1994). At present similar efforts are aimed at introducing monoclonal antibodies directed against plant parasitic nematodes (Schots *et al.*, 1992; Van Engelen *et al.*, 1994). It is anticipated that such an approach might also be effective against fungal pathogens.

2.4.3 Phytoalexins

Often the HR response is accompanied by increased synthesis of phytoalexins. Phytoalexins are defined as low molecular weight antimicrobial compounds with a broad working spectrum, supposedly capable of inhibiting fungal growth. Not only HR is capable of inducing the synthesis of phytoalexins but also biotic elicitors, such as break-down products of cell walls from plant or pathogen (glucans, proteins, glycoproteins, fatty acid derivatives) and abiotic stimuli such as heavy metals, wounding, UV irradiation and chemicals (Darvill & Albersheim, 1984). Phytoalexins are secondary metabolites, produced via several pathways of

which the phenylpropanoid synthetic pathway (Darvill & Albersheim, 1984; Ebel, 1986) has been well studied. Key enzymes in this pathway are phenylalanine ammonia lyase, 4-coumarate: CoA ligase, 6'-hydroxychalcone synthase and chalcone isomerase (for overview see Ebel, 1986). Fungal pathogens have been described capable of inactivating certain phytoalexins, such as *Fusarium solani* f.sp. *phaseoli*, which produces a kievitone hydratase, capable of detoxifying the bean phytoalexins kievitone and phaseollidin (Kuhn and Smith, 1979; Li *et al.*, 1995a). Similarly, pisatin, a phytoalexin produced by pea upon infection by *Nectria haematococca* can be detoxified by this fungus by pisatin demethylase (Van Etten *et al.*, 1989). When the gene encoding this enzyme is transformed into a maize pathogen, *Cochliobolus heterostrophus*, it in turn becomes virulent on pea (Schäfer *et al.*, 1989). However, transformation of a saprophytic fungus *Aspergillus nidulans* with this gene does not render it pathogenic on pea, implying that this enzyme is not sufficient to determine pathogenicity (Schäfer *et al.*, 1989). Other evidence demonstrating that pisatin demethylase activity (*Pda*) contributes to virulence but is not the sole virulence determinant results from the fact that strains of *Nectria haematococca* were identified lacking virulence on pea, but producing the enzyme and possessing sequences hybridizing to the *PdaT9* probe (Mackintosh *et al.*, 1989; Reimmann and Van Etten, 1994). Also transformation of *N. haematococca* which lacked *Pda* with the *Pda* gene did not render these transgenic strains as pathogenic as the wild type *Pda* strains (Van Etten *et al.*, 1994).

The importance of phytoalexins in plant resistance remains at present questionable. Possibly, phytoalexins play a role later on in the infection cycle by slowing down ingress of the pathogen. Certain varieties of grapes, resistant to mildew, possessed higher constitutive levels of resveratrol than their more susceptible relatives, indicating a positive correlation between the presence of this compound and ability to resist fungal attack. The key enzyme in the synthesis of resveratrol is stilbene-synthase. The corresponding gene was identified by Hain and coworkers (1993) and they have shown that overexpression of this enzyme in transgenic tobacco resulted in increased resistance to *Botrytis cinerea*.

2.5 Systemic Defense Responses

In the section on systemic signalling (2.3.2), the various candidates responsible for communication of infected and non-infected tissues were discussed as well as their transduction. In this section the actual systemic defense responses are reviewed. However, it must be stated that almost all research involving systemically induced resistance focus on the

area of these signalling pathways and not per se on the actual defense responses observed at sites far from the initial infection point.

Initially it was shown that local infection of tobacco by TMV or *Peronospora tabacina* leads to immunization of the whole tobacco plant against subsequent viral, bacterial and fungal infection (Dean and Kuc, 1985; Hecht and Bateman, 1964; Kuc, 1982; McIntyre *et al.*, 1981; Ross, 1961a,b; Ward *et al.*, 1991). This phenomenon SAR has since been observed in many plant species (Ryals *et al.*, 1992).

As mentioned earlier, HR is typically used by a host plant to resist pathogen attack. Surprisingly, systemically induced HR has never been reported, indicating that other defense systems are active. Typically, induced resistance is accompanied by *de novo* synthesis of a large number of proteins. These include enzymes involved in the process of lignification and in the synthesis of secondary metabolites such as phytoalexins as well as the PR- proteins. All these processes were described above in detail in the section involving locally induced defense responses and are therefore not mentioned here.

However, of the inducible PR-proteins in tobacco, only the extracellular, class II, isoforms are synthesized systemically. As stated above the class I PR-proteins are far more antifungal *in vitro* than their extracellular, class II, counterparts (see for example Sela-Buurlage *et al.*, 1993). When these two findings are combined it becomes clear that additional factors besides the PR proteins must be involved in systemic defense responses. Identification of those factors might offer alternative strategies for genetic engineering. An example of such a factor is the gene family SAR 8.2. It comprises several highly related cDNAs, isolated through differential screening of a tobacco cDNA library constructed from RNA of induced leaves (Alexander *et al.*, 1992). The cDNA encodes a basic protein of around 50 residues. So far the protein has not been isolated and the function remains unknown, but overexpression in transgenic tobacco either alone or together with the PR-1a from tobacco enhances resistance to *P. nicotianae* and *Peronospora tabacina* (Ryals, pers. comm).

In conclusion, it must be stated that genetic engineering of SAR responses seems unlikely at present. However, exploiting knowledge generated from recognition of elicitors capable of inducing SAR, the signals responsible as well as their signalling pathway, might lead to applications in the future.

FUTURE PROSPECTS

In this review, state of the art on the exploitation of various host defense mechanisms and options that might be used in the near future in genetic engineering of fungal resistance in plants have been presented. It is clear that research concerning the plant/pathogen interface is progressing rapidly. Manipulation of sensing mechanism and the requirements necessary for perception by the host of a potential pathogen offer possibilities for applications. Either an artificial HR generating system is introduced or the plant is triggered to a state of SAR, using its own preexisting programs. A second line of research is aimed more directly at inhibiting pathogen ingress and colonization by overexpressing antifungal compounds. Possibly combination of these two approaches will prove the most effective: on the one hand the stimulation of the plant's own defense systems, before a potential pathogen can cause damage and on the other hand, a simultaneous expression of inhibitory compounds to slow growth even further. The aim should be directed at creating a broad spectrum resistance. Only in this way can modern agriculture exist in a safe environment where yields match the world's demands at a much reduced use of pesticides.

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Chapter 2

Extracts of Leaves of *Nicotiana Tabacum* Cv. Samsun NN) are a Source for Antifungal Proteins

ABSTRACT

Protein extracts, prepared from tobacco leaves (*Nicotiana tabacum* cv. Samsun NN), inoculated with tobacco mosaic virus were calibrated for the various well known groups of pathogenesis-related (PR) proteins. Over 50% of the proteins in such an extract was accounted for. Subsequently, these induced protein extracts were screened *in vitro* for their potential as a source for plant-derived antifungal proteins. Twenty five sporulating fungi were assayed for their sensitivity to an induced protein extract at two stages in their life cycle, namely spores or germlings. Also, inhibition of hyphal growth was monitored of six non-sporulating fungi upon addition of the induced protein extracts. Twenty three fungi were inhibited for 50% or more upon addition of the protein extract. Fifteen fungi were extremely sensitive (0.5-25 µg/well), eight moderately sensitive (50-250 µg/well) and eight were not or only slightly inhibited by 250 µg/well. Most sporulating fungi were far more sensitive if protein extracts were applied to germlings instead of spores. It is concluded that tobacco leaves (*N. tabacum* cv. Samsun NN), inoculated with TMV provide a highly valuable source for the identification of antifungal proteins.

INTRODUCTION

Fungal diseases are one of the major threats to agricultural productivity. Several strategies have been developed, such as crop rotation, the application of chemical fungicides and the breeding of resistant cultivars to control these diseases. Unfortunately, crop rotation is not always an option due to economic problems for farmers specializing in more than a few crops. Application of chemicals has proven very effective in the control of plant pathogens. However, many pathogens are capable of becoming resistant to the chemical compound thus rendering it ineffective. Moreover, the extensive use of agrochemicals has received criticism in recent years, mainly because of their adverse effects on the health of humans and on the environment. The traditional plant breeding techniques to obtain resistant cultivars are very laborious and time consuming. In some cases, resistance genes against specific diseases might not even be available within the species or its relatives. Moreover, new races of the pathogen may emerge with increased virulence.

Since the development of genetic engineering of plants, new strategies for introducing resistance to fungal pathogens are arising (Strittmatter and Wegener, 1993). For example, transgenic tobacco plants constitutively expressing the enzyme stilbene synthase, which is responsible for the production of resveratrol in groundnut, show enhanced resistance to *Botrytis cinerea* (Hain *et al.*, 1993). Similarly, transgenic tobacco plants constitutively expressing either a bean class I chitinase, a bacterial chitinase or a ribosome inhibiting protein from barley show enhanced resistance to *Rhizoctonia solani* (Broglie *et al.*, 1991; Jach, *et al.*, 1992; Logemann *et al.*, 1992; Logemann *et al.*, 1993). More recently, promising results were obtained by constitutive co-expression of a rice basic chitinase and an alfalfa acidic ß-1,3-glucanase to enhance resistance in transgenic tobacco against *Cercospora nicotianae* (Zhu *et al.*, 1994). In our own program it was shown, that simultaneously overexpressing both a class I chitinase and a class I ß-1,3-glucanase from tobacco in transgenic tomato plants enhanced resistance towards *Fusarium oxysporum* f.sp.*lycopersici* (Jongedijk *et al.*, 1995.Van den Elzen *et al.*, 1993).

In search for such antifungal proteins, the phenomenon of induced resistance is exploited. This type of resistance is directed against a broad spectrum of pathogens, including viruses, bacteria and fungi and can be triggered by chemical spraying and with biological factors such as pathogens, elicitors or fungal cell wall components (McIntyre *et al.*, 1981; Sequeira, 1983). Tobacco, *Nicotiana tabacum*, cv. Samsun NN, inoculated with tobacco mosaic virus (TMV) is a well studied example of induced resistance. The triggering of a hypersensitive response results in the induction of resistance, both locally around the sites of infection and systemically

in noninfected parts of the plants (McIntyre *et al.*, 1981). Concomitantly with the occurrence of resistance, *de novo* synthesis of a large number of proteins is induced. These include enzymes involved in the process of lignification and in the synthesis of secondary metabolites such as phytoalexins, as well as a group of proteins known as the pathogenesis-related (PR) proteins. First described in tobacco, PR proteins were initially defined as pathogen-inducible, acidic and relatively protease-resistant proteins occurring in the extracellular spaces, the so called class II proteins (Parent and Asselin, 1984; Van Loon, 1985). However, more recently, serologically related class I isoforms have been identified intracellularly (Linthorst 1991; Stintzi *et al.*, 1993). Traditionally, in tobacco the PR-proteins are divided into 5 groups and in most of these groups at least two classes can be distinguished. The class I representatives of each group are basic in nature and located in the vacuole whereas the class II counterparts are acidic and located extracellularly. Recently, additional groups of PR-proteins have been identified (Van Loon, 1994) of which only PR-11 is of tobacco origin (Melchers *et al.*, 1994). In Table 1, a summary is presented of the various groups PR-proteins currently identified in tobacco. Many of these were shown to exhibit antifungal activity (see references in Table 1) and, therefore, PR-proteins are often believed to play a role in plant defense to pathogen attack.

In our laboratory, an *in vitro* assay has been established to assist in the isolation and identification of antifungal proteins (Woloshuk *et al.*, 1991). Initially, crude protein extracts are tested for antifungal activity. In a later stage, this extract can then be fractionated and the resulting fractions can be monitored for the presence of antifungal activity. Subsequently, fractions containing activity are pooled and subjected to a next round of fractionation. The extract used in the present study is prepared from tobacco leaves, *Nicotiana tabacum* cv. Samsun NN, inoculated with tobacco mosaic virus. As a first step, we calibrated such extracts for the presence of these PR- proteins. Over 50% of the proteins in such extracts could be accounted for and therefore we screened these extracts for their antifungal activity. Over 80% of the assayed fungi appeared sensitive to these protein extracts. These results indicate that TMV inoculated tobacco leaves can serve as a good source for the isolation of proteins with antifungal activity towards a variety of fungi. Undoubtedly, some of such proteins belong to already known groups of PR-proteins, but possibly also novel antifungal proteins can thus be identified.

MATERIAL AND METHODS

Biological Materials

Tobacco plants (*Nicotiana tabacum* cv Samsun NN) were grown in the greenhouse and PR-proteins were induced by inoculation of the leaves of 5- to 6-week-old plants with TMV as described previously (Woloshuk *et al.*, 1991).

All fungi were cultured on potato dextrose agar (Difco) at 25 C, except *Cercospora carotae*, *Colletotrichum coccodes*, *Phoma* spp., *Phytophthora nicotianae*, *Septoria* spp., and *Verticillium dahliae*, which were grown on oat meal agar (Difco) at 25 C. *Phytophthora infestans* was grown on rye agar at 18 C in the dark (Caten and Jinks, 1968). *Alternaria* sp., *Botrytis* sp. and *Phoma* sp. were cultivated under UV. Fungal spores were harvested by flooding the agar plates with sterile water and adjusted to 10,000 spores.ml^{-1}. In the case of non-sporulating fungi, liquid shake cultures were grown in potato dextrose broth at 25 C. To prepare inoculum from these shake cultures, 50 ml broth containing mycelium was harvested and vortexed for 1 minute. After passage through a fine sieve, inoculum density was adjusted to 2500 - 5000 fragments of 1 to 3 cells each, per ml.

Protein Purification and Analysis

Proteins were extracted and purified according to 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 0.5 M sodium acetate, pH=5.2, 0.1% ß-mercaptoethanol, and active charcoal (1 g/100 g leaves). The homogenate was filtered over cheese cloth, centrifuged at 3000 x *g* for 15 min, and the supernatant centrifuged for 15 min at 20,000 x *g*. The resulting supernatant was passed over a Sephadex G-25 (medium coarse; Pharmacia) column (12 x 60 cm) equilibrated with 40 mM NaOAc, pH=5.2. The eluted protein solution was incubated overnight on ice and centrifuged 50 min at 20000 x *g*. The resulting supernatant was used as the protein extract for the *in vitro* fungal screening. A second induced protein extract was prepared identically. The supernatants were concentrated to approximately 2.5 mg/ml. This was done using Microcon concentrators (Amicon) with membranes (MWCO 10 kDa).

The class II proteins PR-1a, PR-2b, PR-2c, PR-3a, PR-3b, PR-4 and PR-5 were purified according to methods described by (Kauffmann *et al.*, 1987a; Kauffmann *et al.*, 1987b;

Legrand *et al.*, 1987; Van Loon *et al.*, 1987). The class I Glu-I and Chi-I according to methods described earlier (Sela-Buurlage *et al.*, 1993). However, after the routine gelfiltration chromatography, Glu-I was purified by one extra step of cation-exchange chromatography: Fractions containing Glu-I were pooled and dialysed to 50 mM potassium phosphate buffer, pH=6.1. This protein solution was applied to a Mono-S (HR 5/5; Pharmacia) column equilibrated in the same buffer. Glu-I was not bound and eluted in an apparently pure state (as judged from SDS-PAA gels). CBP20, AP24 and Chi-V were purified according to methods described earlier (Ponstein *et al.*, 1994; Woloshuk *et al.*, 1991; Melchers *et al.*, 1994)

Protein concentrations were determined using BSA as the standard (Bradford, 1976). Electrophoretic analyses were performed using 12.5% SDS-polyacrylamide gels (SDS-PAGE) (Laemmli, 1970).

The induced protein extracts were calibrated for PR-proteins. For this analysis use was made of Western blotting techniques, using antibodies for the basic, class I, proteins: a) Glu-I: a specific peptide (LFTAPNVVVQDGSRQK) coupled to BSA and for PR-2b/c: PR-N, b) Chi-I,PR-3a/b: the class II chitinase PR-P (or PR-3a, (Legrand *et al.*, 1987), c) CBP20 and PR-4: tomato homologue P2 (Joosten *et al.*, 1990), d) AP24: specific peptide (FAPTNPSGGKCHAIHK) coupled to BSA and PR-5: PR-S (Melchers *et al.*, 1994), e) Chi-V: expressed cDNA in *Escherichia coli*. (Melchers *et al.*, 1994. All antisera were diluted 1:5,000. All antisera were prepared in rabbit and the secondary antibodies consisted of goat-anti-rabbit serum coupled to peroxidase. For detection the ECL-system was used (Ponstein *et al.*, 1994). A concentration series of both crude extracts (for G25-1: 0.25, 0.5, 1, 2 and 4 µg and for G25-2: 1, 2, 5 and 10 µg) as well as purified proteins (10, 30, 50 and 90 ng) were compared to judge the expression level of the proteins present. Both crude extracts were calibrated and all experiments were repeated.

In Vitro Antifungal Assay

Essentially, protocols developed earlier in our lab were followed (Sela-Buurlage *et al.*, 1993; Woloshuk *et al.*, 1991). The assay was performed in a 24-well microtiter dish (Greiner). Potato dextrose agar (250 µl) was pipetted into each well. Five hundred spores in 50 µl, or 125-250 mycelial fragments in the case of non sporulating fungi, were added per well. Protein extracts were applied either immediately or after spores had germinated. In the case where hyphal fragments were used, crude protein extracts were always applied immediately. Before use, protein samples were dialysed against 15 mM potassium phosphate + 20 mM NaCl, pH

6.0 , at 4 C overnight and subsequently filter sterilized through 0.22 µm filters. A range from 0 to 250 µg of induced protein extract was applied in 100 µl per well, resulting in a final volume of 150 µl. Inactivation of proteins was performed by heat treatment (10 minutes 100 C) of the samples. In the case protein extracts were applied to pregerminated spores, percentage lysis of germtube tips was counted one hour after application of the proteins, since we had determined that maximum lysis had then taken place (data not shown). Growth inhibition was monitored when control wells were fully overgrown, typically after 2-3 days depending on the speed of growth of a given fungus. Hyphal growth was stopped by staining the mycelium with 0.02% cottonblue in lactic acid and phenol (1:1 v/w) for 24 hours and subsequent destaining with water. Inhibition of mycelial growth (GI) was rated on a scale from 0 to 4, where GI = 0 is no inhibition, GI = 1 is 10-30% inhibition, GI = 2 is 30-60% inhibition, GI = 3 is 60-90% inhibition and GI = 4 is complete inhibition of fungal growth. Amount of protein extract (expressed in µg/ well) necessary to cause GI=2 are given.

RESULTS

Calibration of Induced Protein Extracts for the Amount of PR-Proteins

In this study it was investigated whether tobacco leaves (*Nicotiana tabacum* cv. Samsun NN) inoculated with tobacco mosaic virus (TMV) can serve as a source for antifungal proteins. From earlier work it was known that PR-proteins are likely candidates since for example ß-1,3-glucanases and chitinases were shown to inhibit fungal growth *in vitro* (Mauch *et al.*, 1988). Two independent extractions of tobacco leaves induced with TMV, were performed to generate protein solutions enriched for acid-soluble (PR)-proteins. Subsequent incubation of the protein extracts overnight on ice yielded a subset of the total protein fraction which was stably solubilized. A calibration was performed of these induced protein extracts, G25-1 and G25-2, to determine the levels of PR-proteins present. For this calibration the class I PR-proteins Glu-I (PR-2), the 32 kDa Chi-I (PR-3), CBP20 (PR-4), AP24 (PR-5) and Chi-V (PR-11) and the class II proteins PR-1a, PR-2b, PR-2c, PR-3a, PR-3b, mixtures of PR-4a and PR-4b and mixtures of PR-5a and PR-5b, were purified. A concentration series of both crude extracts as well as purified proteins (10, 30, 50 and 90 ng) were compared using Western analysis to judge the relative amount of each of the proteins. For the G25-1 extract 0.25, 0.5, 1,

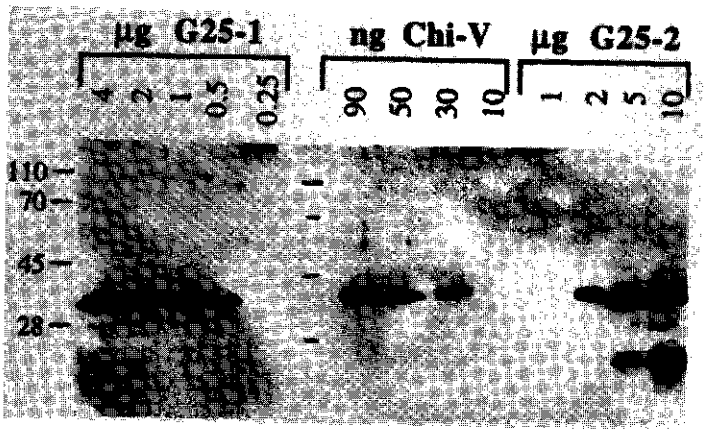

Figure 1. Calibration using Western analysis for the presence of Chi-V in protein extracts of TMV inoculated tobacco. A concentration series of both crude extracts (for G25-1: 0.25, 0.5, 1, 2 and 4 µg and for G25-2: 1, 2, 5 and 10 µg) as well as purified proteins (10, 30, 50 and 90 ng) were compared to judge the expression level of the proteins present. Antibodies were raised in rabbits against the Chi-V protein purified from *Escherichia coli* overexpressing the corresponding cDNA (26). Goat anti-rabbit serum coupled to a peroxidase was used as a second antibody. The ECL-system was used for detection (Ponstein *et al.*, 1994).

2 and 4 µg was used and for the G25-2 extract 1, 2, 5 and 10 µg. An example is shown in Figure 1 were the relative amount of Chi-V is determined in both extracts. The band intensity of around 30-50 ng pure Chi-V equals 0.5 µg G25-1 and 2 µg G25-2, corresponding to 8% in G25-1 and 2% in G25-2.Therefore the calculated average for this protein is 5%. Calculated average results for all proteins tested here are presented in Table 2. In general, the class II proteins were far more abundant then the class I proteins. Of the proteins identified, PR-5, was the most abundant at 12%. The other class II proteins averaged
around 4 percent. Also PR-1a was present at 4%. The other two class II PR-1 proteins, PR-1b and PR-1c, were not individually purified, but using Coomassie staining it was determined that the PR-1a, PR-1b and PR-1c are present in the same relative amounts (data not shown). The class I proteins Glu-I and the 32 kDa Chi-I were present at 5% and 5-10% respectively. The 32 kda Chi-I, when compared on SDS-PAGE with Coomassie staining is present far more abundantly, around 4-fold, than the 34 kda Chi-I (data not shown). CBP20, AP24 and Chi-V were present at 1%, 2% and 3% each. The most abundant proteins in these extracts have been accounted for, adding up to over 50% of the proteins soluble at pH 5.2.

TABLE 2. Calibration of a TMV induced protein extract of tobacco for PR-proteins

	Intracellular			Extracellular		
Group	Class I	%	250 µg G25 [a)]	Class II	% [b)]	250 µg G25
1	PR-1g	nt	nt	PR-1a	4.0	10.0
2	Glu-I	5.0	12.5	PR-2b	2.5-5	2.5-5
				PR-2c	2.5-5	6.3-12.5
3	Chi-I	5-10	12.5-25	PR-3a	2.5-5	2.5-5
				PR-3b	4-5	10-12.5
4	CBP20	1.0	2.5	PR-4	2.5-5	6.3-12.5
5	AP24	2.0	5.0	PR-5	12.0	30.0
11	Chi-V	5.0	12.5			

a) Calculated amount of PR-protein per 250 ug G25. Both extracts were calibrated. In case differences between the two extracts were found, ranges are given.

b) % were determined using Western analysis with purified proteins as reference (25-100 ng).

Screening of Fungi for Sensitivity to an Extract from TMV Induced Tobacco

The two induced protein extracts were assayed for their *in vitro* antifungal activity, using a microtiter dish assay whereby induced protein extracts were applied to a suspension of spores, germlings or hyphal fragments on a layer of potato dextrose agar in wells of a 24-wells microtiterplate. A list of fungi causing some of the most significant losses in agricultural crops was composed. Over thirty species, divided over many genera were assayed. Only non obligate fungi were selected, since obligate fungi can not be grown for an extended period on artificial media. Sporulating fungi were assayed at two stages in their life cycle. The protein samples were applied either directly to the spores or at a later stage after spores had germinated. In the latter case, protein samples were applied after the germtubes had reached a length of 3-5 times the length of the fungal spore. In case of non-sporulating fungi, hyphal pieces were used as a starting inoculum and protein samples were always applied immediately. For each fungus germination rate and speed of growth was determined to allow for application of protein extracts at appropriate timepoints after germination (data not shown). Routinely, if a fungus appeared insensitive to the protein extract, a second species of the same genus was tested, to establish whether the observed insensitivity was common for the whole genus or specific for a particular

TABLE 3. Antifungal activity of a protein extract of TMV induced tobacco leaves on spores and germlings

	Spores[a]		
	non-pregerminated	pregerminated	
	growth inhibition[b]	50% lysis[c]	growth inhibition[b]
Alternaria dauci	50	5	25
Alternaria porri f.sp. solani	25	5	10
Alternaria radicina	25	1-5	5-10
Alternaria solani	50	10	25
Aspergillus flavus	250	> 250	> 250
Aspergillus parasiticus	> 250	>250	> 250
Aspergillus versicolor	nd	0.5	0.5
Botrytis cinerea	5-10	> 250	> 250
Cercospora carotae	25	10-25	5-10
Colletotrichum coccodes	250	> 250	> 250
Colletotrichum tabacum	250	>250	> 250
Fusarium oxysporum f.sp. lycopersici r1	50	5	1
Fusarium solani	50-250	10	1-5
Penicillium digitatum	250	50	50
Phoma destructiva	> 250	50	25
Phoma lingam	25	25	5
Phytophthora infestans	> 250	> 250	50-250
Phytophthora nicotianae nicotianae	250	> 250	250
Pyricularia oryzae	25-50	> 250	50-250
Rhizopus oryzae	> 250	>250	25-50
Rhizopus stolonifer	250	250	50
Septoria lycopersici	25	25	10
Septoria nodorum	250	> 250	50-250
Trichoderma viride	15	< 0.5	< 0.5
Verticillium dahliae	50-250	25	10-25

Assays were performed as described by Sela-Buurlage et al., 1993 and Woloshuk et al., 1991. Each value represents the average of two induced protein extracts which were tested in triplicate repeats.

a) Status of spores at application time.

b) Growth inhibition (GI) was scored on a scale from 0 to 4, whereby 0 is no inhibition and 4 is 100% inhibition. The amount of protein extract (in µg) per well in a final volume of 150 µl, necessary for GI=2, corresponding to 30-60 % inhibition of fungal growth.

c) Percentage lysis of germtube tips was determined 1 hour after application of protein extract. The amount of protein extract (in µg) per well in a final volume of 150 µl, necessary to cause lysis of 50 % of the germtube tips.

nd, not determined.

>250, in case GI=2 is not reached by application of 250 µg.

species.

In all *in vitro* assays, the two induced protein extracts were applied in a concentration series from 0, 1, 5, 10, 25, 50, 100 to 250 µg/well (= 150 µl) and as controls the same amount of protein was applied after heat treatment. All assays were performed in triplicate repeats.

In the case of pregerminated spores, percentage lysis of germtube tips was determined one hour after application of the proteins. The amount of extract necessary for 50% of the germtube tips to be lysed is presented in Table 3. For 10 of the 25 sporulating fungi application of 250 µg induced extract per well was not sufficient to cause lysis of 50% of the germtube tips. However, of the remaining 15 fungi, 11 were very sensitive since already low amounts of induced extract, i.e. 25 µg/well, were capable to cause 50% lysis. For the remaining four fungi between 50 to 250 µg/well was necessary.

After 2-3 days mycelium was stained with lactophenol cotton blue to estimate the amount of growth inhibition, rated on a scale from 0 to 4, whereby 0 represents no growth inhibition and 4 represents complete growth inhibition. The amount of induced protein extract averaged, necessary to cause a growth inhibition of 2 (GI=2, 30-60% inhibition of fungal growth) are presented in Table 3 for the sporulating fungi and Table 4 for the non-sporulating fungi. Of all fungi tested, sporulating and non-sporulating, 15 fungi were extremely sensitive to the extract whereby 0.5-25 µg/well already causes a GI=2. A group of 7 fungi is moderately sensitive, i.e. an amount of 50-250 µg/well is necessary to cause GI=2. The remaining 9 fungi did not show a GI=2 at 250 µg/well and are classified as not sensitive (GI = 0 at 250 µg) or slightly sensitive (GI = 1 at 250 µg).

In some cases variation in sensitivity within one genus was observed, such as in the genera of *Aspergillus, Rhizoctonia, Sclerotinia* and *Septoria*. Some genera appeared sensitive for all species tested, such as *Alternaria, Fusarium, Phoma* , *Rhizopus* and *Phytophthora*. For the genera *Colletotrichum* and *Pythium* both species tested appeared insensitive. If neither of the tested species was sensitive, it was concluded that no antifungal proteins were (sufficiently) present.

DISCUSSION

Tobacco (*N. tabacum* cv. Samsun NN) upon inoculation with tobacco mosaic virus reacts with the increased synthesis of many (PR) proteins. Simultaneously, resistance is induced both locally and systemically against a wide variety of pathogens (Dean and Kuc, 1985;

TABLE 4. Antifungal activity of a protein extract of TMV induced tobacco leaves on non-sporulating fungi

Fungus	Growth inhibition [a)]
Mycosphaerella fijense	10
Pythium sylvaticum	> 250
Pythium ultimum	> 250
Rhizoctonia carotae	5
Rhizoctonia solani	> 250
Sclerotinia homeocarpa	50-250
Sclerotinia sclerotiorum	> 250

Assays were performed as described by Woloshuk et al., 1991, whereby spores were replaced with hyphal pieces as starting inoculum. Each value represents the average of two induced protein extracts which were tested in triplicate repeats.

a) Growth inhibition (GI) was scored on a scale from 0 to 4, whereby 0 is no inhibition and 4 is 100% inhibition. The amount of protein extract (in µg) per well in a final volume of 150 µl, necessary for GI=2, corresponding to 30-60 % inhibition.

nd, not determined.
>250, in case GI=2 is not reached by application of 250 µg.

Hecht and Bateman, 1964; Kuc, 1982; Ross, 1961a; Ross, 1961b). A role in the defense against pathogens is attributed to PR-proteins and indeed many tobacco PR-proteins were shown to possess antifungal activity (Melchers *et al.*, 1994; Ponstein *et al.*, 1994; Sela-Buurlage *et al.*, 1993; Woloshuk *et al.*, 1991). Similarly, PR-protein extracts from the upper leaves of induced tomato plants were shown to contain *in vitro* antifungal activity towards *Phytophthora infestans* (Enkerli *et al.*, 1993). In the engineering of fungal resistance through overexpression of one or more antifungal proteins in a transgenic plant, the identification of such proteins is crucial. An *in vitro* screening assay was developed for this purpose (sela-Buurlage *et al.*, 1993; Woloshuk *et al.*, 1991). In the present study a total extract of TMV induced tobacco leaves was used to screen for the presence of antifungal proteins directed against a wide variety of fungi and to determine whether this extract could serve as a source for antifungal proteins Both inducible and constitutive proteins with antifungal activity can be isolated from protein extracts of TMV-induced tobacco leaves. A calibration of these induced protein extracts was done using Western analysis, with purified proteins as a reference, to

determine to what extent known PR-proteins are present. Over 50% of the proteins present in these induced protein extracts was thus accounted for. Generally, the class II representatives of each group appeared far more abundant than their intracellular, class I, counterparts. It can not be excluded however that the, acidic, class II proteins are more stable under the used conditions that the, basic, class I proteins. Also, upon calibration for the PR-proteins, variation of the relative amounts of the PR-proteins was observed between both extracts. Generally, the intensity and speed of induced host resistance response depends on the amount of inducer, in this case TMV. The higher the concentration of the inducer, the faster and more intensive the host response (Sequeira, 1983). Possibly therefore differences in the level of PR-proteins between both extracts might correlate to the level of initial infection with TMV.

Using an *in vitro* screening assay, many fungi can be screened in a fast and reproducible manner for their sensitivity to protein extracts. It can be expected that different proteins exert their activity at different stages in the life cycle of a fungus. Therefore, antifungal activity of induced tobacco extracts was tested at two stages in the life cycle of the fungi. Firstly, protein extracts were applied to spores and antifungal activity was monitored during germination. A second set of experiments allowed spores to germinate before application of protein extracts. From the results presented in this study it becomes clear that the spore is far less sensitive to the induced protein extracts than the pregerminated spore. However, if pregermination was continued too long, i.e. more than 10 x the length of the spore, before application of the induced protein extracts, the germlings became less sensitive (data not shown). It is anticipated that the germtube is the more likely candidate to encounter plant antifungal proteins *in vivo* than a spore since often at the spore stage the fungus has not entered the host yet. Screening *in vitro* for antifungal activity is therefore best done using pregerminated spores.

For the sporulating fungi (Table 3) the amount of induced protein extract needed to cause 50% lysis of germtube tips is compared to the amount necessary to cause GI=2. It is evident that for all fungi, with the exception of the *Alternaria* sp, more induced extract needs to be applied to cause 50% lysis than GI=2. Assuming that lysis of germtube tips is mainly caused by the (synergistic) action of the hydrolytic enzymes such as chitinases and ß-1,3-glucanases, other antifungal protein(s) are conceivably present capable of causing inhibition of fungal growth. At present it remains unknown what the nature of such (a) protein(s) is (are). Alternatively, low levels of chitinases and ß-1,3-glucanases can slow down growth of a fungus without causing the germtube tip to lyse.

In similar studies on *in vitro* antifungal activity of proteins reported in literature, fungi are generally not pregerminated before application of antifungal proteins. Additionally, in these

kinds of assays the fungi are assayed in a liquid state whereby a nutrient broth containing the fungal spores is applied to a microtiter plate together with the protein samples. Growth inhibition is then measured photospectrometrically (Broekaert *et al.*, 1990; Cammue *et al.*, 1992; Legrand *et al.*, 1987; Terras *et al.*, 1992). It can be expected that hyphal wall formation is different when submerged under excess liquid and hence sensitivity to protein extracts might be altered (either increased or diminished). Another way of monitoring antifungal activity is by placing protein extracts in front of the growing front of a fungus in a petriplate (Broglie *et al.*, 1991; Wessels, 1994). The disadvantage of such a screening assay is the fact that the proteins diffuse away from the initial point of application and therefore require excess application. Also, no quantitative measurements are possible. In our hands, this type of assay was indeed far less sensitive than the one described in this study (data not shown). Indeed, it has been shown that hyphae of *Schizophyllum commune* upon emerging from an aqueous solution into the air, excrete hydrophobic monomeric peptides, hydrophobins, which self-assemble at the outer surface, thus coating the outside of the hyphae (Wessels, 1994). Possibly this hydrophobin layer protects the growing hyphae from (enzymatic) attack. Therefore, an *in vitro* assay was set up in which wells of a microtiter plate are filled with a layer of PDA upon which a spore suspension was pipetted. After appropriate time intervals, induced protein extracts were applied. The amount of liquid on top of the fungus is consequently limited and it can be expected that the hyphal wall formation proceeds normally. Also, the amount of PDA is limited so the protein concentration will not drop more than a factor of [50+100]/[250 + 50 +100] which is 3/8 of the originally applied amount.

It must be stated that antifungal activity of proteins like α- and ß-thionins might be overlooked since they lose their activity if applied on agar containing medium (Florack, pers. comm). For two antifungal proteins from *Raphanus sativus*, RsAFP1 and RsAFP2, similar observations were made (Terras, pers. comm), indicating that the microtiter dish assay also has certain disadvantages. However, of all the fungi tested, over 80% showed sensitivity to the crude protein extracts, indicating the enormous potential of TMV induced tobacco leaves as a source for (novel) antifungal proteins. There was a slight tendency towards the first prepared extract being more antifungal than the second one (data not shown) which was concurrent with the calibration experiments of the protein extracts for the PR-proteins, indicating higher amounts of (induced) proteins.

By adding 250 µg/well as a maximum, some proteins might not have been applied in high enough amounts to exert their antifungal effect. However, addition of higher amounts of protein extracts to a well in some cases caused a nutritional effect such that any antifungal activities present in these extracts are counteracted by the increased growth due to the presence

of high amounts of soluble protein.

For those fungi not sensitive to the TMV induced tobacco extracts, it remains unclear whether the potentially antifungal proteins in the induced extract were too limited in quantity to be active. Additionally, by using a total protein extract as a first screen in the identification of antifungal proteins, possible inhibitory activities, present in these induced protein extracts, might mask antifungal effects. Additional explanations for the observed variation in the level of sensitivity might involve differences in fungal cell wall structure, the amount and type of possible proteases or inhibitory proteins produced by the fungus or variation in the dynamics of growth of various fungi *in vitro*. It remains to be determined for each fungus specifically what is the underlying reason for observed insensitivity.

For the identification and isolation of antifungal (PR) proteins, activity against fungi is monitored *in vitro* during chromatographic fractionation of the induced protein extracts. This approach was successfully used in the identification of AP24, the class I PR-5 protein which was shown to be capable of causing lysis of sporangia of *Phytophthora infestans* (Woloshuk *et al.*, 1991). Similarly, using the *in vitro* assay, we determined which specific proteins and combination of proteins from the PR-2 and PR-3 group exhibited the most pronounced antifungal effect towards *Fusarium solani* , namely the class I chitinase, Chi-I, and the class I ß-1,3-glucanase, Glu-I acting both alone and in synergy (Sela-Buurlage *et al.*, 1993). More recently, a chitinase, CBP20, belonging to the PR-4 group was isolated with antifungal activity towards *F. solani, Trichoderma viride* and *Alternaria radicina* both alone and in synergy with as well Chi-I as Glu-I (Ponstein *et al.*, 1994). Another antifungal PR-protein, the chitinase Chi-V acting alone and in synergy with Glu-I towards *Alternaria radicina, Fusarium solani* and *Trichoderma viride* (Melchers *et al.*, 1994).

In the strategy of genetic engineering it is essential to determine which specific protein(s) are responsible for the observed antifungal effect. Thus the amount of genes needed to be transformed into a plant in order to introduce enhanced resistance can be minimized. Also in such studies *in vitro* assays are a powerful tool to identify antifungal proteins and the most potent combinations thereof.

The data obtained in the *in vitro* assays seem to correlate well with the *in vivo* results in as much that tomato plants constitutively expressing the class I chitinase and the class I ß-1,3-glucanase from tobacco showed enhanced resistance to *Fusarium oxysporum* (Jongedijk *et al.*, 1995; Van den Elzen *et al.*, 1993). Thus, the approach of using total protein extracts to screen *in vitro* for the presence of antifungal proteins appears a useful tool in the search for antifungal proteins and their overexpression in transgenic plants.

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Chapter 3

Extracellular Targeting of the Vacuolar Tobacco Proteins AP24, Chitinase and ß-1,3-Glucanase in Transgenic Plants

ABSTRACT

The *Nicotiana tabacum ap24* gene encoding a protein with antifungal activity toward *Phytophthora infestans* has been characterized. Analysis of cDNA clones revealed that at least three *ap24*-like genes are induced in tobacco upon infection with tobacco mosaic virus. Amino acid sequencing of the purified protein showed that AP24 is synthesized as a preproprotein from which an amino-terminal signal peptide and a carboxyl-terminal propeptide (CTPP) are cleaved off during post-translational processing. The functional role of the CTPP was investigated by expressing chimeric genes encoding either wild-type AP24 or a mutant protein lacking the CTPP. Plants expressing the wild-type construct resulted in proteins properly sorted to the vacuole. In contrast, the proteins produced in plants expressing the mutant construct were secreted extracellularly, indicating that the CTPP is necessary for targeting of AP24 to the vacuoles. Similar results were obtained for vacuolar chitinases and ß-1,3-glucanases of tobacco. The extracellularly targeted mutant proteins were shown to have retained their biological activity. Together, these results suggest that within all vacuolar pathogenesis-related proteins the targeting information resides in a short carboxyl-terminal propeptide which is removed during or after transport to the plant vacuole.

INTRODUCTION

In a variety of plant species, the development of necrosis in response to pathogenic infections is accompanied by the *de novo* synthesis of a large number of proteins. In tobacco one group of induced proteins, known as pathogenesis-related (PR) proteins, has been classified into five families (PR-1 to PR-5) based on their structure and function (for reviews see Bol, 1990; Bowles, 1990; Linthorst, 1991). The function of the PR-1 and PR-4 family of proteins remains to be elucidated. The tobacco PR-2 proteins have ß-1,3-glucanase activity (Kauffmann *et al.*, 1987), whereas PR-3 proteins are chitinases (Legrand *et al.*, 1987). Plant ß-1,3-glucanases and chitinases represent antifungal hydrolases that act synergistically to inhibit fungal growth *in vitro* (Mauch *et al.*, 1988). Recently, a basic 24 kD protein (AP24) of the PR-5 group was identified which has activity against *Phytophthora infestans* (Woloshuk *et al.*, 1991). A similar 24 kD protein inhibitory to *P.infestans* was also purified from pathogen-induced tomato. The tobacco AP24 protein was identified as osmotin, while the tomato AP24 protein was similar to the salt-induced NP24 protein. In addition, other proteins similar to AP24, isolated from seeds of corn, oats, sorghum and wheat, were reported recently to have antifungal activity toward a variety of fungi (Vigers *et al.*, 1991).

Within each family of PR-proteins, except for PR-4, both intracellular and extracellular isoforms have been identified. In general, acidic proteins are found extracellularly while their basic counterparts are localized intracellularly. It has been shown for several basic PR-proteins, namely AP24, chitinase, and ß-1,3-glucanase, that the intracellular location is in the vacuole (Mauch and Staehelin, 1989;Singh *et al.*, 1989; Van den Bulcke *et al.*, 1989). Recently, much research has been directed toward understanding the mechanisms by which vacuolar proteins are selectively targeted in plant cells (Chrispeels, 1991). Briefly, proteins are targeted to the secretory pathway by an N-terminal hydrophobic signal peptide that mediates transport into the endoplasmic reticulum. Proteins lacking specific targeting information are secreted by plant cells (Denecke *et al.*, 1990; Dorel *et al.*, 1989; Itturiaga *et al.*, 1989). Vacuolar targeting requires a positive sorting signal. In mammalian cells, the proper targeting of proteins to lysosomes is dependent on their recognition by receptors for mannose-6-phosphate (Kornfeld and Mellman, 1989). In contrast, plant proteins do not require N-linked oligosaccharides for correct sorting into vacuoles (Sonnewald *et al.*, 1989; 1990; Voelker *et al.*, 1989; Wilkins *et al.*, 1990). A number of studies have demonstrated that the vacuolar sorting signal is part of the primary amino acid sequence of the protein. However, the requirements for vacuolar targeting in plant cells are still poorly understood. For two proteins, barley lectin (Bednarek *et al.*, 1990) and tobacco basic chitinase (Neuhaus *et al.*,1991), it was shown recently that a carboxyl-terminal propeptide (CTPP) is essential for vacuolar targeting. In these propeptides no specific vacuolar targeting determinant was identified. In contrast, a targeting determinant was found in the N-

terminal propeptide of barley aleurain that is necessary and sufficient for efficient delivery of this protein to a plant vacuole (Holwerda *et al.*, 1992). In addition, the N-terminal propeptide of sweet potato sporamin was shown to be necessary for vacuolar targeting (Matsuoka and Nakamura, 1991), but specific targeting determinants of this propeptide have not been reported. Here, we have studied the vacuolar targeting signals of three vacuolar PR-proteins, AP24, chitinase, and ß-1,3-glucanase, in more detail.

The isolation and characterization of cDNA clones and a genomic DNA clone encoding the anti-*Phytophthora* protein AP24 of tobacco, is reported in this article. Furthermore, we analyzed the processing and targeting of the AP24 protein and established, 1) that maturation of AP24 includes N- and C-terminal processing and 2) that the C-terminal propeptide is necessary for correct sorting to the vacuole. Similar results were obtained with the basic PR-proteins chitinase and ß-1,3-glucanase. We demonstrated that removal of a C-terminal propeptide of these proteins results in the secretion of the mature proteins in transgenic tobacco plants. The extracellularly targeted proteins were shown to have retained their biological activity.

MATERIALS AND METHODS

Biological Materials

Phytophthora infestans isolate 88069 was kindly provided by Dr. L. Davidse, Wageningen, The Netherlands. The conditions for maintenance and sporangia formation of the fungus were as described by Woloshuk *et al.* (1991). Tobacco (*Nicotiana tabacum* cv Samsun NN) was grown at 24°C in an artificially illuminated room (12000 Lux at plant height) with a 16-hr photoperiod.

Construction and Screening of cDNA- and Genomic Libraries

Polyadenylated RNA was isolated from TMV-infected Samsun NN plants and double stranded cDNA was made according to the lambda ZAP-cDNA synthesis and cloning system of Stratagene (La Jolla, CA). The tobacco cDNA library (obtained from Dr. H. Linthorst, Leiden University, The Netherlands) was screened for *ap24*-like cDNA clones using the *np*24 gene of tomato as a probe. The *np*24 gene was amplified from genomic DNA of tomato by serial

transcription with Taq-DNA polymerase (Saike *et al.*, 1988) using synthetic oligonucleotide primers LS11 (5'-GTGTGACTTATACTTATGCTGCCAC-3') and LS12 (5'AGAGATCAG-TTGACTATTTGAGGCG-3').

A genomic DNA library in lambda Charon 35 of Samsun NN tobacco was screened for the presence of *ap24* DNA sequences using *ap24* cDNA as a probe (Cornelissen *et al.*, 1987). Out of 5 positive phages, we selected the specific recombinant phage containing the *ap24* gene. A 4.5 kb fragment containing the *ap24* genomic sequence was subcloned from the phage DNA-insert into a pBS-vector.

Cloning Procedures and DNA Sequencing

Wild-type and mutant chitinase constructs were made by the following cloning procedure. A full size tobacco basic chitinase cDNA (Linthorst, 1990b) was cloned into the BamHI-site of vector pMOG181. This vector contains an expression cassette consisting of the cauliflower mosaic virus (CaMV) 35S promoter with a double enhancer, an unique BamHI-site, and the nopaline synthase transcription terminator on an EcoRI-HindIII fragment. The expression unit containing the *wt* chitinase construct was cloned into the binary vector pMOG23 (Sijmons *et al.*, 1990), resulting in plasmid pMOG198. Site directed mutagenesis was used to prepare a ctpp- basic chitinase construct (pMOG189), by using primers SV1 (5'-TTGGAAACTGACTTTAAGTCGACACTATGTA-3'), SV2 (5'-TACATAGTGTCGA CTTA AAGTCAGTTTCCAA-3'), LS19 (5'-TTCCCAGTCACGACGTTGT-3') and LS20 (5'-CAGCTATGACCATGATTACG-3') in an overlapping Taq-polymerase chain reaction (PCR) (Ho *et al.*, 1989). The DNA was checked for the presence of the desired base pair substitutions (see bold type nucleotides) which resulted in an open reading frame lacking 7 codons at the end. The expression unit containing the mutant chitinase construct on a EcoRI-SstI fragment was cloned into pMOG23, to yield pMOG189.

To obtain an open reading frame for *ap24* the synthetic primers LS21 (5'-GCCGGATCCAATTCGGCACATGGGCAACTTGA-3') and LS22 (5'-GTTTATTACAGC AAGGATCCTGATT-3') were used to introduce a startcodon and to tailor this gene with a suitable BamHI cloning site. The *ap24* cDNA was cloned into the BamHI-site of expression vector pMOG180, which contains the CaMV 35S-promoter with a double enhancer, a unique BamHI-site and the nopaline synthase transcription terminator. Subsequently, construct pMOG404 was made by cloning the *ap24* expression unit present on an EcoRI-HindIII fragment into binary vector pMOG23. The ctpp- *ap24* expression construct was made by site directed mutagenesis as described above, using the primers LS19, LS20, LS23 (5'-CTTTTGTCCTTAATAATGGTTAACCTCACCCAAAT-3') and LS24 (5'ATTTGGGTGA-

GGTTAACCATTAAGGACAAAAG-3'). Introduction of one extra nucleotide (T) at position 1117 (Figure 1), resulted in a ctpp- *ap24* cDNA which does not encode the C-terminal extension of 20 amino acids. The mutant *ap24* gene was cloned into pMOG180 and subsequently into pMOG23 to yield pMOG405, using a procedure similar as described for the *wt ap24* gene.

Constructs pMOG412 and pMOG549 were made using the genomic basic glucanase gene of tobacco (Linthorst *et al.*, 1990a). A BamHI-site was introduced into the 5'-end of the gene at position 1521 (accession no. M34086) using PCR with the primers JR50-5 (5'-TAAATAGCTCGTGGATCCTCTTAATTCTCCC-3') and LS19. The genomic basic glucanase gene including its own transcriptional terminator, present on 2.5 kb BamHI-XbaI fragment, was cloned behind the CaMV 35S promoter of expression vector pMOG183. The expression unit containing the 35S-glucanase construct on a SstI-XbaI fragment was cloned into pMOG23 to yield pMOG412. The ctpp- glucanase construct was made by site directed mutagenesis as described above using the primers JR50-5, LS19, JR50Xb (5'-CAACTTTGGGTGATATCGTTAAGTTTGGGAC-3') and JR50Bh (5'-GTCCCAAAC TTAACGATATCACCCAAAGTTG-3'). The mutant glucanase gene does not encode the C-terminal extension of 22 amino acids as a result of nucleotide substitutions which created a new stopcodon (position 3387; accession no. M34086). Cloning of the mutant gene into pMOG183, resulted in a mutant 35S-glucanase expression unit which after insertion in vector pMOG23 gave the final construct pMOG549.

The binary constructs were transferred from *E.coli* to *Agrobacterium tumefaciens* strain MOG101 (octopine Ti helper plasmid) (Melchers *et al.*, in preparation), by triparental mating. Plasmids from the isolated transconjugants were checked by restriction enzyme analysis. Recombinant DNA procedures were performed as described by Maniatis *et al.* (1982). Dideoxy sequencing (Sanger *et al.*, 1977) was performed using a universal M13 DNA primer or with synthetic *ap24* specific oligonucleotides. The sequence of *ap24* was determined from sequencing both DNA strands over their entire length.

Transformation and Analysis of Transgenic Plants

Transgenic tobacco plants were obtained by the standard leaf disc transformation method using kanamycin selection (Horsch *et al.*, 1985). Leaf discs were prepared from top leaves of axenically grown tobacco plants. RNA and protein blot analyses were used to assay the expression of transgenes in the transgenic plants raised. Leaf samples were ground in extraction buffer (50 mM sodium acetate buffer pH 5.0). Protein from the extracellular fluid was isolated according to the procedure described by De Wit and Spikman (1982). Protein concentrations were determined by a Bradford assay (Stratagene quantitative assay kit Cat. no.

201210). Electrophoretic analysis was performed using 12.5 % SDS-polyacrylamide gels (SDS-PAGE) and immunoblotting was done as described previously (Sijmons *et al.*, 1990). Immunoblots were developed using the ECL-detection kit (RPN2105, Amersham) according to the suppliers recommendations. The antisera raised against the tobacco proteins PR-P, acidic PR-5, and basic ß-1,3-glucanase were produced in a rabbit.

Chitinase and ß-1,3-glucanase measurements were done using ^{3}H-labeled chitin and laminarin as substrates (Woloshuk *et al.*, 1991).

Purification and Sequence Analysis of Tobacco AP24

Isolation of the C-terminal peptide of AP24 was done by affinity chromatography on an immobilized anhydrotrypsin column. Tobacco AP24 (2 nmol as determined by N-terminal sequencing), isolated as described before (Woloshuk *et al.*, 1991) was S-pyridylethylated in the liquid phase (Jost *et al.*, 1991) and desalted by reversed-phase HPLC. Subsequently, the protein was digested with trypsin-TPCK (E.C.3.4.21.4; Worthington Biochemical Corporation) in 0.1 M ammoniumbicarbonate pH 8.2 at 37°C at an enzyme/substrate ratio of 1:100 (w/w) for 1 hr. After addition of the same amount of enzyme and acetonitril to 12% (v/v) the incubation was continued for another 3.5 h. A third portion of trypsin was added and the incubation was continued overnight. Cleavage was checked by performing 2 cycles of automatic Edman degradation on 2% of the material. The digestion was stopped by the addition of dilute acetic acid and the sample was dried in a speed-vac.

The immobilized anhydrotrypsin column (volume 1 ml) was used exactly as recommended by the manufacturer (Pierce). The sample was dissolved in 250 µl 'binding buffer' (0.05 M sodium acetate buffer pH 5.0, containing 0.02 M $CaCl_2$ and 0.05% sodium azide) and applied to the column. The column was washed with 20 ml of 'binding buffer', and subsequently bound peptides were eluted with 10 ml 'elution buffer' (0.1 M formic acid pH 2.5). The flow rate was approximately 5 ml/hr and fractions of 0.5 or 1 ml were collected during washing and elution. All fractions were analyzed by HPLC on a Nucleosil 10 C18 reversed-phase column (2 x 150 mm). Peptides were preparatively isolated by reversed-phase HPLC on the same column and sequenced by automatic Edman degradation on an Applied Biosystems model 477A pulse-liquid protein sequencer, coupled on-line to a PTH analyzer. The amino acid composition was determined on a Hewlett Packard amino acid analyzer (model: Aminoquant) using OPA/Fmoc derivatization. Digestion, isolation of peptides and sequence analyses were done by Eurosequence B.V., Groningen, The Netherlands.

RESULTS

Molecular Characterization of cDNA Clones Encoding *AP24*

Nucleotide sequences specific for the anti-*Phytophthora* protein NP24 were amplified from genomic DNA of tomato by serial transcription with Taq-DNA polymerase (Saike *et al.*, 1988) by using two specific oligonucleotide primers based on the *np24* cDNA sequence (King *et al.*, 1988). Screening of a lambda ZAP cDNA library of tobacco mosaic virus (TMV) infected Samsun NN tobacco plants with the amplified tomato *np24* gene as probe resulted in the isolation of nine positively hybridizing clones. Restriction enzyme analysis and (partial) sequence analysis revealed that these clones could be divided into three classes: class A, B and C, represented by one, two and six clones respectively. Within one group no sequence differences were found, whereas between groups insert sequences showed a high degree (>90 %) of identity. To determine which of the three classes of cDNA clones encoded AP24, the protein was partially sequenced. Its N-terminal structure has been determined previously (Woloshuk *et al.*, 1991). Further amino acid sequences were obtained by sequencing peptide fragments of AP24. In total 43 % of the primary structure of AP24 was elucidated, as shown in Figure 1 (underlined amino acid sequences). Comparison of the determined amino acid sequence of AP24 with the primary protein structure deduced from the three classes of cDNAs showed complete identity with the protein encoded by the class B clones. One class B cDNA clone, notably pMOG390, was entirely sequenced and contained a nearly complete AP24 coding region (Figure 1).

Molecular Characterization of the Nicotiana Tabacum *ap24* Gene

A genomic library of *N.tabacum* was screened using pMOG390 as a probe. Five recombinant lambda phages, each containing different restriction fragments hybridizing to the *ap24* cDNA, were purified. The genomic *ap24* clone identical to pMOG390 was selected by a polymerase chain reaction using specific oligonucleotide primers corresponding to the 5'-end coding region and 3'-end non-coding region of *ap24* cDNA. Only one of the five phages showed a very strong amplification of specific sequences with these primers and was selected for further analysis. The complete nucleotide sequence of the *ap24* gene, including the deduced primary structure of the AP24 protein and sequences of the 5'-flanking and 3'-flanking regions of the gene, is shown in Figure 1. Comparison of pMOG390 with *ap24* revealed an identity of the cDNA sequence with nucleotides 442 to 1344 of the *ap24* gene (Figure 1), suggesting that

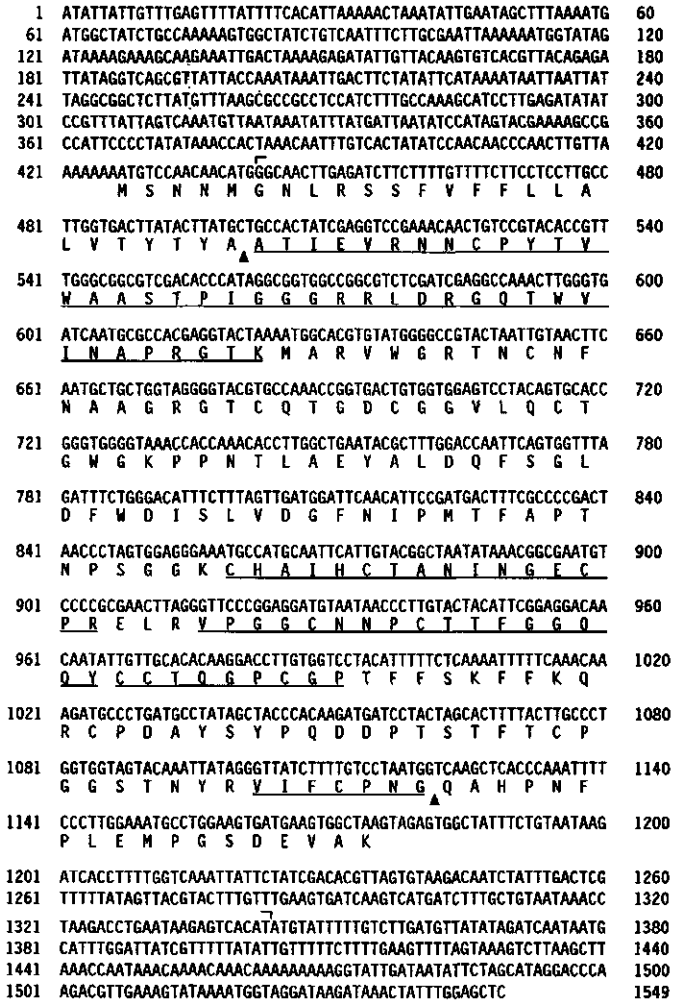

Figure 1. Nucleotide and Deduced Amino Acid Sequence of the *N.tabacum ap24* Gene (GenBank/EMBL Accession No. X65701). The sequenced parts of the AP24 protein are underlined, and processing sites are indicated by arrowheads. The 5'-end and 3'-end of the AP24 cDNA clone pMOG390 (GenBank/EMBL Accession No. X65700), are marked with a bracket symbol (-).

the pMOG390 homologous gene was isolated. Within the 5'-end upstream region a 'CAAT' motif (position 313) and 'TATAAT' motif (position 370) is found. In the 3'-end non coding region a potential polyadenylation signal (5'-AATAAA-3') is present at position 1313. The coding region of the *ap24* gene lacked intron sequences and encoded a precursor protein of 250 amino acids. The AP24 preprotein contained a signal peptide of 25 amino acids that is involved in transport across the membrane of the endoplasmic reticulum. The amino acid sequence of AP24 contained no potential N-linked glycosylation sites since the motif Asn-X-(Ser/Thr) is absent.

Figure 2 shows the C-terminal amino acid sequence alignment of several AP24-like proteins, chitinases and ß-1,3-glucanases. The presence of a C-terminal amino acid extension was reported previously for intracellular proteins, like chitinase and ß-1,3-glucanase of tobacco (Linthorst *et al.*, 1990a, 1990b). Comparison of the AP24-like proteins indicate that all the intracellular proteins (AP24, NP24, and thaumatin) contain extensions between 8 and 21 amino acids beyond the C-terminus of the extracellular PR-5 protein. We hypothesized that these C-terminal extensions may function as a sorting determinant for targeting of these PR-proteins to the vacuole.

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AP24    CPGGSTNYRVIFCPNGQAHPNFPLEMPGS.DEVAK
NP24    CPGGSTNYRVVFCPNGVADPNFPLEMPASTDEVAK
Thau    CP.GSSNYRVTFCPTALELEDE
PR-5    CPPG.TNYRVVFCP

Chi-I   GFYRRYCSILGVSPGDNLDCGNQRSFGNGLLVDTM
Chi-E   GYYRRYCGMLNVAPGDNLDCYNQRNFAQG

Glu-I   SPNKQPKYNINFGVSGGVWDSSVETNATASLVSEM
Glu-E   SPDQRAKYQLNFN
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Figure 2. Comparison of C-terminal amino acid sequences of intra- and extra-cellular mplant proteins. Alignment of homologous proteins at their C-terminus: tobacco AP24 (this work, Neale *et al.*, 1990) with tomato NP24 (King *et al.*, 1988), *Thaumatococcus danielli* thaumatin (Edens *et al.*, 1982) and tobacco PR-(Cornelissen *et al.*, 1986a); basic intracellular tobacco chitinase (Chi-I) with acidic extracellular tobacco chitinase PR-P (Chi-E) (Linthorst, 1990b; Payne *et al.*, 1990); basic intracellular tobacco ß-1,3-glucanase Glu-I) with acidic extracellular tobacco ß-1,3-glucanase PR-N (Glu-E) (Linthorst *et al.*, 1990a; Shinshi *et al.*, 1988). The star (*) indicates the N-linked glycosylation site of Glu-I. C-terminal processing sites are indicated by arrowheads.

The C-terminus of the Intracellular Proteins AP24, Chitinase and ß-1,3-Glucanase is Required for Vacuolar Targeting

To determine the possible role of the C-terminal amino acid extension of the basic PR-proteins AP24, chitinase and ß-1,3-glucanase in vacuolar sorting, we have expressed wild-type (*wt*) and C-terminal mutant constructs of the corresponding genes in transgenic tobacco plants. As shown in Figure 3, the genes of the *wt* constructs were placed under control of the CaMV 35S promoter. Constructs containing the cDNAs of *ap24* and basic chitinase were cloned in front of the nopaline synthase terminator region. The construct containing the genomic basic ß-1,3-glucanase gene retained its natural transcriptional terminator. The mutant constructs were engineered by introduction of a translational stopcodon into each of the *wt* constructs (details see Methods section). As a result, the mutated genes code for proteins lacking the C-terminal extension as shown in Figure 2. Transgenic tobacco plants containing *wt* -and mutant constructs were analyzed for expression of specific proteins. Figure 4 shows a Western blot analysis of different leaf samples including extracellular washing fluid (EF), leaf material remaining after removal of EF ("-EF") and total leaf protein fraction. In transgenic plants expressing the different *wt* constructs, the encoded proteins AP24, chitinase and ß-1,3-glucanase (pMOG404, pMOG198, and pMOG412 respectively) were targeted intracellularly. These proteins were present in the total (c) and "-EF" fractions (b) but not in the EF-fraction (a) of the corresponding transgenic plants. The intracellular targeting of AP24, chitinase and ß-1,3-glucanase in transgenic plants is in agreement with their natural location. In contrast, transgenic plants expressing the mutant constructs (pMOG405, pMOG189, and pMOG549) produced proteins that were found predominantly in the EF-fraction (a). The small amounts of protein present in the "-EF" fractions was probably residual protein not removed in the EF-fraction. Comparing the level of protein in the "-EF" fractions with that in the total fractions indicates that all three modified proteins are targeted extracellularly. Control plants showed no expression of PR-proteins except for an extracellular chitinase protein (PR-P) which was detected in the EF-fraction only. We found that mutant AP24 protein, which lacks 20 amino acids, migrates on a SDS-gel like *wt* AP24. This observation suggests that after removal of the signal peptide of AP24 a second processing step occurs where a peptide fragment of about 2 kD is removed.

Maturation of the AP24 Tobacco Protein Involves N- and C-terminal Processing

Post-translational processing of AP24 was studied in more detail by characterization of the mature AP24 protein. C-terminal and internal parts of AP24 were analyzed by using peptide

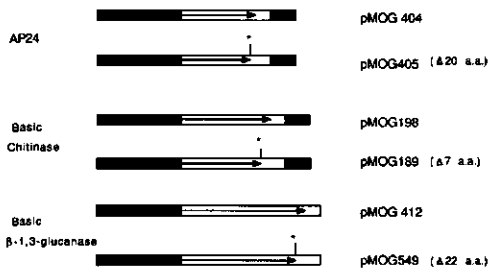

Figure 3. Schematic drawing of the chimeric gene constructs. Expression constructs of the wild-type *ap24* cDNA (pMOG404) and Carboxyl-Terminal Mutant *ap24* cDNA (pMOG405); the wild-type (pMOG198) and Carboxyl-Terminal Mutant (pMOG189) basic chitinase cDNAs; and the wild-type (pMOG412) and Carboxyl-Terminal Mutant (pMOG549) basic ß-1,3-glucanase gene. All genes are under control of 35S-promoter (hatched box). The black box represents the nos-terminator region. The star (*) indicates the site were an additional stop codon is created via site-directed mutagenesis by overlap extension using the polymerase chain reaction. The number of deleted C-terminal amino acids is given in brackets. Chimeric gene constructs are present in a binary vector next to the NPTII-gene.

fragments isolated from a tryptic digest of the protein. The C-terminal peptide of AP24 was specifically isolated via affinity chromatography on an immobilized anhydrotrypsin column (Ishii *et al.*, 1983; Kumazaki *et al.*, 1986; Yokosawa and Ishii, 1979). The partial amino acid sequence obtained from AP24 is shown in Figure 1 (underlined amino acid sequences). The composition of the sequenced peptides was confirmed by amino acid compositional analysis (data not shown). The primary structure of the C-terminal peptide appeared to correspond to the amino acid sequence deduced from nucleotides 1102 to 1123 (Figure 1), indicating that AP24 is C-terminally processed. Thus, processing of the tobacco AP24 protein includes the removal of both a N-terminal signal peptide of 25 amino acids and a C-terminal propeptide (CTPP) of 18 amino acids. The mature AP24 (207 a.a.) has a calculated molecular mass of 22,336 Da.

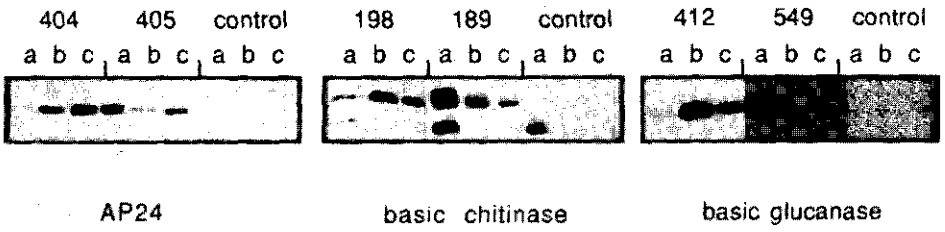

Figure 4. Expression analysis of transgenic tobacco plants containing different wild-type (pMOG404, pMOG198, pMOG412) and mutant constructs (pMOG405, pMOG189, pMOG549). Extracellular washing fluid (a), leaf material remaining after removal of EF (b), and total leaf protein fraction (c), were isolated from leaves of transgenic -and control plants. One microgram of protein was loaded per lane. Immunoblot analysis was performed using antibodies raised against: PR-5 for detection of the serologically related protein AP24 in pMOG404 and pMOG405 transgenics; acidic chitinase PR-P for detection of basic chitinase in pMOG198 and pMOG189 transgenics; basic ß-1,3-glucanase for analysis pMOG412 and pMOG549 transgenic plants.

Biological Activity of Extracellularly Targeted Proteins

The extracellularly targeted proteins were tested for their characteristic activity. The AP24 protein isolated from the EF-fraction of transgenic plants showed lysis activity on *P.infestans* sporangia similar to the *wt* AP24 protein purified from TMV-induced tobacco leaves (data not shown). Tobacco plants expressing either the *wt* -or mutant chitinase construct showed an 8- to 10-fold increase in chitinase enzyme activity over that of control plants. Expression of the *wt* -or mutant glucanase construct gave a 10- to 15-fold increase in ß-1,3-glucanase enzyme activity. Table 1 shows the results of the analysis of the EF-fractions for chitinase and ß-1,3-glucanase enzyme activity. They indicated that both the extracellularly targeted chitinase and glucanase proteins were enzymatically active. Collectively, these data demonstrate that the extracellular targeted mutant proteins AP24, chitinase and ß-1,3-glucanase have retained their activity, which suggests that these proteins are folded properly.

Table 1. Chitinase and ß-1,3-Glucanase enzyme activity of extracellularly targeted proteins in EF of transgenic plants.

Transgenic		Chitinase[a] cpm/µg EF	Glucanase[b] nkat/µg EF
pMOG198	*wt* chitinase	320	22
pMOG189	mutant chitinase	2414	25
pMOG412	*wt* ß-1,3-glucanase	211	23
pMOG549	mutant ß-1,3-glucanase	261	303
Control		238	13

[a] Chitinase activity of the EF-protein fraction was determined by measuring the amount of soluble radioactivity released from ^{3}H-chitin after 30-min. incubation. [b] ß-1,3-Glucanase activity of the EF-protein fraction was determined using laminarin as a substrate.

DISCUSSION

In this study we have determined the molecular structure of the tobacco *ap24* gene and analyzed the amino acid sequences required for vacuolar targeting of AP24, a protein with antifungal activity toward *P.infestans*. The isolation of three types of tobacco cDNA clones encoding AP24-like proteins indicates that *ap24* is part of a small gene family. The cDNA sequence of *ap24* is identical to the nucleotide sequence of the tobacco osmotin cDNA determined by Neale *et al.* (1990), except for a translationally silent difference (C instead of T

residue) at position 1002. Differences reported by Neale and co-workers (1990) with the previously determined sequence of osmotin published by Singh *et al.* (1989) were hereby confirmed. This report shows the complete sequence of the *ap24* gene which was isolated from the tobacco genome. All previous literature reports show partial cDNA sequences of *ap24* (Neale *et al.*, 1990; Singh *et al.*, 1989) and an aberrant coding sequence lacking three codons in the middle of the open reading frame (Singh *et al.*, 1989). The nucleotide sequence of *ap24* contains no introns and encodes a polypeptide of 250 amino acids. In contrast to Singh *et al.* (1989), we propose that the AUG initiation codon at position 427 is used for translation of the *ap24* gene and not the AUG-codon present at position 439 (Figure 1). The sequence (5'-AAAAAUGTC-3') surrounding the first AUG initiation codon is more in agreement with the consensus sequence 5'-AACAAUGGC-3' (Lütcke *et al.*, 1987) than the sequence (5'-CAACAUGGG-3') surrounding the next AUG-codon, which is located four codons downstream.

We have determined a total of 43 % of the amino acid sequence of AP24 isolated from TMV-treated tobacco plants. The protein encoded by *ap24* is identical to the partial amino acid sequence of the antifungal protein AP24. This observation strongly suggest that AP24 and the vacuolar protein osmotin are identical proteins. Our protein sequence data demonstrate that post-translational processing of AP24 involves removal of a 25 amino acids signal peptide and a 18 amino acids C-terminal propeptide. Comparative sequence analyses between intracellular and extracellular isoforms of chitinases, ß-1,3-glucanases and PR-5 proteins have shown previously that in general intracellular proteins contain a C-terminal extension compared to their extracellular homologue (Cornelissen *et al.*, 1986a; Linthorst *et al.*, 1990a, 1990b; Van den Bulcke *et al.*,). To examine the role of these C-terminal extensions, we have expressed *wt* and C-terminal mutant constructs in transgenic plants. We have demonstrated for AP24, basic chitinase and basic ß-1,3-glucanase, that deletion of C-terminal regions of 20, 7 and 22 amino acids respectively, resulted in the secretion of these proteins in transgenic plants. We found that the C-terminal propeptide of AP24, basic chitinase and ß-1,3-glucanase is necessary for efficient sorting of these proteins to vacuoles. Our results regarding basic chitinase are in accordance with data published by Neuhaus and coworkers (Neale *et al.*, 1990). Recently, they reported that basic chitinase of tobacco contains a CTPP of 7 amino acids, which is essential for vacuolar targeting and is removed during transport to the vacuoles. It was shown previously for a class of vacuolar proteins, including barley lectin and ß-1,3-glucanases of tobacco and *Nicotiana plumbaginifolia*, that they are processed to their mature form by the removal of a glycosylated CTPP (Raikhel *et al.*, 1987; Shinshi *et al.*, 1988; Van den Bulckev 1989; Wilkins *et al.*, 1990). The propeptide of barley lectin has been shown to be necessary for targeting of the protein to the vacuole (Bednarek *et al.*, 1990). For the ß-1,3-glucanases we have shown here that a CTPP is also required for vacuolar targeting. The presence of a vacuolar targeting

signal at the C-terminal end of the basic tobacco proteins AP24, chitinase and ß-1,3-glucanase resembles the situation found in barley lectin (Bednarek *et al.*, 1990). The propeptide sequences of these proteins display no obvious consensus sequence that contains potential vacuolar targeting information. This suggests that either different sorting signals exist or, as proposed by Chrispeels (1991) that the vacuolar targeting signal is formed by physicochemical and/or structural properties of the propeptide. Recently, it was demonstrated that the CTPP from barley lectin and tobacco chitinase is sufficient to redirect a secreted protein, cucumber chitinase, to plant vacuoles of tobacco (Bednarek and raikhel, 1991; Neuhaus *et al.*, 1991). It remains to be shown whether a C-terminal propeptide is sufficient to correctly target other heterologous protein to the plant vacuole as well. In line with our observations, we hypothesize that all vacuolar PR-proteins contain a C-terminal propeptide that is necessary for proper targeting of these proteins and is removed during or after transport to the plant vacuole. This hypothesis predicts that a vacuolar targeting signal is present at the C-terminal end of basic PR-1, which contains a C-terminal extension compared to its acidic counterpart (Cornelissen *et al.*, 1986b), as shown in Figure 5.

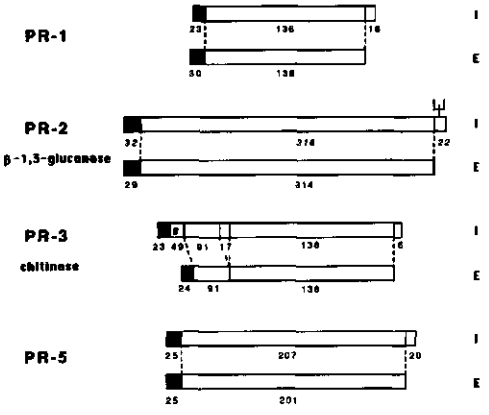

Figure 5. Schematic representation of the different domains of the intracellular and extracellular PR-proteins of tobacco. For each group of PR-proteins the intracellular (I) and extracellular (E) isoforms were aligned. The N-terminal signal peptide in all proteins is indicated by shading. Regions of high similarity in intracellular and extracellular isoforms are connected by broken lines, thus indicating the C-terminal extensions in the intracellular (basic) proteins and the hevein-domain (#) at the N-terminus of the basic chitinase. The N-linked glycan group of the CTPP of the basic ß-1,3-glucanase is depicted by the branched structure. The number of amino acid residues of the different domains is given.

The accumulation of the extracellularly targeted proteins AP24, chitinase and ß-1,3-glucanase in the extracellular washing fluid of transgenic tobacco leaves indicates that these secreted basic proteins are relatively stable. Moreover, it was shown that the secreted hydrolytic enzymes, basic chitinase and basic ß-1,3-glucanase, have retained their enzymatic activity, while the secreted AP24 protein possessed its characteristic lysis activity. The observation that vacuolar PR-proteins can be targeted extracellularly, opens the possibility to study the contribution of these proteins to fungal resistance.

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Chapter 4

Only Specific Tobacco (*Nicotiana Tabacum*) Chitinases and ß-1,3-Glucanases Exhibit Antifungal Activity

ABSTRACT

Different isoforms of chitinases and ß-1,3-glucanases of tobacco (*Nicotiana tabacum* cv. Samsun NN) were tested for their antifungal activities. The class I, vacuolar, chitinase and ß-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 ß-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 ß-1,3-glucanase. The class II ß-1,3-glucanases showed no inhibitory activity in any combination. In transgenic tobacco plants producing modified forms of either a class I chitinase, a class I ß-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 ß-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* germlings *in vitro*, comparable to the purified vacuolar class I proteins. Mixing of the EF fractions from these plants revealed synergism in inhibitory activity against *F. solani*, similar to EF from plants expressing both secreted enzymes.

INTRODUCTION

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 the pathogen having triggered the hypersensitive response. For example, inoculation of tobacco (*Nicotiana tabacum* cv. Samsun NN) with tobacco mosaic virus (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 pathogenesis-related (PR) proteins, including chitinases and ß-1,3-glucanases (for reviews see Bol *et al.*, 1990; Bowles, 1990; Linthorst, 1991). In tobacco, three classes of chitinases 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 class II chitinases, in literature known as PR-3a and -3b (formerly PR-P and -Q), are very homologous to class I chitinases, but lack the hevein domain (Linthorst *et al.*, 1990b; Payne *et al.*, 1990a) and are located extracellularly. Very recently, a third class of tobacco chitinase genes has been described (Lawton *et al.*, 1992) encoding proteins homologous to chitinases in cucumber and *Arabidopsis* (Metraux *et al.*, 1989; Samac *et al.*, 1990), but not related to class I or II isoforms. Based on their primary structures, three major classes of ß-1,3-glucanases have been identified (Payne *et al.*, 1990b; Ward *et al.*, 1991a). Class I contains basic, vacuolar isoforms (Glu-I), whereas class II is formed by acidic, extracellular isoforms. In literature, the latter proteins are also known as the pathogenesis-related (PR) proteins PR-2a, -2b and -2c (formerly PR-2, -N and -O). Class I and II ß-1,3-glucanases 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 - 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 ß-1,3-glucan, substrates for chitinases and ß-1,3-glucanases respectively (Wessels and Sietsma, 1981). *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 ß-1,3-glucanases 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 chitinases and ß-1,3-glucanases and fungal resistance have led to speculations about a role for these hydrolytic enzymes in systemically induced resistance (Mauch and Staehelin, 1989).

Here we study the antifungal activity of the class I and II chitinases and ß-1,3-glucanases from tobacco. To this end, the various chitinases and ß-1,3-glucanases were

purified to homogeneity and tested, either alone or in combinations, for their ability to inhibit *in vitro* growth of *Fusarium solani*. In addition, tobacco was transformed with modified gene constructs of the class I chitinase and class I ß-1,3-glucanase and constructs containing both modified genes, resulting in extracellular targeting of these enzymes. It appeared that these proteins retained their enzymatic activity and that extracellular washing fluid (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.

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 5-6 week old plants were inoculated with tobacco mosaic virus (TMV) as described previously (Woloshuk *et al.*, 1991). Seven days after inoculation leaves were harvested and stored at-80°C.

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 PDA at 25°C in the light. Spores were harvested from 3-6 week old plates by flooding the plate with water. The spore concentration was adjusted to 10,000 sp/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 0.5 M NaOAc, pH 5.2, 0.1 % (v/v) ß-mercaptoethanol, and active charcoal (1 g per 100 g leaves). The homogenate was filtered through four layers of cheese cloth and centrifuged at 3,000*g* for 15 min. The supernatant was centrifuged for 50 min at 20,000*g*. The final supernatant was passed over a Sephadex G-25 (medium coarse; Pharmacia) column (12 x 60 cm) equilibrated in 40 mM NaOAc, pH 5.2. The eluted protein solution was incubated overnight on ice before centrifuging 50 min at 20,000*g*. The resulting supernatant was loaded onto an S-Sepharose (Fast Flow, Pharmacia) column (5 x 5 cm), equilibrated in 40

mM NaOAc, 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 chitinase activity were pooled and concentrated by ultrafiltration through YM10 membranes (Amicon). The same was done for the fractions containing glucanase activity. The chitinase pool was brought to 20 mM sodium bicarbonate and further dialyzed against 20 mM sodium bicarbonate, pH 8.3. Class I chitinases were allowed to adsorb for 1 hour to a matrix of 50 ml 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 20 mM sodium bicarbonate and 100 ml 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 Superdex-75 gelfiltration column (HR 10/30; Pharmacia). Gelfiltration was carried out in 50 mM potassium phosphate buffer, pH 7.0, containing 0.2 M sodium chloride at a flow rate of 0.5 ml/min. The fractions showing chitinase 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). Fractions were separated on 12.5% SDS gels and the 32 and 34 kD chitinases were pooled accordingly. The pool of glucanase activity was loaded onto a gelfiltration column as above. Glucanase activity eluted at an apparent molecular mass of 5-10 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 x 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-O, which was pooled based on glucanase activity measurements. In some instances Mono Q (FPLC) ion exchange chromatography was performed to enhance the separation of PR-3a and PR-3b. All the class II proteins were further purified by gelfiltration 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 using 12.5% SDS-polyacrylamide gels (SDS-PAGE) according to the method of Laemmli (1970). In the case of native gels, SDS was omitted.

Chitinase and ß-1,3-glucanase activity measurements were performed using [^{3}H] chitin and laminarin as substrates respectively (Kaufmann *et al.*, 1987; Molano *et al.*, 1977).

Chitinase activity was expressed in counts per minute per microgram of protein. The activity of ß-1,3-glucanase was expressed in nanokat per milligram protein, where a nkat was the amount of nanomols glucose released per second.

Transformation and Analysis of Transgenic Plants

Transgenic tobacco plants were obtained as described by Melchers *et al.* (1993). Isolation of extracellular washing fluid (EF) was done as described by de Wit & Spikman (1982). Extracellular targeted enzymes were purified from the EF as described for the proteins, isolated from TMV inoculated leaf material (see Protein Purification). Specific activities of ß-1,3-glucanase and chitinase 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). Per well 250 µl PDA was pipetted. Five hundred *F. solani* spores in 50 µl were added per well. The spores were pregerminated six to seven hours at 25°C. Protein samples were dialyzed two to four hours to 50 mM potassium phosphate buffer, pH 6, at 4°C and subsequently filtersterilized through 0.22 µm filters. Protein concentrations were determined and adjusted appropriately. Per well 100 µl protein sample was pipetted, resulting in a total volume of 150 µl. Denaturation of enzymes was done by boiling the samples for ten minutes. At two hours after the initiation of incubation, the fungus was monitored microscopically for possible effects of the added proteins. After three days fungal growth was stopped by staining the mycelium with lactophenol cotton blue. Subsequently the plates were destained with water.

RESULTS

Purification of Chitinases and ß-1,3-Glucanases

The 32 kD and 34 kD class I chitinases (Chi-I), the class II chitinases PR-3a and -3b (Chi-II), the 33 kD class I ß-1,3-glucanase (Glu-I) and the class II ß-1,3-glucanases PR-2a, -2b

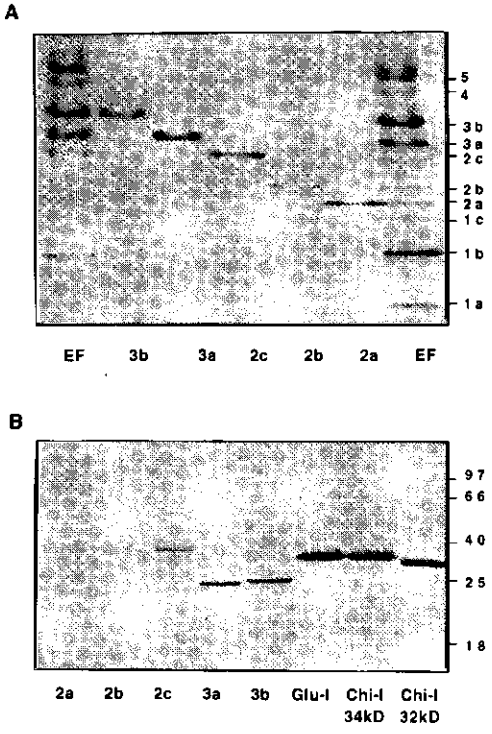

Figure 1. Native (A) and SDS-PAGE (B) of proteins purified from TMV infected Samsun NN tobacco. EF = proteins from extracellular washing fluid from TMV infected leaves. Glu-I = class I B-1,3-glucanase; Chi-I = class I chitinase. Molecular masses are indicated in kD.

and -2c (Glu-II) 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 extracellular washing fluid (EF) of TMV infected leaves were loaded and used as markers. The purity of the proteins was checked by SDS-PAGE (Fig. 1B). Upon purification, the specific activity of each of the chitinases was determined on tritiated chitin. For the determination of the specific activity of ß-1,3-glucanase, 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 class I chitinases (10,000 to 11,000 cpm/µg for the 32 kD Chi-I and 17,000 to 19,000 cpm/µg for the 34 kD Chi-I) was ten to fifteen times higher than that of the class II chitinases (1,000 to 1,100 cpm/µg). In agreement with Kaufmann *et al.* (1987), the specific activities of the class I ß-1,3-glucanase and the extracellular PR-2c were comparable (300 to 500 nkat/mg). The specific activities of PR-2a and PR-2b were

fifty to seventy fold lower (5 to 10 nkat/mg).

Table 1. Specific Activities of Purified Chitinases and ß-1,3-Glucanases of Tobacco

Enzyme		Specific Activity [a]
Chi-I	(32 kD)	10.000-11.000 cpm/µg
Chi-I	(34 kD)	17.000-19.000 cpm/µg
Chi-II	(PR-3a)	1.000-1.100 cpm/µg
Chi-II	(PR-3b)	1.000-1.100 cpm/µg
Glu-I		400-500 nkat/mg
Glu-II	(PR-2a)	5-10 nkat/mg
Glu-II	(PR-2b)	5-10 nkat/mg
Glu-II	(PR-2c)	400-500 nkat/mg

[a] Chitinase and ß-1,3-glucanase 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 *Fusarium 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 two hours, 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 chitinases and up to 10 µg for each of the ß-1,3-glucanases. Of the chitinases, only the class I chitinases were capable of causing lysis of *F. solani* germlings. Application of 5 µg Chi-I per well showed strong lysis activity (50 % for 5 µg 32 kD Chi-I; 80 % for 5 µg 34 kD Chi-I). Lower amounts of these proteins showed intermediate levels of lysis. The class II chitinases PR-3a and PR-3b exhibited no lysis activity at all, even when up to 50 and 30 µg per well respectively, was added to the wells. Testing of the ß-1,3-glucanases 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 class II ß-1,3-glucanases showed no lysis activity

even at the highest concentration of 10 µg per well.

At three days after addition of the proteins, 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 chitinases and in the right panel for the ß-1,3-glucanases respectively. 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 Glu-I showed intermediate growth inhibition and with 1 µg Glu-I per well, no fungal growth was observed. The class II ß-1,3-glucanases 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 ten minutes. By this treatment the antifungal activities were completely abolished.

Synergism between Class I Chitinases and ß-1,3-Glucanases

Mauch and coworkers (1988) have shown that combinations of chitinases and ß-1,3-glucanases are capable of synergistically inhibiting fungal growth *in vitro*. Here, we tested which specific combinations of enzymes exhibited synergistic antifungal activity. In Figure 2B results are shown of combinations of specific chitinases and ß-1,3-glucanases. As class I chitinase, the 32 kD isoform was used. Neither 0.1 nor 0.5 µg 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 Glu-II. No or intermediate effect was observed with 0.1 or 0.5 µg Glu-I, respectively. However, the combination of 0.1 µg Chi-I with 0.1 µg Glu-I caused complete tip lysis of germlings of *F. solani* two hours after addition of the enzymes and complete growth inhibition after three days. The class II ß-1,3-glucanases, 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 since the specific activity of this enzyme on laminarin is comparable to that of Glu-I (Table I). Mixing class II chitinases with 0.1 µg class I ß-1,3-glucanase showed no effect. However, an enhanced growth inhibition was observed in combination with 0.5 µg Glu-I, as compared to 0.5 µg 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 Glu-I. The effect was significantly less than with Chi-I and no lysis was observed. The Chi-II PR-3b exhibited a similar, but slightly more pronounced effect as PR-3a: only in combination with Glu-I growth inhibition was observed without lysis activity and the antifungal effect was far less than the combination of Chi-I and Glu-I.

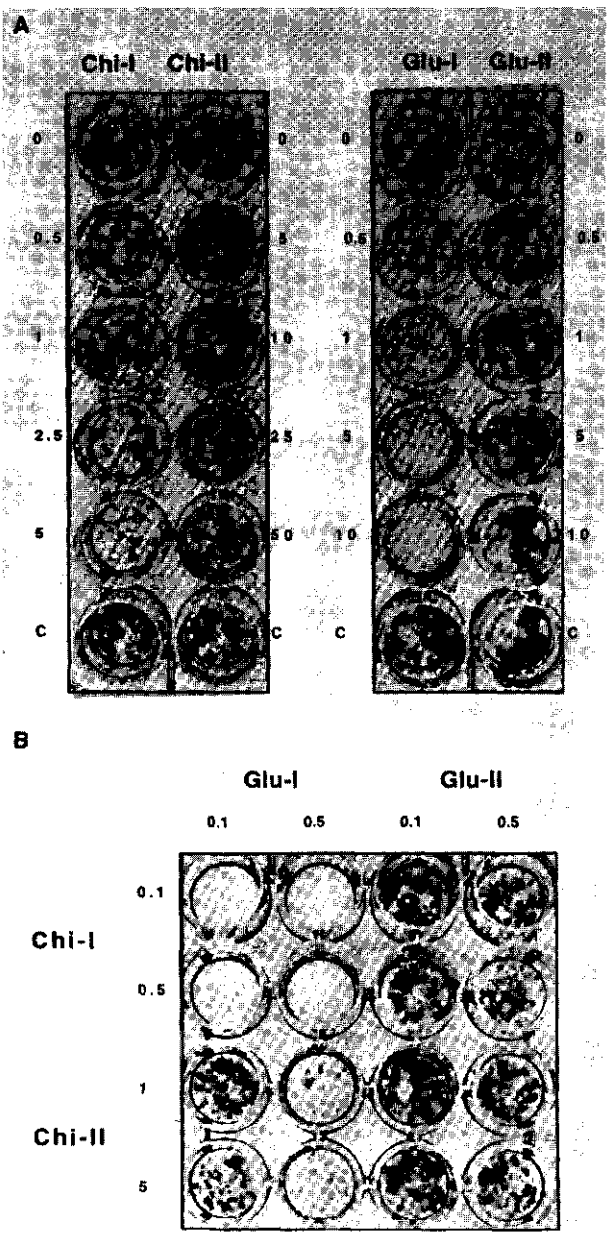

Figure 2. Effect of A) purified chitinases and ß-1,3-glucanases and B) combinations thereof on *in vitro* growth of *Fusarium solani*. Amounts of protein (in microgram) per well are indicated. Chi-I = 32 kD class I chitinase; Chi-II = PR-3a; Glu-I = class I B-1,3-glucanase; Glu-II = PR-2c; C = highest amount of protein after 10 minutes boiling.

The Antifungal Effect of Extracellularly Targeted Class I Chitinase and Class I ß-1,3-Glucanase

Class I chitinases and ß-1,3-glucanases are synthesized as preproproteins from which an amino-terminal signal peptide and a carboxyl-terminal propeptide (CTPP) are cleaved off during post-translational 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 constitutively expressing either a chimeric, modified Chi-I gene, a chimeric, modified Glu-I gene or both. The modifications involved the introduction of a stopcodon 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 extracellular washing fluid (EF) of the transgenic plants showed that the specific activity of the extracelllarly targeted class I chitinase and of the ß-1,3-glucanase was not affected (data not shown).

In order 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. Per well 5 µg of EF proteins was added to

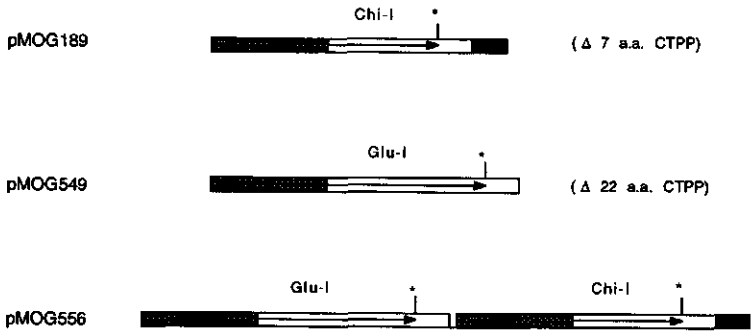

Figure 3. Schematic drawing of the gene constructs. Expression constructs of the carboxyl-terminal mutants Chi-I cDNA (pMOG 189), Glu-I gDNA (pMOG 549) and construct containing both (pMOG 556). All genes are under control of 35S-promoter (hatched box). The black box represents the nos-terminator region. Construct pMOG 549 contains the genomic Glu-I gene with its own terminator. The star (*) indicates the presence of an additional stopcodon which results in deletion of a carboxyl-terminal propeptide (CTPP) from the protein. Chimeric gene constructs are present in the binary vector pMOG402 next to the NPTII-gene.

F.solani germlings. Typical results are shown in Figure 4A. The EF from control plants did not show any effect on the *F. solani* germlings. In contrast, the EF from plants having both enzymes targeted extracellularly, exhibited almost complete lysis after two hours and almost full growth inhibition of the fungus after three days. The EF from plants containing secreted Chi-I or Glu-I alone had an intermediate effect on *F. solani*. Combinations of EF's were also tested to determine whether the effect of the double construct could be mimicked by mixing the EF of both single constructs. Results are shown in Figure 4B. Mixing 2.5 µg protein of the EF fraction of control plants with an equal amount of EF from either a retargeted Chi-I or a Glu-I transgenic plant had a minor inhibitory effect. However, combining EF of these transgenic plants showed clear inhibition of fungal growth. The denatured EF fractions had no effect on the fungus.

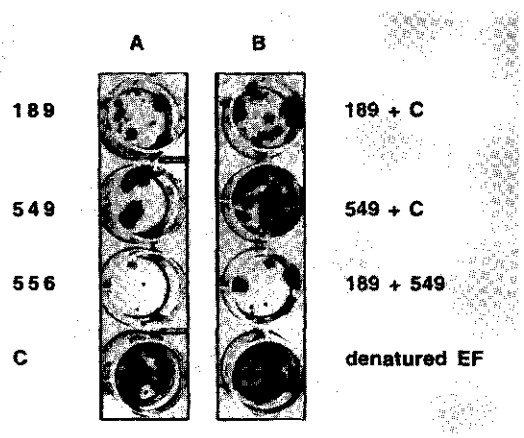

Figure 4. Effect of EF fractions A) and mixtures of EF fractions B), harvested from transgenic plants on *in vitro* growth of *Fusarium solani*. Per well a total of 5 microgram protein was added. pMOG 189 = extracellularly targeted Chi-I; pMOG 549 = extracellularly targeted Glu-I; pMOG 556 = extracellularly targeted Chi-I and extracellularly targeted Glu-I; control = regenerated nontransformed tobacco plant; denatured EF = EF from a pMOG 556 plant boiled for 10 minutes.

DISCUSSION

To assess the *in vitro* antifungal activity of various chitinases and ß-1,3-glucanases 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 kD and 34 kD class I chitinases were found to be approximately ten to fifteen 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 class I ß-1,3-glucanase 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 fifty to seventy fold lower specific activity, despite their over 90% identity in primary structure with PR-2c (Linthorst *et al.*, 1990a; Ward *et al.*, 1991a). Similar results have been reported by Kaufmann *et al.* (1987).

To determine the effects of the purified proteins on the growth *in vitro* of *Fusarium solani*, a microtiter dish assay was used. Both class I ß-1,3-glucanase and class I chitinases exhibited high antifungal activities. Combinations of Glu-I and Chi-I revealed that these two purified enzymes acted synergistically in the inhibition of fungal growth. The class II chitinases 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 class II ß-1,3-glucanases showed any antifungal activity, neither alone nor in combination with chitinases. 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 *per se* inhibitory 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 enzymatic and antifungal activities of the different chitinases and ß-1,3-glucanases. This finding contributes to our understanding 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 chitinase and ß-1,3-glucanase activities, both locally and systemically. At the same time, resistance is induced, directed towards a broad range of pathogens, including fungi. The observation that chitinases and ß-1,3-glucanases exhibit antifungal activities *in vitro* has lead to speculations as to a direct antifungal role of these hydrolytic enzymes in systemically induced fungal resistance (Boller, 1988; Mauch and Staehelin, 1989). Recently it has been shown that in TMV infected tobacco, the messengers for class II ß-1,3-glucanases and class II chitinases are induced both locally around the site of infection and systemically in the non-infected 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 chitinases or ß-1,3-glucanases fulfil a direct antifungal role in systemically induced fungal resistance. This

does not rule out an indirect role of the enzymes 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 their turn trigger the defense system. Indeed, Keen and Yoshikawa (1983) have shown that ß-1,3-glucanases from soybean are capable of releasing elicitor active carbohydrates from fungal cell walls. Besides their possible role in defense, ß-1,3-glucanases and chitinases may have functions in a number of developmental processes in plants as well. Glucanases 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 chitinase 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.*, 1991). In this context it is noteworthy, that during the early period of imbibition of barley seeds, two embryo-associated chitinases are selectively released (Swegle *et al.*, 1992) and that in *Saccharomyces cerevisiae*, a chitinase is required for cell separation during growth (Kuranda and Robbins, 1991).

Transgenic plants have been made expressing constitutively a modified class I chitinase, a class I ß-1,3-glucanase or both. In these plants the modified gene products were targeted extracellularly. The specific activities of the targeted chitinase and ß-1,3-glucanase 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 ß-1,3-glucanase and chitinase 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. Mixing EF's of the plants containing the single constructs showed synergism similar to that observed with the purified proteins. Almost complete lysis of the germling tips and growth inhibition of the fungus was 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 class I bean chitinase. Many fungi penetrate their host through the stomates and never actually enter the plant cell, but reside in the extracellular space. Targeting of the most antifungal chitinases and ß-1,3-glucanases to the extracellular space might be an effective way of increasing the resistance of the transgenic plants against fungal infection. Future experiments with transgenic plants expressing Chi-I and Glu-I extracellularly will include analyzing fungal disease resistance and the determination whether the *in vitro* synergistic

antifungal effect of these enzymes is also observed *in vivo*.

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Chapter 5

A Novel Pathogen- and Wound-Inducible Tobacco (*Nicotiana Tabacum*) Protein with Antifungal Activity

ABSTRACT

A novel pathogen- and wound-inducible antifungal protein of 20 kD was purified from tobacco Samsun NN leaves inoculated with tobacco mosaic virus. The protein, designated CBP20, was purified by chitin affinity chromatography and gel filtration. *In vitro* assays demonstrated that CBP20 exhibits antifungal activity toward *Trichoderma viride* and *Fusarium solani* by causing lysis of the germ tubes and/or growth inhibition. In addition it was shown that CBP20 acts synergistically with a tobacco class I chitinase against *F. solani*, and with a tobacco class I ß-1,3-glucanase against *F. solani* and *A. radicina*. Analysis of the protein and corresponding cDNAs revealed that CBP20 contains a N-terminal chitin-binding domain which is present also in the class I chitinases of tobacco, the putative WIN1 and WIN2 proteins of potato, and several plant lectins. The C-terminal domain of CBP20 showed high identity to tobacco PR-4a and PR-4b, tomato PR-P2 and potato WIN1 and WIN2. CBP20 is synthesized as a preproprotein which is processed into the mature protein by the removal of a N-terminal signal peptide and a C-terminal propeptide, most likely involved in the vacuolar targeting of the protein. The intracellular localization of CBP20 and its induction upon TMV-infection and wounding indicate that CBP20 is the first class I PR-4 type protein purified.

INTRODUCTION

In plants resistance against pathogens can be induced by a variety of biotic and abiotic elicitors (Sequeira, 1983). For example, infection of tobacco with a necrotizing strain of tobacco mosaic virus (TMV) leads to the induction of resistance against a broad range of pathogens including fungi, bacteria and viruses. Resistance is induced both locally around the site of infection and systemically in non-infected parts of the plant. Concomitant with resistance, the synthesis of a large number of proteins including the so-called pathogenesis-related proteins (PRs) is induced. The PRs form a group of proteins that are pathogen-induced in the infected parts of the plant, although they may be synthesized in other parts of the plant constitutively or during specific developmental stages (Van Loon, 1990). PR proteins have been classified into five different groups (reviewed by Bol *et al.*, 1990 and Linthorst, 1991). Most groups can be subdivided into two or more classes. Generally, class I proteins are localized in the vacuole of the plant cell, whereas class II proteins are present extracellularly. The class I and class II proteins are related both structurally and immunologically but differ in their induction patterns (Brederode *et al.*, 1991; Ward *et al.*, 1991).

Two of the five groups of PR proteins have known enzymatic functions: the PR-2 group consists of ß-1,3-glucanases (Kauffmann *et al.*, 1987) and the PR-3 proteins exhibit chitinase activity (Legrand *et al.*, 1987). A third class of PR-2 proteins (Payne *et al.*, 1990b) and two new classes of PR-3 proteins (Lawton *et al.*, 1992; Collinge *et al.*, 1993) have been described recently. It has been shown that chitinases and ß-1,3-glucanases from various plant sources are able to inhibit fungal growth *in vitro* (Mauch *et al.*, 1988). This is also true for the class I chitinases and ß-1,3-glucanases from tobacco. However, both the class II hydrolases seem to lack detectable amounts of antifungal activity in *in vitro* assays (Sela-Buurlage *et al.*, 1993). As yet the biochemical basis for the difference in the antifungal effect of class I and class II proteins is not known. In the case of the tobacco PR-3 polypeptides the main structural difference is the presence in the class I proteins of a chitin-binding domain and a hinge region which are both absent in the mature class II proteins (Shinshi *et al.*, 1990; Linthorst *et al.*, 1990b, Linthorst, 1991; Collinge *et al.*, 1993). Class III chitinases seem to lack antifungal activity as well (Vogelsang and Barz, 1993). Chitin-binding proteins like hevein (Van Parijs *et al.*, 1991), stinging nettle lectin (Broekaert *et al.*, 1989) and some antimicrobial peptides purified from *Amaranthus caudatus* seed (Broekaert *et al.*, 1992) have been shown to exhibit antifungal activity. These chitin-binding proteins all lack detectable levels of chitinase activity. In contrast to the lectins mentioned above, wheat germ agglutinin, consisting of four hevein domains in tandem (Raikhel and Wilkins, 1987), was reported to lack antifungal activity (Schlumbaum *et al.*, 1986; Chrispeels and Raikhel, 1991).

In this paper we report on the purification of a chitin-binding protein of 20 kD from

tobacco Samsun NN. Structural and immunological data indicate that this protein belongs to the PR-4 group. The polypeptide is located intracellularly like all tobacco class I proteins. The pathogen- and stress-induction pattern of CBP20 mRNA resembles the induction pattern of class I PR proteins rather than the pattern of the class II PR proteins. Consequently CBP20 was classified as a class I PR-4 protein. The purified protein was shown to inhibit growth of several fungi and to act synergistically with both a tobacco class I chitinase and a class I ß-1,3-glucanase.

METHODS

Biological materials

Tobacco (*Nicotiana tabacum* cv Samsun NN) was grown at 24°C in an artificially illuminated room (12000 Lux at plant height) with a 16-hr photoperiod.

Purification of the class I chitinases and CBP20

Proteins were extracted from tobacco leaves, 7 days after infection with TMV (Woloshuk *et al.*, 1991). The protein extract was desalted by passage through a G-25 column and partly separated by cation-exchange chromatography as described by Woloshuk *et al.*, 1991. Fractions containing chitinase activity were pooled and concentrated by ultrafiltration through a YM10 (Amicon) membrane (MWCO 10 kD). The concentrated solution was brought to 20 mM $NaHCO_3$. The pH of the protein solution was adjusted to 8.3 by the addition of 1 M NaOH and the proteins were further dialyzed to 20 mM $NaHCO_3$ (pH 8.3). Chitin affinity purification was performed essentially as described by Broekaert *et al.* (1988). Proteins were allowed to adsorb to a matrix (50 mL) of regenerated chitin (Molano *et al.*, 1977) equilibrated in 20 mM $NaHCO_3$, for 1 hour at 4°C under continuous stirring. A column was poured and unbound proteins were washed off by passage of 100 mL 20 mM $NaHCO_3$ (pH 8.3). The column was further washed with 100 mL 20 mM NaAcetate (pH 5.2) and bound proteins were eluted by the passage of 20 mM HAc (pH 3.5). Fractions of about 4 ml each were collected at a flow rate of 1 ml/min.

Fractions containing chitin-binding proteins were further purified by gel filtration chromatography. The chitin eluate was concentrated (to about 1 mL) and loaded in several runs onto a Superdex-75 column (HR 10/30; Pharmacia) equilibrated in 50 mM $KHPO_4$ buffer pH

7.0, containing 0.2 M NaCl. Gel filtration was carried out at 0.5 ml per minute in the same buffer and fractions (0.5 ml) were collected. The gel filtration column was calibrated with BSA (68 kD), carbonic anhydrase (29 kD) and cytochrome c reductase (12.5 kD). Two protein peaks at apparent molecular weights of 15 to 15.5 kD and 8 kD containing the class I chitinases and CBP20, respectively, were obtained. The two peaks were pooled separately and rechromatographed (usually twice). The basic chitinases were purified as previously described (Sela-Buurlage *et al.*, 1993).

Purification of PR-4a,b

The proteins that did not bind to the S-Sepharose column (see above) were dialyzed to 20 mM Tris-HCl (pH 8.0) and allowed to flow through a Q-Sepharose column equilibrated in the same buffer (Woloshuk *et al.*, 1991). The flow through was collected, concentrated and dialyzed to 25 mM diethanolamine-HCl (pH 9.7). The resulting protein solution was loaded onto a Mono P column (Pharmacia) equilibrated in the same buffer. Bound proteins were eluted with a decreasing pH gradient produced by a 10% solution of Polybuffer 96, pH 6.0. PR-4a,b (formerly called PR-r_1,r_2) was readily eluted as deduced from the electrophoretic pattern of the protein fractions on 10% native gels. The PR-4a,b containing fractions were concentrated and passed through the Superdex 75 gel filtration column as described above. PR-4a,b containing fractions were pooled and used for antifungal assays.

Protein analysis

Extracellular fluids were isolated as described by De Wit and Spikman (1982). "-EF" and "total" fractions were prepared as described by Melchers *et al.* (1993).

Protein concentrations were determined according to Bradford (1976) with BSA as the standard. Chitinase activity measurements were carried out with dye-labeled CM-chitin (Wirth and Wolf, 1990). Mixtures of substrate (100 µL of a 2 mg/mL solution per assay) in 50 mM $KHPO_4$ buffer (pH 6.4) and appropriate amounts of enzyme were incubated at 37°C for 30 minutes in a final volume of 200 µL. The reaction was terminated by the addition of 100 µL 1 M HCL, causing precipitation of the non-degraded substrate. The reaction vials were cooled on ice for 10 minutes and centrifuged (5 min Eppendorf centrifuge). Two hundred µL of the resulting supernatant was pipetted into a microtiter dish (96 wells) and the absorbance at 540 nm was read. The absorbance (expressed in OD units) was taken as a measure for enzyme activity. Lysozyme activity measurements were carried out in 50 mM $KHPO_4$ buffer (pH 6.0) as

described by Selsted and Martinez (1980).

Electrophoretic analysis was performed by the use of 10% native or 12.5% SDS-polyacrylamide (SDS-PAA) gels (Laemmli, 1970). Gels were either stained with Coomassie Brilliant Blue G-250 (Neuhoff *et al.*, 1988) or transferred to nitrocellulose filters to allow for immunological detection. The transfer buffer consisted of 48 mM Tris, 39 mM glycine, 20% (v/v) methanol and 0.0375% (w/v) SDS. Immunodetection was performed according to the ECL Western blotting protocol provided by Amersham, UK. The antiserum to the PR-4a,b analogue from tomato (PR-P2) was kindly provided by Matthieu Joosten, Wageningen, The Netherlands (Joosten *et al.* 1990). The antisera against PR-3a from tobacco (Linthorst *et al.*, 1990b) and PR-P2 from tomato were produced in rabbits. The antisera were diluted 1:5,000 and 1:1,000, respectively.

Protein Sequence Determinations

Purified CBP20 was used to determine the amino acid sequence of the mature protein. Internal sequences were obtained after digestion of 10 µg with N-chlorosuccinimide/urea (Lischwe and Ochs, 1982) or V_8 protease (Boehringer Mannheim, Cleveland *et al.*, 1977), respectively. Trypsin digestion were carried out on 40 µg purified CBP20 as described earlier (Yokosawa and Ishii, 1979; Ishii *et al.*, 1983; Kumazaki *et al.*, 1986).

To obtain the amino acid sequences, digested protein samples were separated on 17.5 % SDS-PAA gels as described by Moos *et al.* (1988) and electroblotted to PVDF membranes according to Matsudaira (1987). Proteins were visualized by Coomassie Brilliant Blue R-250 staining (Matsudaira, 1987). Protein bands of interest were cut out and sequenced by Eurosequence, Groningen, The Netherlands, using Edman degradation on an Applied Biosystems 477A protein sequencer.

Antifungal assays

Fusarium solani, *Trichoderma viride* and *Alternaria radicina* were maintained and *in vitro* assays performed as described by Sela-Buurlage *et al.* (1993).

Screening of a cDNA Library and DNA sequence analysis

A tobacco cDNA library, prepared from polyadenylated RNA isolated from TMV-

infected Samsun NN tobacco leaves was made using a ZAP-cDNA synthesis kit (Stratagene Cat #200400, 200401) and was kindly provided by Dr. Huub J.M. Linthorst (Linthorst *et al.*, 1991). One microgram of DNA isolated from the tobacco lambda-ZAP library was used in a PCR with oligonucleotides LS20 (5'-CAGCTATGACCATGATTACG-3') and LS46 (5'-CTCGAATTCGG-A/T-CCIACIGG-A/T-CC-G/A-TA-G/A-AAAGCIGTCCA-3') to amplify a partial CBP20 cDNA fragment. The PCR product was cloned after digestion with *Eco*RI into a pBS-vector to yield clone pMOG684. From this clone a specific CBP20 probe (187 bp) was amplified in a PCR using the oligonucleotides LS48 (5'-CTCGAATTCGGCACGAGGATCCTCTATTTC-3') and LS49 (5'-CTCGAATTCCACTG-CACTGGCTTTGGCAGC-3'). Recombinant DNA procedures were performed as described by Maniatis *et al.* (1982). Nucleotide sequence of the different cDNA clones was determined using the double-stranded DNA sequencing method (Chen and Seeburg, 1985).

Northern and Southern blot analysis

Ten micrograms of tobacco genomic DNA was digested with either *SstI*, *PstI*, *Hind*III or *Eco*RI restriction endonucleases and fragments were separated on an 1% agarose gel and transferred onto Hybond N+ membranes. Hybridization was performed with either the ^{32}P-labeled cDNA of clone *cbp*20-44 or PR-4a and the membranes were washed in 0.5 x SSC containing 0.1% SDS at 55 °C. Total RNA was isolated from the leaves of healthy Samsun NN tobacco plants and from stressed plants using phenol extraction and LiCl precipitation Verwoerd *et al.* 1989). Fifteen micrograms of RNA, denatured by glyoxal treatment, was separated on an 1.5 % agarose gel, blotted and crosslinked onto a GeneScreen membrane, and hybridized to a ^{32}P-labeled insert of clone *cbp*20-44. The Northern blot was washed in 0.1 x SSC at 65°C.

RESULTS

Identification and Characterization of a Novel Chitin-Binding Protein

Seven days after inoculation of Samsun NN tobacco plants with tobacco mosaic virus (TMV) proteins were extracted from the infected leaves. The leaf extract was passed through a Sephadex G-25 column (Figure 1, lane A) and a cation exchange column (Figure 1, lane B). Chitin-binding proteins were isolated by the subsequent employment of affinity

chromatography. Proteins were allowed to bind to insoluble chitin at pH 8.3 at 4°C. Bound proteins were eluted by lowering the pH of the mobile phase to 3.5. Three proteins of 20 kD, 32 kD and 34 kD were present in the eluate (Figure 1, lane C). The 32 and 34 kD proteins were identified as the two class I chitinases which are known to be induced in tobacco upon infection with TMV (Legrand *et al.*, 1987) and known to reversibly bind to chitin (Broekaert *et al.*, 1988, Sela-Buurlage *et al.*, 1993).

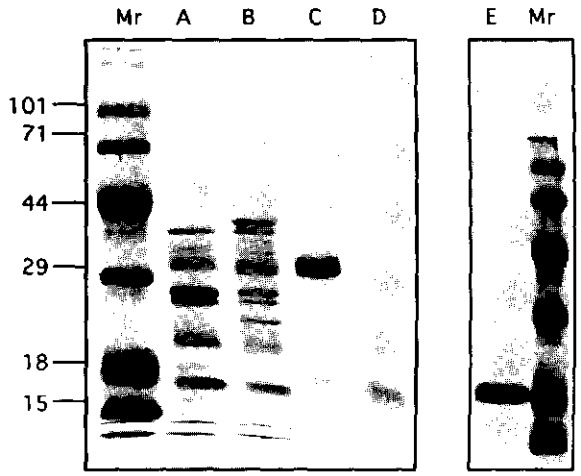

Figure 1. Purification of CBP20. Protein samples were taken at different stages in the purification procedure of CBP20 and analyzed on 12.5% SDS-PAA gels. A sample of the crude desalted leaf extract is shown in lane A. Protein pools obtained after S-Sepharose cation exchange chromatography, chitin affinity chromatography and gel filtration chromatography are shown in lanes B, C and D, respectively. In lane E, 3 µg purified protein is loaded. The lanes indicated by Mr show prestained markers. The corresponding molecular weights are as indicated.

The third protein of about 20 kD appeared to be a novel chitin-binding protein. This protein (further referred to as CBP20) was purified to homogeneity by three rounds of gel-filtration chromatography. The class I chitinases eluted at an apparent molecular weight of 15 to 15.5 kD, CBP20 at an apparent molecular weight of 8 kD. Usually, base line separation occurred after the second passage. The CBP20 containing fractions resulting after third passage were pooled and appeared to be electrophoretically pure (Figure 1, lane E). Usually 100 µg pure CBP20 was obtained from about 400 g TMV-infected tobacco leaves (containing about 100 mg protein). In extracts of healthy tobacco leaves no chitin-binding proteins were detected (data not shown).

Since CBP20 bound to chitin, it seemed obvious to assay this protein for chitinase activity. To this end we used the chitinase assay described by Wirth and Wolf (1990). Some

activity was found to be associated with CBP20: 0.03 to 0.05 ODU per µg protein (data not shown). However, this activity is extremely low compared to the activity measured for the class I chitinases (around 3 ODU per µg). Since some chitinases exert lysozyme activity, we tested CBP20 for such an activity as well. Activity measurements indicated that CBP20 exhibited some lysozyme activity: 0.01 ODU per µg protein. This specific activity is in the same range as the lysozyme activity associated with the class I chitinases but about 1000-fold lower than the specific activity for hen egg lysozyme measured under the same reaction conditions.

CBP20 Inhibits Fungal Growth *In Vitro*

The antifungal activity associated with several chitin-binding proteins prompted us to test the effect of CBP20 on fungal growth. To this end 24-well microtiter plates were used (Sela-Buurlage *et al.*, 1993). Spores of either *Trichoderma viride, Fusarium solani* and *Alternaria radicina* were pipetted onto potato dextrose agar and allowed to germinate for 6 to 16 hours. Purified and filter-sterilized protein solutions were added to the pregerminated spores. The ability to lyse the fungus was studied one hour after the addition of protein. Growth inhibition was scored 2 to 3 days later. *T. viride* appeared to be the most sensitive fungus. Almost complete lysis of the germ tubes was observed immediately after the addition of small amounts (1 µg/well = 6.7 µg/mL) CBP20 (data not shown). Consequently, growth was severely inhibited (data not shown). Hyphal tips of *F. solani* showed no lysis in the presence of up to 10 µg CBP20 per well, although swelling of the hyphal tips was observed microscopically (data not shown). However, some effect of purified CBP20 (1 µg/well) was observed on the growth of *F. solani* (Figure 2). The addition of CBP20 (up to 5 µg/well) to spores of *A. radicina* did not result in lysis either. Contrary to *F. solani* , growth of *A. radicina* was not affected in the presence of CBP20 (data not shown).

Since chitin-binding proteins are known to act synergistically with chitinases (Broekaert *et al.*, 1989; Hejgaard *et al.*, 1992), and chitinases and ß-1,3-glucanases are known to act synergistically as well (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993), we studied the effect of CBP20 in combination with these two enzymes. The addition of 0.5 µg class I ß-1,3-glucanase alone resulted in a small amount of lysis (< 5%) and some growth inhibition in case of *F. solani* (Figure 2). The combination of 1 µg CBP20 and 0.5 µg ß-1,3-glucanase showed a strong lysing activity (about 70%). Also a severe effect on growth was visible (Figure 2). From these data we conclude that CBP20 and the class I ß-1,3-glucanase act synergistically. Addition of 0.5 µg class I chitinase alone did not cause lysis of the germ tubes nor interfered with the growth of *F. solani* (Figure 2). The combination of 1 µg CBP20 and 0.5 µg class I chitinase did not cause lysis either, but inhibited the growth of *F. solani* in a synergistic manner (Figure

2). The effect of the latter combination was even more potent than the combination of CBP20 and the ß-1,3-glucanase.

The addition of 0.5 µg/well class I ß-1,3-glucanase alone inhibited growth of *A. radicina* substantially, whereas 0.1 µg class I ß-1,3-glucanase was ineffective in inhibiting fungal growth. Combinations of CBP20 (5 µg/well) and ß-1,3-glucanase (0.1 µg/well) inhibited the growth of *A. radicina* (about 35%) resulting in more condensed growing mycelia (data not shown). Apparently, both proteins acted synergistically. Combining 5 µg CBP20 with a class I chitinase (0.5 µg/well) did not result in enhanced growth inhibition compared to the effect of the class I chitinase alone. Thus, in case of *A. radicina* no synergism was observed between CBP20 and the class I chitinase. In all cases heat-inactivation of the protein mixtures eliminated their antifungal effect.

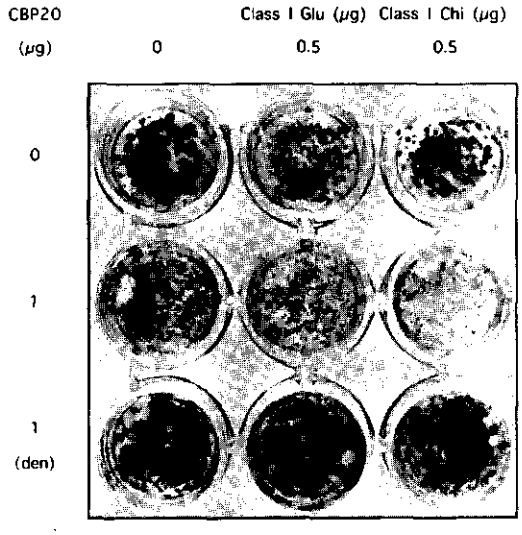

Figure 2. Antifungal activity of CBP20. The effect of purified CBP20, and combinations of CBP20 with a class ß-1,3-glucanase (Glu) or a class I chitinase (Chi) on the *in vitro* growth of *Fusarium solani* . Amounts of protein (in µg) per well are indicated. The effect of the protein combinations should be compared to the effect of the individual proteins (left column and top row) and heat-denatured ("den")control samples (bottom row).

CBP20 is C-terminally Processed

To further characterize CBP20, amino acid sequences were determined.The protein was separated on a 12.5% SDS-PAA gel and electroblotted to a PVDF membrane to allow for N-terminal sequencing. However, no sequence data became available, probably due to the

presence of a modified Gln residue interfering with Edman degradation (see below).

An internal sequence was obtained after digesting CBP20 with the Glu-C specific endoproteinase (V_8protease) from *Staphylococcus aureus* (Cleveland *et al.*, 1977). The major reaction product was a 17 kD peptide which gave the following amino acid sequence (CBP-PEP1): Tyr-(Ala/Gly)-Ser-Pro-Ser-Gln-Gly-X-Gln-Ser-Gln-(Arg)-Ser-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Ala-Gln-Asn. From the specificity of the protease it can be predicted that a Glu residue precedes the first given amino acid residue. The amino acid indicated as X is most likely a Cys residue since no special care was taken to alkylate the Cys residues prior to sequence analysis. The amino acid residues given in brackets were not characterized unequivocally. A second internal amino acid sequence was obtained after chemical digestion of CBP20 with N-chlorosuccinimide (Lischwe and Ochs, 1982). A band of 9-11 kD was used for the sequence determination (CBP-PEP2): Thr-Ala-Phe-Tyr-Gly-Pro-Val-Gly-Pro-(Pro/Arg)-Gly-Arg-Asp-Ser-X-Gly-Lys-(Gly)-Leu-?-Val-Thr-Asn. Since N-chlorosuccinimide cleaves at the C terminus of tryptophan residues it is assumed that this amino acid precedes the given sequence. The amino acid indicated as X is again most likely a cysteine residue as argued above. At the position of the question mark no amino acid identification could be made. A third amino acid sequence representing the C-terminus of CBP20 was obtained as follows. Four nmol CBP20 was digested with trypsin and the C-terminal peptide was purified from the digest via affinity chromatography on an immobilized anhydrotrypsin column (Yokosawa and Ishii, 1979; Ishii *et al.*, 1983; Kumazaki *et al.*, 1986). Sequencing of this peptide revealed the following amino acid sequence (CBP-PEP3): (Gly)-(His)-Leu-Ile-Val-Asn-Tyr-Glu-Phe-Val-Asn-Cys-Gly-Asp-Asn, which is presumably preceded by a Lys or an Arg residue.

In order to isolate clones corresponding to CBP20 a lambda ZAP cDNA library of tobacco leaves infected with TMV was screened. A partial cDNA fragment was amplified from a total lambda ZAP-cDNA library by using the oligonucleotides LS20 and LS46 in a polymerase chain reaction.The oligonucleotide LS20 was complementary to the pSK-vector sequence of the lambda ZAP arm at the 5'-end of the cDNA insert, and oligonucleotide LS46 was based on the amino acid sequence of fragment CBP-PEP2. The amplified DNA fragment (407 bp) was cloned as a *Eco*RI fragment into the *Eco*RI linearized vector pBlueScript to yield clone pMOG684. The nucleotide sequence of the cloned *Eco*RI fragment confirmed that a partial CBP20 cDNA clone was isolated. To ensure the use of a CBP20 specific DNA probe a 187 bp PCR-fragment was amplified from plasmid pMOG684 using the oligonucleotides LS48 and LS49. This PCR fragment, encoding the N-terminal part of CBP20 up to the Gly-rich region present in CBP-PEP1, was subsequently used to screen a tobacco cDNA library.

The screening for CBP20-like cDNAs resulted in the isolation of several positive clones. Five clones containing 800bp cDNA inserts were sequenced. The derived sequences indicated

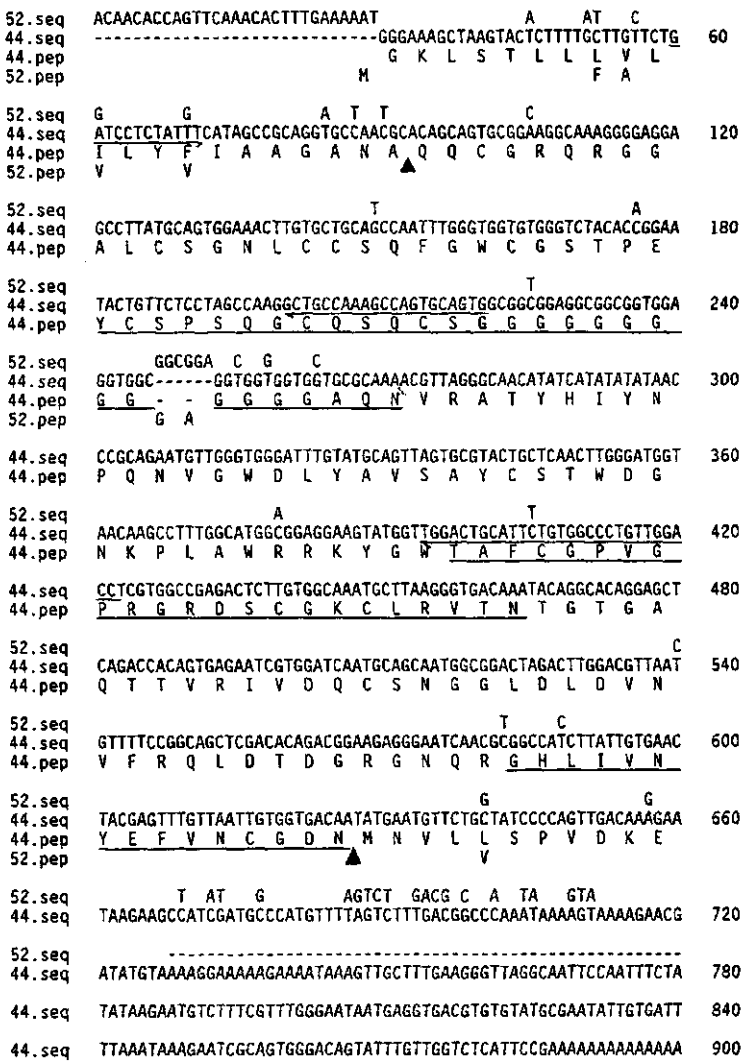

Figure 3. Sequence of *cbp*20 clones. Two *cbp*20 clones (designated *cbp*20-44 and *cbp*20-52) were isolated and sequenced (44.seq and 52.seq respectively). The deduced amino acid is also given (44.pep and 52.pep respectively). Nucleotides and deduced amino acid residues of *cbp*20-52 are only given when they differed from the *cbp*20-44 sequence. The peptide sequences obtained after V_8 protease treatment (CBP-PEP1), NCS digestion (CBP-PEP2) and trypsin digestion (CBP-PEP3) are underlined. The putative N-terminal signal peptide cleavage site and the C-terminal cleavage site of the propeptide are indicated by arrowheads. The primers used in the PCR reactions are underlined.

that two types of cDNA clones had been isolated. The nucleotide sequence and the deduced amino acid sequence of the two different types of cDNA clones are shown in Figure 3. Clone *cbp*20-44 represents a nearly full length cDNA. Since the deduced amino acid sequence exactly matched the determined sequences CBP-PEP1, CBP-PEP2 and CBP-PEP3 (Figure 3), we concluded that *cbp*20-44 is a true CBP20 cDNA clone. Clone *cbp*20-52 encodes a protein containing two additional amino acid residues (Gly, Ala) in the Gly rich region compared to CBP-PEP2 (Figure 3).

Both cDNAs code at their 5' end for a putative signal peptide that may be involved in transport of the protein across the membrane of the endoplasmic reticulum. The cleavage site would be between Ala-22 and Gln-23 analogous to the cleavage sites of other PR proteins, including ß-1,3-glucanases (Shinshi *et al.*, 1988; Linthorst 1990a), PR-1 proteins (Cornelissen *et al.*, 1987), chitinases (Linthorst *et al.*, 1990b; Payne *et al.*, 1990a), and class II PR-4 proteins (Linthorst *et al.*, 1991). The resulting N-terminal Gln residue is readily modified probably resulting in ineffective Edman degradation of the mature protein (see above). As mentioned above, CBP-PEP3 represents the C-terminus of the mature CBP20 protein. However, as compared to this peptide the deduced amino acid sequence extends for another eleven residues.

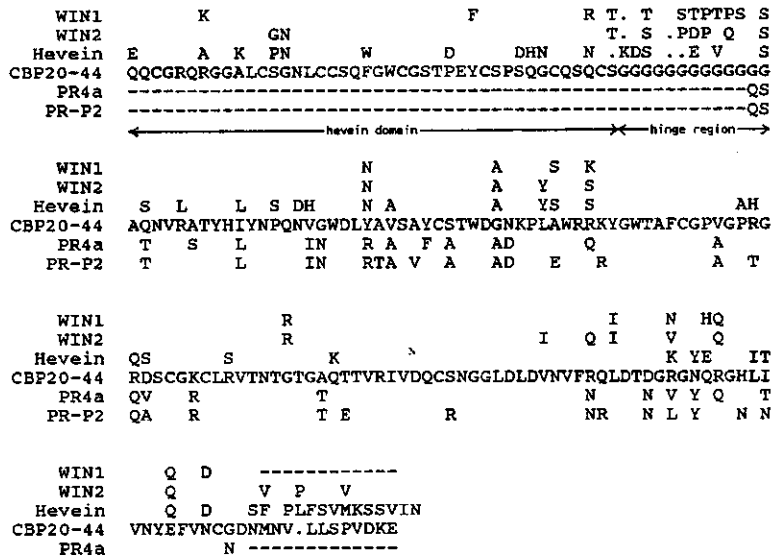

Figure 4. Sequence comparison of several PR-4 proteins. The sequence of proCBP20 is given and compared to several PR-4 proteins. Amino acid residues of tobacco PR-4a and tomato PR-P2 (Linthorst *et al.*, 1991), proHevein (Broekaert *et al.* 1990), the putative proWIN1 and proWIN2 proteins (Stanford *et al.*, 1989) different from those in proCBP20 are indicated. Gaps introduced to optimize the alignment of the proteins are indicated as dots (.), whereas amino acids lacking from the sequences are indicated by hyphens (-).

Apparently mature CBP20 is synthesized as a preproprotein from which the N-terminal signal peptide and the C-terminal propeptide (CTPP) are cleaved off. The CTPP most likely functions as a vacuolar targeting signal since the CTPPs of class I chitinases, class I ß-1,3-glucanase and AP24 were shown to be involved in vacuolar targeting (Neuhaus *et al.*, 1991; Melchers *et al.*, 1993).

CBP20 is a Class I PR-4 Protein

Comparison of the deduced amino acid sequence of CBP20 with sequences stored in the SwissProt protein sequence database revealed striking similarity with the primary structures of the putative, wound-inducible proteins encoded by the *win1* and *win2* genes from potato (Stanford *et al.*, 1989) and of prohevein from the rubber tree (Broekaert *et al.*, 1990). Prohevein is the precursor of hevein, a small lectin found in the latex of the rubber tree. A comparison of these four proteins is shown in Figure 4. In all cases the N-terminal signal peptide (not included in Figure 4) is followed by a chitin-binding domain which is connected to a C-terminal domain by a hinge region. High identity of the CBP20 proprotein sequence was found with the putative WIN1 and WIN2 proproteins and prohevein (86.9%, 87.5% and 70.5%, respectively). In contrast to the hinge regions of prohevein and the putative WIN proteins, the hinge region of CBP20 is extremely Gly-rich. In clone *cbp*20-52, 14 out of 15 amino acid residues are Gly and in clone *cbp*20-44 even all 13 residues are Gly (Figure 4). In addition to the above similarities, the N-terminal sequence of CBP20 was found to be very similar to the chitin-binding domain of tobacco class I chitinases (Linthorst *et al.*, 1990b; Shinshi *et al.*, 1990) and stinging nettle lectin (Broekaert *et al.*, 1989) (data not shown). In view of the chitin-binding capacity of CBP20, this similarity is not unexpected.

A strong identity in primary structure was also observed between the C-terminal domain of the CBP20 proprotein and the class II PR 4 proteins PR-4a and PR-4b (78.7% and 78.7%, respectively; Figure 4) from tobacco and PR-P2 (75%) from tomato (Linthorst *et al.*, 1991). This observation prompted us to investigate the immunological relationship between the class II PR-4 proteins and CBP20. To this end the pool of chitin-binding proteins (Figure 1, lane C) and class II PR-4 proteins from tobacco were incubated with antiserum raised against PR-P2 from tomato. The antiserum recognized the purified class II PR-4 proteins as well as CBP20 (Figure 5, lane A and C, respectively). No cross-reactions were observed with the class I chitinases which were present in the pool of chitin-binding proteins (Figure 5, lane B). Similar results were obtained with antiserum specific for the tobacco class II PR-4 proteins (data not shown).

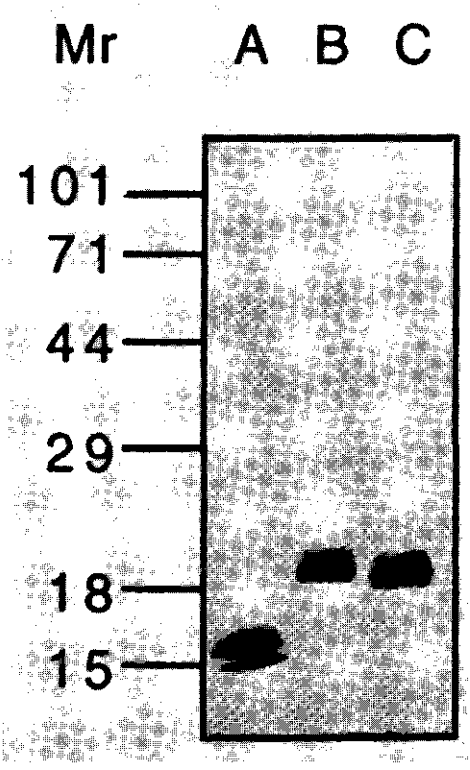

Figure 5. Immunological identification of CBP20. CBP20 containing fractions were used to screen for immunological cross reactivity. Samples of purified PR-4a and PR-4b, the chitin eluate and purified CBP20 were separated on 12.5% SDS-PAA gels (lanes A, B, and C, respectively) and electroblotted to nitrocellulose membranes. Immunodetection was performed with the antiserum to the PR-4 protein of tomato (PR-P2). The lane Mr shows prestained markers. The corresponding molecular weights are as indicated.

The serological relation between CBP20 and the class II PR-4 proteins made it possible to study the cellular localization of CBP20 in TMV-infected tobacco plants. Primary infected and healthy tobacco leaves were used for the isolation of extracellular fluids (EF). Leaves from which the EF had been removed and freshly harvested leaves were used to prepare extracts further referred to as "-EF" and "T" (total), respectively. Immunoblots of these protein samples showed a clear induction of CBP20 as a result of TMV-infection (compare lanes A and D in Figure 6). It was furthermore shown that CBP20 was present in T and -EF extracts prepared from TMV inoculated leaves (Figure 6, lanes D and E) and not in the EF isolated from these leaves (Figure 6, lane F). This indicates that CBP20 is intracellularly located. A protein of 14 to 15 kD present in the protein samples T and EF (lanes D and F) cross-reacted with the antiserum

as well. This protein band was induced by TMV (compare Figure 6, lanes A and D) and represents the class II (acidic) PR-4 proteins from tobacco which are known to be located extracellularly (Linthorst *et al.*, 1991).

The identity in primary structure of CBP20 and the class II PR-4 proteins from tobacco, as well as the serological relationship between these proteins, and the intracellular localization of CBP20 led us to the conclusion that CBP20 is a class I PR-4 protein.

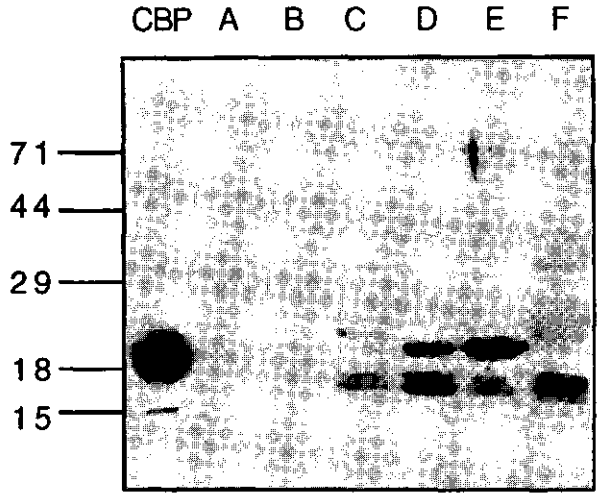

Figure 6. Cellular localization of CBP20. The antiserum to the tomato PR-4 protein PR-P2 was used to determine the cellular localization of CBP20. Uninfected (lanes A, B and C) and TMV-infected (lanes D, E and F) tobacco plants were sampled for total soluble leaf protein (lanes A and D), the extracellular washing fluid (lanes C and F), and soluble proteins remaining after the removal of the EF fraction (lanes B and E). Samples of 5 µg protein were separated by 12.5% SDS-PAA gel electrophoresis and electroblotted to a PVDF membrane. Immunodetection was performed with the antiserum to tomato PR-P2 (diluted 1:1,000). A purified sample of CBP20 served as reference as well as a prestained molecular weight standard. The corresponding molecular weights are as indicated.

CBP20 is Encoded by a Small Gene Family

The cDNA insert of the *cbp* 20-44 clone and a class II PR-4 clone (Linthorst *et al.*, 1991) were hybridized to blots containing tobacco DNA digested with four different restriction enzymes. The Southern blot hybridized with the CBP20 cDNA showed a maximum of two hybridizing fragments in each lane (Figure 7, right panel). This suggests that at least two copies of the CBP20

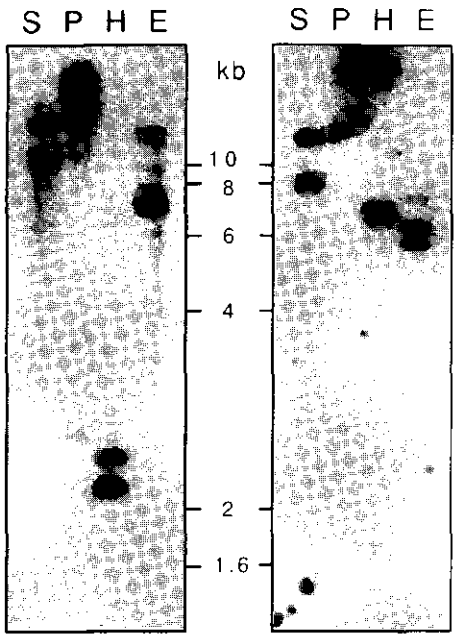

Figure 7. Determination of the size of the PR-4 gene family. Tobacco DNA was digested with four restriction enzymes: SstI (S), *Pst*I (P), *Hind*III (H) and *Eco*RI (E). The resulting fragments were separated on an 1 % agarose gel and blotted to Hybond N+. Fragments were hybridized with either a ^{32}P-labeled cDNA of clone PR-4a (left panel) or *cbp*20-44 (right panel). Molecular weight standards are as indicated.

gene are present in tobacco. The Southern blot hybridized with the class II PR-4 clone indicates the presence of two to four class II PR-4 genes (Figure 7, left panel) which is in agreement with a previous report by Linthorst and coworkers (1991). Since distinct DNA fragments hybridized with the probes, it was concluded that CBP20 and class II PR-4 genes were specifically detected and that no strong cross-hybridization occurred.

Accumulation of CBP20 mRNA upon TMV-infection, Wounding, and Other Forms of Stress

The induction of CBP20 by TMV and the high similarity of the polypeptide with the putative, wound-inducible proteins WIN1 and WIN2 led us to investigate whether the expression of CBP20 mRNA is responsive to stress. Samsun NN tobacco plants were subjected to different stress conditions and leaf samples were taken 3 days after inoculation with TMV, 2 days after wounding, 1 day after ethephon treatment, and 1 day after UV light irradiation. At these time

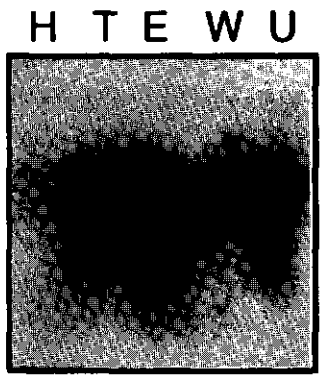

Figure 8. Induction pattern of CBP20 mRNA. Messenger RNA was isolated from healthy tobacco plants (H) and tobacco plants stressed by inoculation with TMV (T), ethephon treatment (E), wounding (W), or UV light treatment (U). The amount of CBP20 specific mRNA was determined by hybridizing with a ^{32}P-labeled probe.

points, maximal expression of the class I PR-2 and PR-3 genes was reported by Brederode and coworkers (1991). The leaf samples were extracted and analyzed on a Northern blot. Figure 8 shows that the expression of *cbp*20 in non-stressed tobacco leaves is not detectable. TMV-infection of tobacco leaves induced the expression of *cbp*20 gene to high levels (Figure 8, lane T). Treatment with ethephon resulted in an even higher level of CBP20 mRNA (Figure 8, lane E). Moderate induction of CBP20 expression was found after wounding the leaves or UV light treatment (Figure 8, lanes W and U, respectively). Thus the stress induction pattern of *cbp*20 matches the stress induction pattern of other class I PR proteins (Brederode *et al.* 1991).

DISCUSSION

Characterization of CBP20

In this paper we describe the purification and characterization of a novel stress-inducible, antifungal protein from TMV-infected tobacco leaves. Its native and denatured molecular weights, 8 and 20 kD, respectively, indicate that the protein is probably purified as a monomer. Since the 20 kD protein binds to chitin it is referred to as chitin-binding protein (CBP20). Low levels of chitinase and lysozyme activities are associated with the protein. Furthermore, the protein inhibits the growth of various fungi tested, especially in the presence of a tobacco class I chitinase or a tobacco class I ß-1,3-glucanase.

Based on partial protein sequences obtained from the purified protein (CBP-PEP1 and CBP-PEP2), DNA primers were designed to isolate cDNA clones corresponding to CBP20. Two types of clones were isolated. The deduced primary structure of the protein encoded by

one type (*cbp*20-44) matches exactly the three peptide sequences that were determined for CBP20. The other type encodes a protein which differs from *cbp*20-44 by 5 conservative amino acid changes and a small insertion of two amino acids. Four of the five amino acid changes are within the N-terminal signal peptide and consequently absent in the mature protein. The insertion is in the hinge region of the protein which consists of thirteen Gly residues. The finding of two types of cDNA clones is in agreement with the observation that CBP20 is encoded by a small family of at least two genes.

CBP20 is both structurally and immunologically related to the pathogenesis-related class II proteins PR-4a and PR-4b of tobacco and PR-P2 of tomato. From these observations and its intracellular localization we conclude that CBP20 is a class I PR-4 proteins. Interestingly, the relationship between the class I and class II PR-4 proteins is analogous to the relationship between the class I and class II PR-3 proteins (Shinshi *et al.*, 1990; Linthorst *et al.*, 1990b): the class I proteins of both the PR-3 and PR-4 group consist of a N-terminal chitin-binding domain linked to a C-terminal domain by a hinge region, whereas the class II proteins of both groups consist of the C-terminal domain only. In tobacco both class I and class II PR-proteins are induced under various stress conditions. However, their induction patterns are not identical (Brederode *et al.*, 1991). A striking difference is the reaction upon wounding. Whereas the class I proteins are strongly induced upon wounding, the class II proteins are not. CBP20 is induced upon wounding and also otherwise the induction pattern of this protein resembles that of the class I PR proteins. This further substantiates the conclusion that CBP20 is a class I PR protein.

Comparison of CBP20 To Other Chitin-Binding Proteins

The chitin-binding domain of CBP20 shows extensive identity to the chitin-binding domains of other proteins like the class I chitinases (Linthorst *et al.*, 1990b), hevein (Broekaert *et al.*, 1990), stinging nettle lectin (Lerner and Raikhel, 1992) and the putative WIN proteins from potato (Stanford *et al.*, 1989). Except for the class I chitinases and stinging nettle lectin, identity is not restricted to the chitin-binding domain: the C-terminal domain of CBP20 is also homologous to the deduced C-terminal domain of prohevein (Broekaert *et al.*, 1990) and the putative WIN proteins (Figure 4).

Tomato leaves infected by *C. fulvum* synthesize a basic chitin-binding, intracellularly located 20 kD protein cross-reacting with the antiserum to PR-P2 (data not shown). In contrast to the monomeric nature of CBP20, the native tomato protein appears to be a dimer. We anticipate that this protein is homologous to CBP20. Tamarillo fruits (*Cynhomandra betacea*, also a Solanaceous species) has been shown to contain 25 kD chitin-binding lectins (Xu *et al.*,

1992). Since the amino acid composition of these proteins and CBP20 is comparable it might turn out that these lectins are also representatives of the class I PR-4 group of proteins.

Targeting and Processing of Class I PR-4 Proteins

Immunological studies indicate that CBP20 is localized intracellularly. Like the tobacco class I PR-2 (Van den Bulcke *et al.*, 1989), and PR-5 (Singh *et al.*, 1987) proteins, and the bean class I PR-3 (Boller and Vögeli, 1984) protein, CBP20 is likely to be localized in the vacuole. Transport of the immature CBP20 across the membrane of the endoplasmatic reticulum is facilitated by the N-terminal signal peptide. Unique to the class I PR proteins is the presence of a CTPP that is involved in intracellular (vacuolar) targeting of these proteins and which is cleaved off during maturation (Shinshi *et al.*, 1988; Neuhaus *et al.*, 1991; Melchers *et al.*, 1993). In the case of CBP20 we showed that the CTPP was removed during maturation of the protein as well. From the sequence comparison of CBP20 and prohevein one would expect that hevein is located intracellularly and processed in a manner comparable to CBP20. However, next to the expected 20 kD protein, prohevein has been shown to be processed into a 5 kD N-terminal chitin-binding domain (hevein) and a 14 kD C-terminal domain (Lee *et al.*, 1991). All three proteins appear to be localized intracellularly. Although we never obtained any indication of this kind of processing taking place in tobacco we cannot exclude the possibility that proCBP20 is also processed in a way similar to prohevein. About the nature of the mature WIN1 and WIN2 proteins from potato one can only speculate, since these proteins have not been described in the literature. The putative WIN2 protein presumably is localized intracellularly due to the presence of a CTPP (Stanford *et al.*, 1989). Mature proteins arising from proWIN2 may be either the full length 20 kD protein or the 5 kD and 14 kD N- and C-terminal domains of the protein. Since the putative WIN1 protein seems to lack a CTPP it is likely that this protein is extracellularly localized. It thereby resembles the class IV chitinases consisting of a N-terminal signal peptide and a hevein domain connected to a C-terminal domain by a hinge region (Collinge *et al.*, 1993).

Antifungal Activity of Chitin-Binding Proteins

The association of antifungal activity with several chitin-binding proteins led us to look into the antifungal effect of CBP20. The chitin containing fungi *T. viride*, *F. solani* and *A. radicina* were used to study the effect of CBP20. *T. viride* appeared to be the most sensitive fungus. Low amounts of CBP20 (6.7 µg /mL = 1 µg/well) resulted in lysis and total growth

inhibition of this fungus. This amount of CBP20 is in the same range as that of PR-4 type proteins from barley grain needed for growth inhibition of *T. harzianum* (Hejgaard *et al.*, 1992). These authors state that the basic PR-4 type proteins used in their studies lack a chitin-binding domain, yet binds to chitin. If so, it should be regarded as a class II type PR-4 protein. Interestingly, we found that the class II PR-4 type proteins from tobacco lack antifungal activity (data not shown). The amount of CBP20 needed for growth inhibition of *T. viride* is lower than the amount of hevein and stinging nettle lectin (both about 45 µg/mL) needed for 50% growth reduction of *T. hamatum* (Van Parijs *et al.*, 1991) and *T. viride* , respectively (Broekaert *et al.*, 1989). However, differences in the extraction and/or assay procedures may account for these effects. To account for the difference in extraction, CBP20 was isolated in a buffer in which ß-mercapto-ethanol had been replaced by 10 mM thiourea (Van Parijs *et al.*, 1991; Broekaert *et al.*, 1992). Further purification of the protein was performed and antifungal assays were run with *F. solani* as the test fungus. It appeared that CBP20 obtained in this way had a 2 to 5 fold stronger antifungal effect than CBP20 obtained in the usual way. Apparently, CBP20 is partly inactivated by the addition of ß-mercapto-ethanol.

The germ tubes of *F. solani* were less sensitive to CBP20 as compared to the germ tubes of *T. viride*. However, growth inhibition was clearly observed in the presence of 30 µg/mL (= 4.5 µg/well). This again seems to be lower than the amount of hevein needed (600 to 1250 µg/mL) for 50% growth inhibition of two *Fusarium* species (Van Parijs *et al.*, 1991). By the use of *F. solani* as the test fungus it was shown that CBP20 interacts synergistically with both a class I ß-1,3-glucanase and a class I chitinase of tobacco. Binding of CBP20 to the chitin matrix of the cell wall apparently renders the fungus more sensitive to enzymes capable of hydrolysing cell wall components.

Since chitin containing fungi are very sensitive to chitin-binding chitinases (Sela-Buurlage *et al.*, 1993) it was essential to achieve complete separation of the class I chitinases and CBP20 before performing antifungal assays. This was achieved by repeated gel-filtration. Nevertheless control experiments were incorporated to rule out the possibility that traces of class I chitinases were responsible for the observed antifungal effects. This appeared not to be the case (data not shown). The most direct way to prove that CBP20 itself exhibits antifungal activity rather than contaminating class I chitinases is the synergistic activity of these two chitin-binding proteins on *F. solani*.. In case the chitinase activity in the CBP20 preparation would result from contaminating chitinases, and CBP20 itself was not antifungal at all, then no synergistic effect against *F. solani*. would be expected by mixing 1 µg CBP20 with 0.5 µg class I chitinase (Sela-Buurlage *et al.*, 1993).

Biological Role of CBP20

From the results presented in this paper it is clear that CBP20 is induced after several forms of stress regimes including pathogen attack and wounding and exhibits antifungal activity. Potato is known to react to wounding by inducing the expression of two wound-inducible genes (Stanford *et al.*, 1989) which are both highly homologous to CBP20. It would be interesting to know whether these genes are also induced by pathogen attack. Assuming that this is the case it seems likely that CBP20 and related proteins play (amongst others) a role in plant defense. Since chitin is present in many fungal cell walls and absent from plant cell walls, chitinases and chitin-binding proteins are excellent and specific defense barriers for plants. The importance of chitinases is probably well illustrated by the diversity of chitinases which are induced upon pathogen attack (Legrand *et al.*, 1987; Lawton *et al.*, 1992): chitin-binding proteins with low and high levels of chitinase activity, and several chitinases that are not able to bind to chitin yet hydrolyse (partly solubilized) chitin. By producing so many apparently harmless plant defense enzymes, a large spectrum of substrate molecules (present in the fungal cell wall) may be hydrolysed into a spectrum of reaction products which in turn may function as a-specific elicitors in the plant defense reaction.

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Chapter 6

In Vitro Antifungal Activity of Tobacco Class I Chitinase and Class I ß-1,3-Glucanase is Solely Dependent on Synergy

ABSTRACT

The vacuolar class I chitinase (Chi-I) and class I ß-1,3-glucanase (Glu-I), isolated from tobacco (*Nicotiana tabacum* cv. Samsun NN) leaves, inoculated with tobacco mosaic virus, have been shown previously to possess potent antifungal activity *in vitro* towards *Fusarium solani* f.sp. *phaseoli* both individually and in synergy (Sela-Buurlage *et al*, 1993). Routinely, purity of proteins was ensured with SDS-PAGE, Western analysis and enzymatic assays. Transgenic tobacco plants were generated constitutively expressing either a modified Chi-I or Glu-I gene lacking the carboxyl-terminal propeptide, which resulted in extracellular targeting of Chi-I* or Glu-I* (Melchers *et al*, 1993). Here, Chi-I* and Glu-I* were isolated from intercellular fluids from these transgenic plants. Molecular weights and enzymatic activities, on artificial substrates, of Chi-I* and Glu-I* remained unchanged, indicating that targeting had not influenced the nature of these proteins. However their *in vitro* antifungal activity was decreased, compared to Chi-I and Glu-I, when applied separately on *F.solani.* Antifungal activity was restored by amending with 1% of the other hydrolase, demonstrating the dependency of antifungal activity of these two proteins on synergy.

INTRODUCTION

In tobacco (*Nicotiana tabacum* cv Samsun NN) inoculation with tobacco mosaic virus leads to the induction of resistance against a broad range of pathogens including fungi, bacteria and viruses. Resistance is induced not only locally around the site of infection, but also systemically in non-inoculated parts of the plant (Ross, 1961*a*, *b*). Accompanying the induction of resistance is *de novo* synthesis of a large number of proteins, the so called pathogenesis-related (PR) proteins (for reviews: see Bol *et al.*, 1990; Linthorst, 1991; Stintzi *et al.*, 1993). Their biological function is for the majority unknown with the exception of two types of enzymes with hydrolytic activity, namely the chitinases and ß-1,3-glucanases. Their substrate, chitin and ß-1,3-glucan respectively, are major components of cell walls of many fungi (Wessels & Sietsma, 1981). The PR-2 group consists of ß-1,3-glucanases (Kauffmann *et al.*, 1987) and, based on their primary structure, can be divided into at least three classes (for review: see Simmons, 1994). Class I consists of basic, vacuolar isoforms, whereas class II contains 3 extracellularly located, acidic proteins PR-2a, -2b, -2c, formerly known as PR-2, -N, and -O. To date, only one class III enzyme has been identified (Payne *et al.*, 1990b). The PR-3 proteins were traditionally described as a group of proteins exhibiting chitinase activity (Legrand *et al.*, 1987). In tobacco, three classes of PR-3 proteins have been identified based on the structural analysis of their genes (Shinshi *et al.*, 1990). Class I contains two basic, vacuolar isoforms (Chi-I) of 32 and 34 kD in size. Class II consists of two acidic extracellular isoforms (Chi-II), PR-3a and -3b, formerly known as PR-P and PR-Q. Recently, two new classes of PR-3 have been described (Lawton *et al.*, 1992; Collinge *et al.*, 1993).

In vitro, growth of a number of fungi is inhibited by a chitinase from bean (Schlumbaum *et al.*, 1986) or combinations of chitinases and ß-1,3-glucanases from pea (Mauch *et al.*, 1988). Within the group of PR-2 and PR-3 proteins from tobacco there is a large variation in level of antifungal activity. Of both groups the class II proteins exhibited little or no antifungal activity. In contrast, the vacuolar, class I proteins (Glu-I and Chi-I) were shown to possess high levels of antifungal activity *in vitro* against *Fusarium solani*. Synergy between specifically Chi-I and Glu-I proteins from tobacco was demonstrated (Sela-Buurlage *et al.*, 1993). Corresponding genes were isolated and transgenic tobacco plants were generated overexpressing single genes. Additional stop codons had been introduced into the C-terminal regions of both the Chi-I and Glu-I genes, which resulted in removal of the vacuolar targeting signals (Neuhaus *et al.*, 1991b) and subsequently targeting of either of the proteins to the intercellular space (Melchers *et al*, 1993). Molecular weights of the mature Chi-I* and Glu-I* proteins remained the same as those of Chi-I and Glu-I. Intercellular fluids (IF) of these transgenic plants were shown to possess antifungal activity *in vitro* towards *Fusarium solani*.

(Sela-Buurlage *et al.*, 1993) and were shown to act synergistically. In the present study we have isolated both Chi-I* and Glu-I* from the IF of the transgenic plants overexpressing single genes to determine whether such proteins have the same enzymatic and antifungal activity as when they are isolated from tobacco plants inoculated with TMV. Enzymatic activities of both proteins appeared unchanged upon extracellular targeting. Surprisingly, both proteins, if applied individually, showed decreased antifungal activity compared to the same proteins isolated from TMV inoculated tobacco. However, amending with 1% of the other hydrolase was sufficient to cause high percentage of lysis of germtube tips and growth inhibition of *F. solani* was observed. It is concluded that the antifungal activity of Chi-I and Glu-I solely depends on synergy between these proteins.

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).

Fusarium solani f.sp. *phaseoli* was kindly provided by Dr. Th. Boller, Botanical Institute of the University of Basel, Switzerland. The fungus was maintained on V8 agar at 20^{0}C in the light. Spores were harvested from 1- to 3-week-old plates by flooding the plate with buffer to be tested. The spore concentration was adjusted to 10,000 sp/mL.

Protein Purification and Analysis

Chi-I and Glu-I were purified from TMV inoculated tobacco leaves according to methods described previously by Sela-Buurlage *et al.*. (1993). However, Glu-I was purified by one extra step of cation-exchange chromatography. Glu-I was pooled after gelfiltration chromatography and dialyzed to 50 mM potassium phosphate buffer, pH=6.1. This protein solution was applied to a Mono-S (HR 5/5; Pharmacia) column equilibrated in the same buffer. Glu-I ran through the column in an apparently pure state (as judged from SDS-PAA gels). For purification of Chi-I* and Glu-I* transgenic tobacco plants were used, expressing single genes under control of the cauliflower mosaic virus 35S promoter. The intercellular fluid was isolated

from transgenic plants according to de Wit and Spikman (1982). The IF containing the Chi-I* protein was dialyzed to 20 mM sodium acetate, pH 5.2, and applied to a Mono S column (HR 5/5; Pharmacia), equilibrated in the same buffer. Bound proteins were eluted from the Mono S column by a linear gradient (40 ml) of 0 to 160 mM NaCl in the above buffer (1 ml/min). Fractions were analyzed on 12.5% SDS gels and the Chi-I* was pooled accordingly. A similar approach was used for the IF containing the Glu-I*.

Protein concentrations were determined by the method of Bradford (1976) using BSA as the standard.

Chitinase activity measurements were carried out with dye-labelled CM-chitin as described by Wirth and Wolf (1990). Mixtures of substrate (100 µL of a 2 mg/mL solution per assay) in 50 mM $KHPO_4$ buffer (pH 6.4) and appropriate amounts of enzyme were incubated at 37°C for 30 minutes in a final volume of 200 µL. The reaction was terminated by the addition of 100 µL 1 M HCL, causing precipitation of the non-degraded substrate. The reaction vials were cooled on ice for 10 minutes and centrifuged (5 min Eppendorf centrifuge). Two hundred µL of the resulting supernatant was pipetted into a microtiter dish (96 wells) and the absorbance at 540 nm was read. The absorbance, expressed in ODu (OD units), was taken as a measure for enzyme activity.

ß-1,3-Glucanase activity measurements were performed using laminarin as a substrate following methods described by Kaufmann *et al.* (1987). The activity of ß-1,3-glucanase was expressed in nanokat per milligram protein.

In Vitro Antifungal Assay

Antifungal assays were performed as described by Sela-Buurlage *et al.*, (1993).

RESULTS

Purification of Chi-I* and Glu-I* from Intercellular Fluid of Transgenic Plants

Routinely, Chi-I and Glu-I were purified from tobacco leaves (*Nicotiana tabacum* cv. Samsun NN) inoculated with tobacco mosaic virus (TMV). The degree of purity is determined to be at or above 95% using PAGE, Western and enzymatic analyses (Sela-Buurlage *et al.*, 1993). The possibility of copurification of minor amounts of either Chi-I or Glu-I contaminants however could never be excluded. Since the corresponding genes had been

identified, transgenic plants constitutively expressing single genes coding for one of these proteins were created to facilitate isolation procedures. However, in tobacco, PR-proteins are easily induced at greenhouse growing conditions. So, also in the case of transgenic plants expressing a single gene it could never be excluded that other (endogenous) PR-proteins were present in the purified protein samples.

In order to obtain highly pure Chi-I and Glu-I, transgenic tobacco plants were used overexpressing single gene constructs whereby the transgene product had been targeted extracellularly. Through introduction of an additional stop codon in the C-terminus of these genes, the propeptides, containing the vacuolar targeting signals, were removed, resulting in targeting of the proteins to the apoplast (Melchers *et al.*, 1993; Neuhaus *et al.*, 1991b). Previously it was shown that IF harvested from these transgenic plants possessed *in vitro* antifungal activity towards *Fusarium solani* f.sp.*phaseoli.* Mixing of these IFs resulted in an enhanced antifungal activity, similar to IF harvested from transgenic tobacco overexpressing Chi-I* and Glu-I* simultaneously (Sela-Buurlage *et al.*, 1993).

In the present study both Chi-I* and Glu-I* were purified from IF from transgenic plants expressing single genes to determine whether targeting of these proteins to the apoplastic space had any effect on the enzymatic or antifungal activity. Any endogenous Chi-I or Glu-I possibly induced in these transgenic plants by greenhouse growing conditions, are located in the vacuole and not extracellularly. In the IF of these plants, all endogenous PR-proteins are acidic in nature and since the transgene products are basic in nature, ionexchange chromatography could successfully be applied, resulting in highly purified Chi-I* and Glu-I* transgene products. To ensure purity, a final gel filtration step was performed.

Enzymatic Activity of Chi-I* and Glu-I*

On SDS-PAGE and immunoblots the transgene products Chi-I* and Glu-I* behaved identically to Chi-I and Glu-I proteins, isolated from tobacco leaves inoculated with TMV (data not shown). Also, the molecular weights of Chi-I* and Glu-I* were identical to those of Chi-I* and Glu-I* respectively.

Subsequently, enzymatic assays were performed using artificial substrates. For Chi-I* and Chi-I dye labelled soluble CM-chitin was used (Wirth and Wolf, 1990), whereby the amount of released dye represents the chitinase activity and the absorbance, expressed in ODu (OD units), was taken as its measure. Results are summarized in Table 1. No significant differneces were observed in the specific activities between Chi-I and Chi-I*. In the case of Glu-I and Glu-I* laminarin was used as a substrate. The activity of the ß-1,3-glucanases was expressed in nanokat per milligram protein. Again no significant differences could be detected

in the level of glucanase activity between these two enzymes.

Table 1. Specific Activities of Purified Chi-I, Chi-I*, Glu-I and Glu-I*

Enzyme	Substrate	Chitinase Activity (Odu/μg)	ß-1,3-Glucanase Activity (nkat/mg)
Chi-I	RBV-chitin	1.57 ± 0.11	
Chi-I*	RBV-chitin	1.83 ± 0.09	
Glu-I	Laminarin		880 ± 65
Glu-I*	Laminarin		941 ± 112

Chitinase and ß-1,3-glucanase activities were determined as described in "Materials and Methods". Ranges from three independent experiments are listed.

Comparison of Chi-I, Chi-I*, Glu-I and Glu-I* for Antifungal Activity *in vitro*

An *in vitro* system is used to determine the antifungal effect of the plant hydrolases on *Fusarium solani*. Spores of the fungus are harvested in water and applied to wells of a microtiter plate containing PDA as a solid medium. After spores had germinated for seven hours, enzymes were applied and percentage germtube tips showing lysis was monitored one hour after addition of the proteins. After 2 days fungal growth was stopped by staining the mycelium with lactophenol cotton blue. The amount of fungal material was taken as a measure of growth.

A first round of experiments involved the assaying of Chi-I, Chi-I*, Glu-I and Glu-I* for their individual antifungal activity. Up to 10 μg/well for each protein was tested. Results are shown in Table 2 for percentage germtube tips showing lysis and Figure 1 for growth inhibition, as determined after 2 days. At 0.5 μg/well Chi-I 15% of the germtube tips were lysed. When 5 microgram of Chi-I was applied, about 50% of the germtube tips were lysed. At 10 μg/well of Chi-I no increase in lysis was observed compared to 5 μg/well. In contrast, 1 μg/well Chi-I* caused no lysis and only at 2.5 μg/well Chi-I* about 15% lysis was observed. A similar level of lysis was observed after application of 10 μg/well Chi-I*. After 2 days, growth inhibition by the Chi-I and Chi-I* proteins was determined. In the well where 2.5 μg of

Table 2. Effect of purified Glu-I, Glu-I*, Chi-I* and Chi-I, on lysis of germtube tips of *F. solani*

Enzyme	Percentage lysis of germtube tips			
µg/well	Glu-I	Glu-I*	Chi-I*	Chi-I
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
0.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	1 ± 1	0 ± 0	1 ± 1	24 ± 9
2.5	12 ± 6	2 ± 2	4 ± 7	44 ± 22
5	20 ± 10	2 ± 3	7 ± 7	64 ± 14
10	38 ± 13	7 ± 10	13 ± 14	78 ± 6

Chitinase and ß-1,3-glucanase activities were determined as described in "Materials and Methods". Ranges from three independent experiments are listed.

Chi-I had been applied, almost no fungal growth was observed. If the same amount of Chi-I* was applied, no growth inhibition was observed (Fig 1.). To achieve complete inhibition of fungal growth, a 4-fold higher amount of Chi-I* needed to be applied, compared to Chi-I.

Similarly, Glu-I and Glu-I* were compared for their antifungal activity. Again, a concentration range from 0 to 10 µg/well was assayed. If 2.5 µg/well Glu-I was applied, 15% of the germtube tips were lysed. At 5 µg/well 30% showed lysis and at the maximum 10 µg/well of Glu-I half of the population showed lysis of germtube tips. Little or no lysis was observed if up to 5 µg/well of Glu-I* was applied, and even at 10 µg/well only 20% of the germtube tips were lysed. After 2 days 10 µg/well of Glu-I caused significant growth inhibition, whereas 10 µg/well of Glu-I* caused no growth inhibition at all.

Antifungal Activity *in vitro* of Chi-I* and Glu-I* Mixtures on *Fusarium solani*

Although on SDS-PAGE, Western analysis and in enzymatic assays the transgene products behaved identically to the TMV derived proteins, antifungal activity appeared severely decreased. As mentioned above, although in the standard purification of Chi-I and Glu-I from TMV inoculated tobacco gel electrophoresis, immunoblotting procedures and enzymatic assays were used to check for (cross) contamination, absolute pure Chi-I or Glu-I could not be ensured.

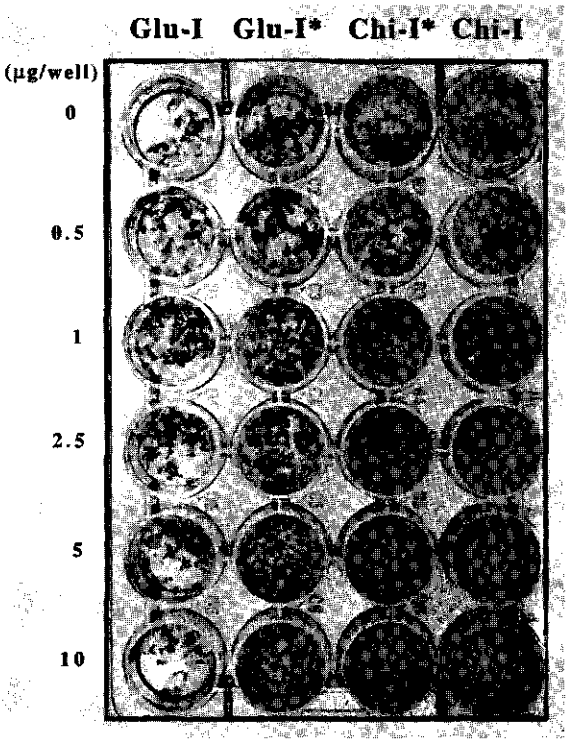

Figure 1. Effect of Chi-I, Chi-I*, Glu-I and Glu-I* on *in vitro* growth of *Fusarium solani*. Growth inhibition was scored after 2-3 days. Mycelium was stained with lactophenol blue. Amounts of protein (in µg per well) are indicated.

Since it was shown that Chi-I and Glu-I act synergistically (Sela-Buurlage *et al.*, 1993), mixing experiments were performed to determine whether the antifungal activity of Chi-I* or Glu-I* could be demonstrated. Results are presented in Table 3 for the amount of germtube tips showing lysis and in Figure 2 for growth inhibition. For example, if 0.5 µg/well Chi-I* was mixed with 0.005 g/well Glu-I*, 30% lysis and severe growth inhibition were observed. Similarly, when 0.5 µg/well Glu-I* was mixed with 0.01 µg/well Chi-I*, over 80% of germtube tips showed lysis. Again severe growth inhibition was observed. These results demonstrate that 1 % (w/w) of Chi-I* in Glu-I* and *vice versa* was sufficient to restore antifungal activity to levels observed with Chi-I and Glu-I, isolated from TMV inoculated tobacco. Finally, if a minimum of 0.1 µg of both of the hydrolases is mixed over 90 % of the germtubes tips are lysed and severe growth inhibition is observed, which is in accordance with earlier observations (Sela-Buurlage *et al.*, 1993).

Table 2 Effect of specific Chi-I* and Glu-I* mixtures on percentage lysis of germtube tips of Fusarium solani

Percentage lysis of germtube tips

		*Chi-I**					
	µg/well	*0*	*0.005*	*0.01*	*0.05*	*0.1*	*0.5*
	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3 ± 5
	0.005	0 ± 0	0 ± 0	2 ± 2	12 ± 9	21 ± 11	31 ± 13
*Glu-I**	*0.01*	0 ± 0	0 ± 0	6 ± 4	19 ± 12	27 ± 19	46 ± 22
	0.05	0 ± 0	31 ± 15	46 ± 15	49 ± 8	68 ± 2	75 ± 6
	0.1	0 ± 0	45 ± 13	68 ± 19	89 ± 8	90 ± 7	95 ± 4
	0.5	0 ± 0	76 ± 3	91 ± 6	96 ± 3	96 ± 4	99 ± 1

DISCUSSION

Previously it was shown that Chi-I and Glu-I are potent antifungal agents *in vitro* towards *Fusarium solani* both alone and synergistically (Sela-Buurlage *et al.*, 1993). The degree of synergistic action was shown to be high since low amounts (0.67 µg/ml) of each protein were able to cause severe lysis of germtube tips and complete growth inhibition. If proteins were applied separately, antifungal activity was less compared to the mixture. Routinely, Chi-I and Glu-I were purified from tobacco leaves (*Nicotiana tabacum* cv. Samsun NN) inoculated with tobacco mosaic virus (TMV). The degree of purity was determined to be at or above 95% using PAGE, Western and enzymatic analyses and no cross contamination was detected using these techniques. However, since in TMV inoculated tobacco both Chi-I and Glu-I are induced to high levels (Sela-Buurlage *et al.*, 1995), the possibility of copurification of the two basic proteins could never be excluded. Since the corresponding genes had been identified, transgenic plants constitutively expressing single genes coding for one of these proteins were created. Greenhouse growing conditions are capable of inducing PR-protein synthesis and thus also in the case of transgenic plants overexpressing the single proteins, minor contamination of endogenous proteins could not be excluded. More suitable

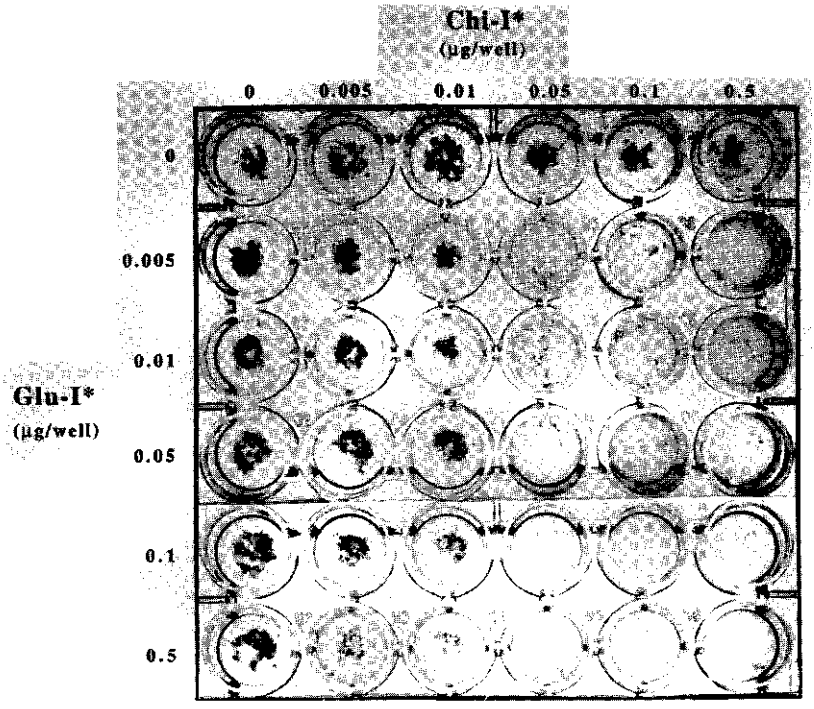

Figure 2. Effect of mixtures of Chi-I*Glu-I* on *in vitro* growth of *Fusarium solani*. Growth inhibition was scored after 2-3 days. Mycelium was stained with lactophenol blue. Amounts of protein (in µg per well) are indicated.

were transgenic plants expressing either Chi-I* or Glu-I* mutant genes. encoding proteins lacking a vacuolar targeting signal, resulting in targeting of these proteins to the apoplast (Melchers *et al.*, 1994). Intercellular fluids harvested from these transgenic plants harboring single gene constructs, were shown to possess both alone and in synergy in *vitro* antifungal activity towards *F. solani* (Sela-Buurlage *et al.*, 1993). In the present study the transgene products, Chi-I* and Glu-I* respectively, were purified from these IFs easily and without the opposite hydrolase. It was investigated whether this targeting extracellularly had any effect on the nature of these proteins. On SDS-PAGE and Western analyses, proteins behaved identically to those isolated from TMV inoculated tobacco plants. Also, enzymatic activities of Chi-I* and Glu-I* remained unchanged upon targeting extracellularly, as determined on the respective artificial substrates, chitin and laminarin.

Most strikingly however were the *in vitro* antifungal assays with *Fusarium solani* which seemed to indicate a decreased effect compared to the proteins, Chi-I and Glu-I, isolated from TMV inoculated tobacco leaves. The antifungal activity of Chi-I* was 5-fold lower than that of

Chi-I. In the case of Glu-I* the effect was even more pronounced. In the assays described here there was more than a 5-fold decrease, but compared to earlier studies (Sela-Buurlage *et al.*, 1993), a more than 10-fold decrease was noted. The discrepancy between the antifungal activity of Glu-I in the present study and the earlier one probably results from stricter pooling of fractions containing Glu-I, but lacking Chi-I. Additionally, an extra cation-exchange chromatographic step was applied in the present study.

The most obvious explanation for the observed decrease in antifungal activity of Chi-I* or Glu-I* compared to Chi-I or Glu-I would be that by targeting the proteins extracellularly antifungal activity had been abolished even though it was shown that enzymatic activity had not changed (Table 1). However, by performing mixing experiments with Chi-I* and Glu-I* it is shown how trace amounts of cross contamination are sufficient to ensure severe lysis (Table 3) and growth inhibition (Fig 2). It is demonstrated that the relative amounts of both hydrolases determine the level of antifungal activity. A lower amount of one of the hydrolases can be compensated for with higher amounts of the other. In earlier studies, we demonstrated that IF harvested from plants expressing either Chi-I* or Glu-I* possessed antifungal activity in synergy, both also if applied individually (Sela-Buurlage *et al.*, 1993), indicating that possibly minor contaminations must have been present also in these IFs. The requirements for purification to 100% homogeneity of Chi-I and Glu-I from TMV inoculated tobacco pass the limits of biochemical skills. It is only through the use of specific transgenic plants for isolating purified proteins that the true nature of antifungal activities of individual proteins can be established.

If results such as in this study are extrapolated to the *in planta* situation, it questions the strategies used in genetic engineering whereby a single chitinase is overexpressed. On the use of an individual chitinase to control fungal growth in the plant, positive results have been described. Toyoda *et al.* (1991) show that microinjection of a bacterial chitinase into epidermal cells of barley effectively digests the haustoria of *Erysiphe graminis* f.sp *hordei* . However, the amount of chitinase administered may have been very high and out of physiological range. Overexpression of a bacterial chitinase in microbes such as *Rhizobium meliloti*, *Pseudomonas fluorescens* and *Escherichia coli* has been described as a method for biological control, either by using extracts of such transgenic microbes or by directly applying them to soil (Koby *et al.*, 1994; Shapira *et al.*., 1989; Sitrit *et al.*, 1993). Broglie and coworkers (1991) demonstrated enhanced resistance towards *Rhizoctonia solani* in transgenic canola and tobacco plants overexpressing a bean chitinase. These transgenic canola plants were studied cytochemically after infection with *Rhizoctonia solani* and it was determined that *in vivo* growth and morphology of this fungus in transgenic plants was markedly distorted (Benhamou *et al.*,

1993). Similarly, enhanced resistance towards the same pathogen was observed in transgenic tobacco overexpressing either a bacterial chitinase (Jach *et al.*, 1992; Logemann *et al.*, 1993) or a tobacco class II chitinase, PR-3b (Lawton *et al.*, 1993). On the contrary, the work of Samac and Shah (1994) indicates that the contribution of chitinases alone in inducible defense responses might be very limited. In *Arabidopsis* plants, antisense expression of the predominant class I chitinase did not lead to a significant increase in susceptibility to the pathogen *Botrytis cinerea*, although it must be added that the antisense RNA strategy was not completely effective in suppressing induced chitinase expression. Overexpression of the tobacco class I chitinase in transgenic tobacco plants did not increase resistance towards *Cercospora nicotianae* (Neuhaus *et al.*, 1991a). In accordance with the latter results, in our lab transgenic tomato plants expressing either Chi-I or Glu-I were as sensitive to *Fusarium oxysporum* f. sp. *lycopersici* race 1 as control plants (Jongedijk *et al.*, 1995). It was demonstrated that the simultaneous constitutive expression of Chi-I and Glu-I in transgenic tomato plants provides an enhanced level of resistance to the same fungus (Jongedijk *et al.*, 1995; Van den Elzen *et al* ., 1993). Similarly, Zhu and coworkers (1994) demonstrated that the observed level of resistance to *Cercospora nicotianae* was higher in the hybrid progeny possessing both a rice basic chitinase and an alfalfa acidic glucanase compared to the parent tobacco plants expressing only one of the genes. The *in vitro* data presented in the present study show that caution must be taken during the interpretation of antifungal effects of chitinases and glucanases individually at least *in vitro* and possibly also *in vivo*, since their synergy is extremely high. It might well be that although the effects are studied of a single chitinase in a transgenic plant, in fact endogenous ß-1,3-glucanase(s) are present to support the hydrolytic activity of the chitinase.

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Chapter 7

Exposure of Macroconidia of *Fusarium solani* f.sp. *phaseoli* to Chitinase Decreases the Sensitivity of their Germtube to Subsequent Chitinolytic Attack

ABSTRACT

Class I chitinases (Chi-I) and class I ß-1,3-glucanases (Glu-I), purified from tobacco (*Nicotiana tabacum* cv. Samsun NN) exhibit synergistic antifungal activity *in vitro* on germlings of *Fusarium solani* f.sp. *phaseoli*, resulting in lysis of germtube tips and inhibition of mycelial growth (Sela-Buurlage *et al.*, 1993). Extending the pregermination time of macroconidia of *F. solani* f.sp. *phaseoli* prior to hydrolase treatment, increases the sensitivity of the emerging germtube to the hydrolases. Seven hours of pregermination is sufficient to cause lysis of almost all germtube tips after treatment with a mixture of Chi-I and Glu-I. At shorter pregermination times a higher concentration of hydrolases is required to obtain maximum lysis of germlings. If the hydrolases are applied before the macroconidia have germinated, no lysis of germtube tips is observed. Germination percentage and rate of germtube growth is equal, irrespective of the presence of antifungal hydrolases. If these enzymes, after incubation for 7 hours with germinating macroconidia, are transferred to pregerminated macroconidia, extensive lysis of germtube tips still occurs, indicating that the germinating *F. solani* f.sp. *phaseoli* conidia do not inactivate hydrolases. Pre-exposure of the macroconidia to (low doses of) hydrolases for 3 hours is sufficient to decrease the sensitivity of the germtube tip to subsequent challenge with normally lethal concentrations of hydrolase. Our results suggest that the decreased sensitivity of macroconidia of *F. solani* f.sp. *phaseoli* to chitinase is due to adaptation of the growing germtube.

INTRODUCTION

In plant-pathogen interactions where a characteristic hypersensitive response occurs, resistance is induced both locally at the sites of infection and systemically in noninfected parts of the plants. Resistance is induced against a broad range of pathogens, including viruses, bacteria and fungi (McIntyre *et al.*, 1981; for overview see Stintzi *et al.*, 1993). In tobacco, concomitantly with the occurrence of resistance, a large variety of proteins is synthesized *de novo*. These include enzymes involved in lignification, the synthesis of secondary metabolites and pathogenesis-related (PR) proteins. Initially described in tobacco, PR proteins were defined as pathogen-inducible, acidic and relatively protease-resistant proteins occurring in the extracellular spaces of plants (Parent and Asselin, 1984; Van Loon, 1985). However, more recently, serologically related isoforms have been identified intracellularly (for reviews see: Bol *et al.*, 1990; Bowles, 1990; Linthorst, 1991; Stintzi *et al.*, 1993).

Based on similarities in primary structure and serological relationships, PR proteins of tobacco can be divided into different groups. Each group is subdivided into at least two classes. Class I proteins of each group are basic in nature and located in the vacuole while class II proteins are acidic and located extracellularly. Of group 2, the class II proteins PR-2a, -2b and -2c (formerly known as PR-2, -N and -O) and the class I isoforms exhibit ß-1,3-glucanase activity (Kauffmann *et al.*, 1987). Of group 3 the class II proteins PR-3a and -3b (also known as PR-P and PR-Q) and the 32 kDa and 34 kda class I isoforms are chitinases (Legrand *et al.*, 1987). The polysaccharides chitin and ß-1,3-glucan, substrates for chitinases and ß-1,3-glucanases respectively, are major components of the cell walls of many fungi (Wessels and Sietsma, 1981). Of the PR-group 2 and 3 hydrolases, only the class I proteins (Chi-I and Glu-I) were shown to possess antifungal activity *in vitro* against *Fusarium solani* f. sp. *phaseoli*. The extracellular class II enzymes of these two groups exhibited no antifungal activity (Sela-Buurlage *et al*, 1993). Synergy between Chi-I and Glu-I has been demonstrated (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993). An apparent lack of substrate for chitinases in plants and concomitantly occurrence of *de novo* synthesis of chitinases and ß-1,3-glucanases and fungal resistance have led to speculations about a pivotal role for these hydrolytic enzymes in plant defense (Mauch and Staehelin, 1989). Recently we have demonstrated that transgenic tomato plants simultaneously expressing the tobacco Chi-I and Glu-I show enhanced resistance to *Fusarium oxysporum* f. sp *lycopersici* race 1 (Jongedijk *et al.*, 1995; Van den Elzen *et al.*, 1993). Similarly, constitutive co-expression of a rice basic chitinase and an alfalfa acidic ß-1,3-glucanase enhanced resistance in transgenic tobacco against *Cercospora nicotianae* (Zhu *et al.*, 1994).

In classic disease-resistance breeding programs it has often been observed that new

variants of the fungus emerge that are able to overcome the introduced resistance genes. Generally, monogenic resistances are more easily overcome than polygenic ones (Poehlman, 1986). Here, an *in vitro* study was designed to determine whether fungi would be able to overcome the action of chitinase and ß-1,3-glucanase. Evidence is presented that the plant hydrolases remain antifungal during the complete period of germination and are not inactivated by *F. solani* f.sp. *phaseoli.* When macroconidia are germinated in the absence of Chi-I and Glu-I, emerging germtube tips are fully lysed upon addition of a mixture of these hydrolases. Our studies suggest that, in the presence of chitinase and ß-1,3-glucanase, the growing fungal cell wall might be modified and germtubes emerging in the presence of these hydrolases become insensitive.

MATERIALS AND METHODS

Biological Materials

Tobacco plants (*Nicotiana tabacum* cv Samsun NN) were grown in pots in a greenhouse. For induction of PR proteins, leaves of 5- to 6-week-old plants were inoculated with TMV as described previously (Woloshuk *et al.*, 1991)

Fusarium solani f.sp. *phaseoli* was maintained on V8 agar at 20° C in daylight. Macroconidia were harvested from 1- to 3-week-old sporulating plates by flooding the plate with water. The concentration was adjusted to 10,000 sp/ml.

Protein Purification and Analysis

Glu-I was purified from TMV inoculated tobacco leaves basically according to methods previously described by Sela-Buurlage *et al.*, 1993. For purification of Chi-I* transgenic tobacco plants were used, expressing single genes under control of the cauliflower mosaic virus 35S promoter. Since additional stop codons had been introduced into the C-terminus of both genes, these proteins were targeted extracellularly (Melchers *et al.*, 1993). Intercellular washing fluid was isolated from transgenic plants according to methods described by de Wit and Spikman (1982). The IF, containing the Chi-I* protein, was dialysed against 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). Fractions were analyzed on 12.5% SDS-PAA

gels and the Chi-I* was pooled accordingly. Gel filtration was performed using a SD75 Superdex column (Pharmacia) and again fractions were analyzed as above. A similar approach was used for the IF containing the Glu-I*. One additional step of cation-exchange chromatography was added: Glu-I* containing fractions which were pooled after gel filtration chromatography were dialysed to 50 mM potassium phosphate buffer, pH=6.1. The remaining protein solution was applied to a Mono-S (HR 5/5; Pharmacia) column equilibrated in the same buffer. Glu-I* ran through the column and was pure as judged from SDS-PAA gels.

Protein concentrations were determined by the method of Bradford (1976) using BSA as the standard.

Chitinase activity measurements were carried out with dye-labelled CM-chitin as described by Wirth and Wolf (1990). Mixtures of substrate (100 µl of a 2 mg/ml solution per assay) in 50 mM $KHPO_4$ buffer (pH 6.4) and appropriate amounts of enzyme were incubated at 37°C for 30 minutes in a final volume of 200 µl. The reaction was terminated by the addition of 100 µl 1 M HCl, causing precipitation of the non-degraded substrate. The reaction vials were cooled on ice for 10 minutes and centrifuged (5 min Eppendorf centrifuge). Two hundred µl of the resulting supernatant was pipetted into a microtiter dish (96 wells) and the absorbance at 540 nm was read. The absorbance (expressed in OD units) was taken as a measure for enzyme activity.

In vitro Antifungal Assay

Antifungal assays were performed as described by Sela-Buurlage *et al.*, (1993). 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* f.sp. *phaseoli* macroconidia in 50 µl water were added per well. The macroconidia were pregerminated for 0 to 7 h at 25°C. Protein samples were diluted in 50 mM potassium phosphate, pH 6 and subsequently filter-sterilized through 0.22 µm filters (Millepore). Protein concentrations were determined and adjusted appropriately. One hundred microliters of hydrolase sample was pipetted into each well, resulting in a total volume of 150 µl. The effect of 50 mM potassium phosphate, pH 6 was determined by adding 100 µl buffer to the germlings. If hydrolases were added in two doses, appropriate amounts were diluted in 50 µl, in order to obtain equal volumes compared to the standard assay conditions. One hour after application of the hydrolases, the fungus was monitored microscopically for percentage of lysed germtube tips. After 2 to 3 days fungal growth was stopped by staining the mycelium with lactophenol cotton blue and subsequently, the medium was destained with water. The amount of fungal material was taken as a measure for growth inhibition. All assays were performed in triplicate.

RESULTS

In Vitro Germination of *Fusarium solani* f.sp. *phaseoli*

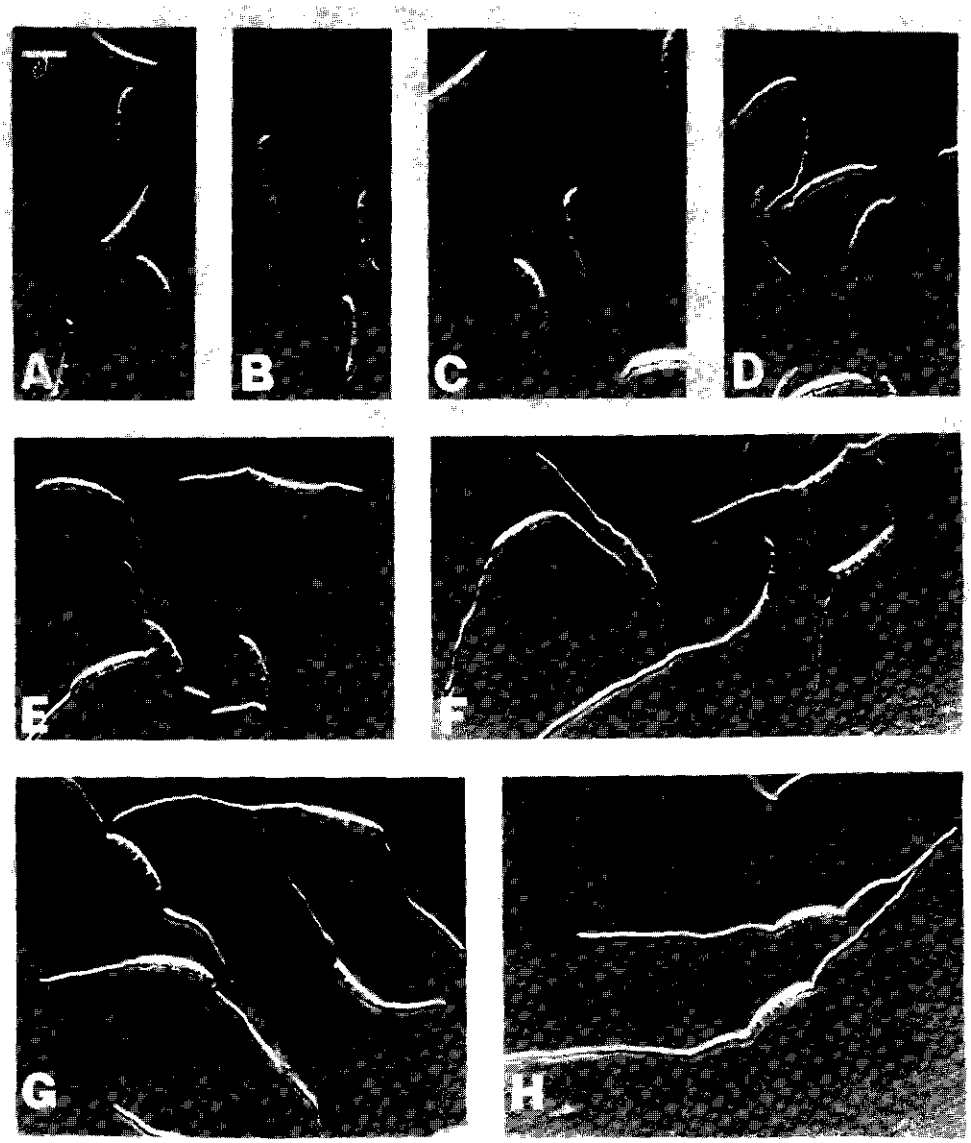
Typically *F. solani* f. sp. *phaseoli* produces a mixture of micro- and macroconidia. Macroconidia germinate much faster than the microconidia, resulting in a very asynchronous population. If maintained at 25°C in daylight on V8 agar this specific strain of the fungus only produces macroconidia. Macroconidia of the fungus were germinated on solid medium for appropriate periods of time after which hydrolase mixtures were added. Percentage of lysed germtube tips was monitored as well as inhibition of fungal growth.

The germination rate of macroconidia of *F.solani* f. sp. *phaseoli* in our *in vitro* assay was determined. Different phases of germination over a period of 7 hours are shown in Fig 1. In the absence of hydrolases, over 90% of the macroconidia showed an emerging germtube 2 hours after incubation (hrs p.i.) with a maximum length of 25% of the length of a macroconidium. At 3 hrs p.i.the germtube was about half the length of a macroconidium, increasing in length up to 1x the length at 4 hrs p.i., 1-2x at 5 hrs p.i., 2-3x at 6 hrs p.i. and over 3x the length of a macroconidium at 7 hrs p.i. To exclude the possibility that the preincubation volume might influence the sensitivity of the germinating macroconidia, regardless of the presence of a hydrolases, pregermination was also performed in 140 µl of water in stead of the standard 50 µl. After 7 hrs p.i., percentage germination and germtube length was similar to those obtained under standard conditions and shown in Fig 1.

Sensitivity of Macroconidia of *Fusarium solani* f.sp. *phaseoli* treated with a Mixture of Chi-I and Glu-I during Germination

Hyphal cell walls of *F. solani* f.sp. *phaseoli* contain chitin and ß-1,3-glucan (Wessels and Sietsma, 1981) and are sensitive to chitinases and ß-1,3-glucanases (Mauch *et al*, 1988). We tested chitinases and ß-1,3-glucanases purified from TMV-infected tobacco leaves. The class I chitinase (Chi-I) and class I ß-1,3-glucanase (Glu-I) caused lysis of hyphal tips of germinating macroconidia and inhibited fungal growth. *F. solani* f. sp. *phaseoli* is highly sensitive to a mixture of Chi-I and Glu-I, due to the high synergistic activity of these enzymes (Sela-Buurlage *et al.*, 1993). In these *in vitro* assays, macroconidia were pregerminated 7 hrs

Figure 1. germination phases of macroconidia of *Fusarium solani* f.sp. *phaseoli*. Length of germtube at A) 0 hr, B) 1 hr, C) 2 hrs, D) 3 hrs, E) 4 hrs, F) 5 hrs, G) 6 hrs, H) 7 hrs of incubation


A
B
C
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E
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G
H

before addition of the hydrolases, since this proved to be the most sensitive assay system. Preliminary experiments indicated that addition of Chi-I and Glu-I to non-germinated macroconidia was far less effective in inhibiting fungal growth (data not shown). In order to study *in vitro* whether indeed *F. solani* f. sp. *phaseoli* was capable of resisting the hydrolytic action, a specific mixture of Chi-I and Glu-I was applied at various time points of the germination process. These enzymes were isolated from transgenic tobacco plants constitutively expressing single genes encoding these proteins, Chi-I* and Glu-I*. The genes had been modified in such a way that proteins were targeted to the extracellular space (see *Materials and Methods* for details). The isolated proteins Chi-I* and Glu-I* were identical in their amino acid sequence and their specific enzymatic activity compared to the mature form of class I chitinase and ß-1,3-glucanase respectively. Addition of 1% Glu-I* (w/w) to Chi-I*, C/G, showed increased antifungal activity compared to Chi-I* alone (Sela-Buurlage *et al.*, Chapter 6). This particular mixture was used in the present study.

The susceptibility of *F. solani* f.sp. *phaseoli* was studied at various phases during the germination process by addition of the C/G mixture. Either 0, 1, 2.5 or 5 µg of C/G was added to germinating *F. solani* f.sp. *phaseoli* macroconidia from 0 to 7 hrs p.i. At each time point the percentage of germination and length of the germtube was determined. No significant differences in either the percentage of germination or the length of the germtube were observed irrespective whether germination was performed in the presence or absence of the C/G mixture (data not shown).

Percentage lysis of the germtube tips was scored1 hr after addition of the C/G mixture (Fig 2A). A pregermination period of at least 4 hrs was required to cause 10% lysis at 1 µg. The higher the concentration of C/G mixture added, the higher the percentage of lysed germtube tips. Extension of the pregermination period resulted in increased percentages of lysis. Addition of 5 µg C/G mixture at 6 to 7 hrs p.i. resulted in almost complete lysis of germtube tips. Shorter pregermination times could be compensated for by adding higher concentration of C/G mixture. For example, 1 µg added at 7 hrs p.i. caused 50% lysis. Roughly similar percentages were found when 2.5 µg was added at 5 hrs p.i. or when 5 µg was added at 4 hrs p.i.. Thus, percentage of lysis of germtube tips is dependent on pregermination time and concentration of C/G mixture.

The degree of fungal growth inhibition was scored after 2 days. Representative results are shown in Fig 2B. Only limited growth inhibition was observed when 2.5 or 5 µg of C/G mixture was applied at 3 hrs p.i. With longer pregermination times, growth inhibition increased accordingly, resulting in almost complete inhibition after applying 5 µg C/G mixture at 5 hrs p.i. or 2.5 µg C/G mixture at 6 hrs p.i.. Clearly, shortening the period of pregermination renders the fungus less sensitive to the hydrolases. Moderate sensitivity of the germinating macroconidia to the C/G mixture was observed when macroconidia were

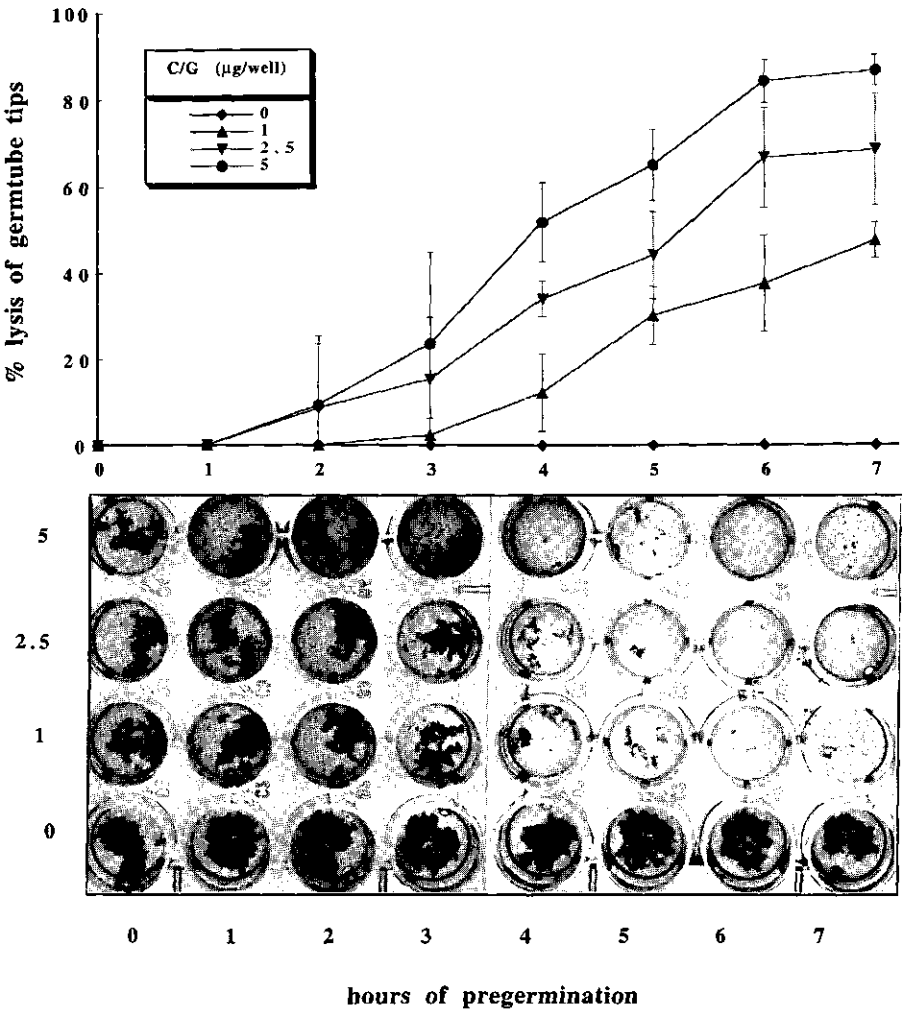


Figure 2. Decreased sensitivity of *Fusarium solani* f.sp. *phaseoli* macroconidia to a C/G mixture depends on germination phase. Effect of 0 (•), 1 (▯), 2.5 (♦) or 5 (Δ) µg of a C/G mixture applied at different phases on A) percentage lysis of germtube tips one hour after application and B) growth inhibition after 2 days. Bars indicate the standard deviation.

pregerminated for 3 to 5 hrs, while high sensitivity was observed after 6 to 7 hrs of pregermination. The question arose whether the hydrolases, when applied at the beginning of the germination process, would still have unchanged antifungal activity 7 hrs p.i.

Germinating *Fusarium solani* f.sp. *phaseoli* Macroconidia Do Not Inactivate Antifungal Activity of the C/G Mixture

Experiments were performed to determine whether the C/G mixture, after incubation with the germinating macroconidia had retained its antifungal activity or that compound(s) had been secreted by the macroconidia capable of inactivating or inhibiting the antifungal activity. In order to investigate these options, 0, 2.5 or 5 µg of the C/G mixture was incubated with germinating macroconidia for 7 hrs on solid PDA medium (+). As a control, equal amounts of enzyme mixture were incubated on PDA without germinating macroconidia (-). After incubation at 25°C for 7 hours, the supernatant was harvested and passed through a 0.22 µm filter to remove the germlings (+). Chitinase activity of these harvested mixtures was compared by incubating the mixtures on RBV-chitin (Wirth & Wolf, 1990). Specific enzymatic activity, expressed in ODu/µg, was identical irrespective of incubation with (1.68 ± 0.1 ODu/µg) or

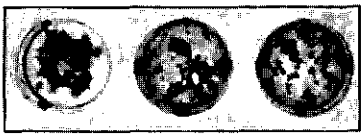
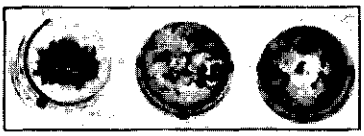
	% LYSIS			GROWTH INHIBITION		
Preincubation of C/G mixture	**amount (µg/well)**			**amount (µg/well)**		
	0	**2.5**	**5**	**0**	**2.5**	**5**
with macroconidia	0 ± 0	36 ± 3	54 ± 7			
without macroconidia	0 ± 0	36 ± 5	53 ± 5			

Figure 3. Germinating macroconidia of *Fusarium solani* f.sp. *phaseoli* do not influence the antifungal activity of a C/G mixture after transfer to pregerminated macroconidia. Percentage lysis of germtube tips is shown in middle panel and growth inhibition in the right panel.

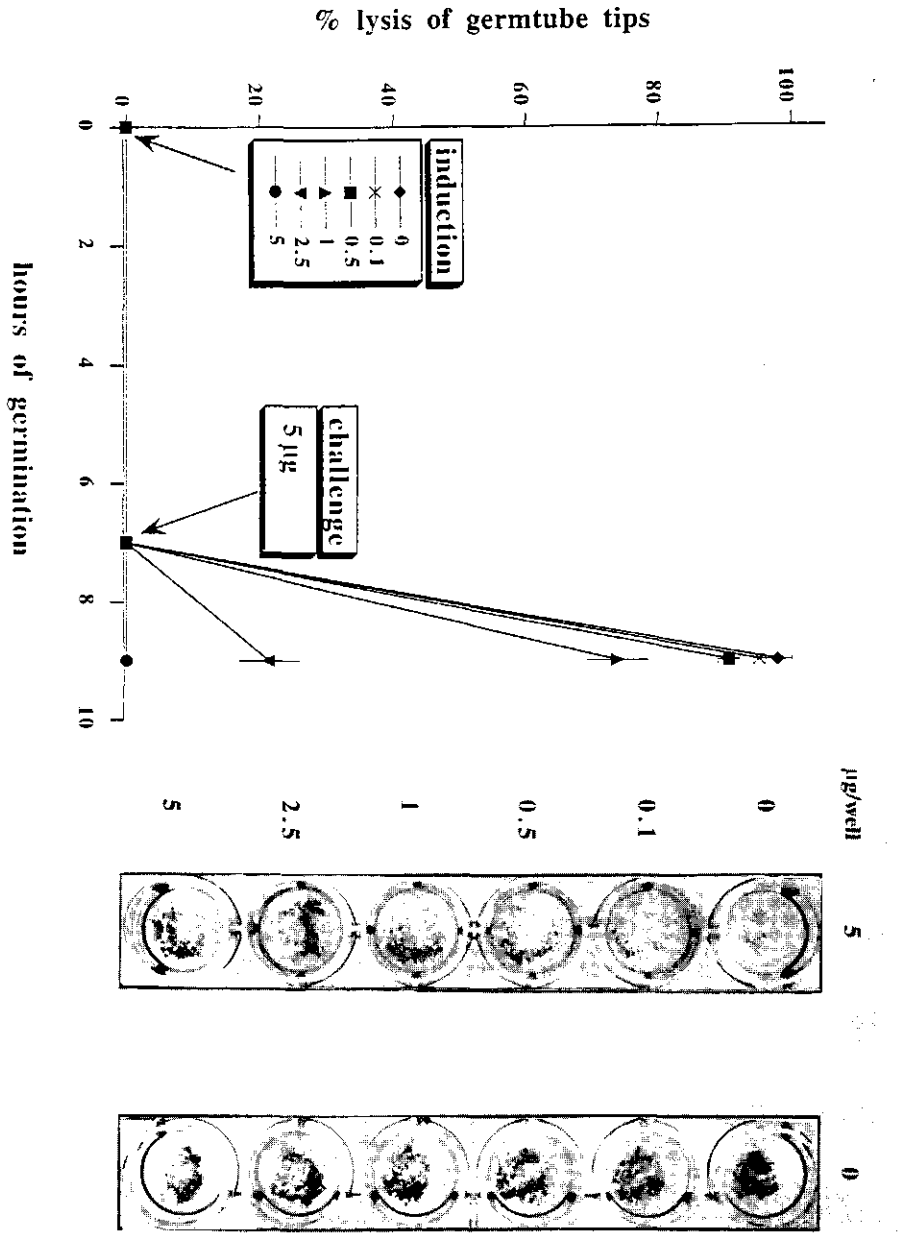


Figure 4. Treatment of macroconidia of *F. solani* f.sp. *phaseoli* with C/G mixtures influences the sensitivity of their germtube to subsequent chitinolytic attack. 0, 0.1, 0.5, 1, 2.5 or 5 µg of a C/G mixture was added at 0 hrs p.i. At 7 hrs p.i. 5 µg C/G (challenge) was added and percentage lysis of germtube tips was scored at 8 hrs p.i. (left pannel). Growth inhibition (right pannel) was scored after 2 days. Error bars indicate the standard deviation.

without (1.80 ± 0.04 ODu/µg) germinating macroconidia. Subsequently, both the (+) and (-) mixture were compared for their capability to cause lysis of germtube tips and growth inhibition of *F. solani* f.sp. *phaseoli*. Results are shown in Fig 3. Antifungal activity, observed both as lysis of germtube tips as well as growth inhibition, was identical when equal amounts of enzymes were applied, irrespective of having been incubated on germinating macroconidia (+) or on PDA (-). These results clearly indicated that the hydrolytic enzymes had not been inactivated by the germinating macroconidia, but had retained their antifungal activity 7 hrs p.i. in the presence of germinating macroconidia. Hence, a possible hypothesis for the decreased sensitivity of *F. solani* f.sp. *phaseoli* might be that the germinating macroconidia have adapted to C/G mixture.

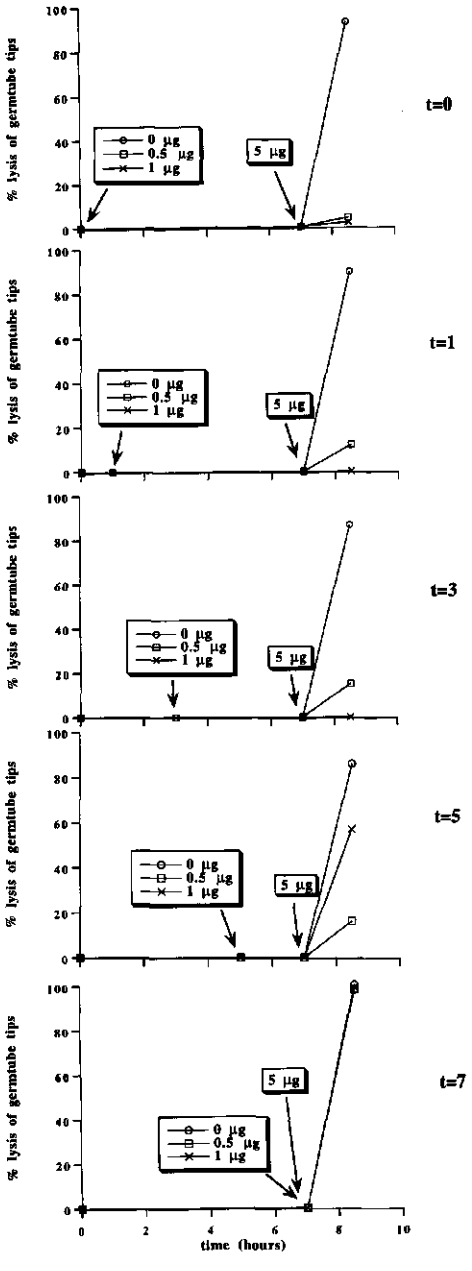
Germinating Macroconidia of *Fusarium solani* f.sp. *phaseoli* Adapt to the Presence of the C/G Mixture

The hypothesis of fungal adaptation was investigated in further detail by adding a double dose of C/G at two different stages of germination. In this way any possible loss of antifungal activity of the hydrolase mixture would be compensated for by addition of the second dose. Additionally, the amount of C/G required for the macroconidia to become insensitive, could be determined.

Various amounts of C/G, 0, 0.1, 0.5, 1, 2.5 or 5 µg, were added at 0 hrs p.i. and a second dose (0 or 5 µg) was applied at 7 hrs p.i.. Percentage of lysis of germtube tips was scored at 8 hrs p.i. Results are shown in Fig 4A and B. A dramatic decrease in percentage of lysis of germtube tips was observed if more than 0.5 µg of the mixture was applied at 0 hrs p.i. Five µg of enzyme mixture had to be added at 7 hrs p.i. to ensure maximum antifungal activity. If 1 µg of mixture was added at 0 hrs p.i., even an amount of 5 µg at 7 hrs p.i. could not cause complete lysis and growth inhibition. If 5 µg was applied at 0 hrs p.i., almost no lysis and growth inhibition could be achieved by adding 5 µg at 7 hrs p.i. This is very striking since at the highest concentration, approximately 10 µg of active hydrolase mixture is present.

Clearly, the fungus was able to overcome the action of the hydrolases if applied at 0 hrs p.i. We were interested to determine when during the germination process the fungus

Figure 5. Treatment of germinating macroconidia of *F. solani f.sp. phaseoli* f.sp. *phaseoli* with C/G mixtures influences the sensitivity of their germtube to subsequent chitinolytic attack. 0 (o), 0.5 (□) or 1 (x) µg of a C/G mixture was added at A) 0 hrs p.i., B) 1 hrs p.i., C) 3 hrs p.i., D) 5 hrs p.i., E) 7 hrs p.i. At 7 hrs p.i. 5 µg C/G (challenge) was added and percentage lysis of germtube tips was scored at 8 hrs p.i.


% lysis of germtube tips
0 µg
0.5 µg
1 µg
5 µg
t=0
t=1
t=3
t=5
t=7
time (hours)

loses the ability to adapt and becomes sensitive to the hydrolase mixture. Therefore, 0, 0.5 or 1 µg of the enzyme mixture was added either at 0 hrs p.i., as above, or at 1, 3, 5 or 7 hrs p.i. At 7 hrs p.i. either 0 or 5 µg of C/G mixture was added to the wells and percentage germtube tips showing lysis was scored 1 hr later. Results are shown in Fig 5. If buffer was applied before 7 hrs p.i., the germlings were sensitive to both 2.5 and 5 µg challenge. If 0.5 µg of enzyme was used as trigger at 0, 1 or 3 hrs p.i. germlings were insensitive to 5 µg challenge. If this triggering amount was added at 5 hrs p.i., some lysis was observed. The most obvious results were obtained by using 1 µg as a triggering amount. If this amount was applied up to 3 hrs p.i., the germlings were insensitive to 5 µg added at 7 hrs p.i.. Addition of 1 µg at 5 hrs p.i. was too late for the germlings to adapt and thus high antifungal activity was observed upon subsequent challenge at 7 hrs p.i.

DISCUSSION

Previous experiments, performed in our lab, indicated that Chi-I and Glu-I enzymes, isolated from tobacco leaves infected with TMV exhibited strong antifungal activity in a synergistic fashion against *Fusarium solani* f.sp. *phaseoli* (Sela-Buurlage *et al*, 1993). In these assays, macroconidia of *F. solani* f.sp. *phaseoli* were always pregerminated 6 to 7 hours before enzymes were added to the germlings, since sensitivity to these hydrolases was the highest if pregermination was allowed. Surprisingly, when enzymes were added before germination was initiated, the fungus was not sensitive at all (unpublished results). Four different explanations for this observation are possible. Firstly, the hydrolases might be instable during the course of the *in vitro* assay and therefore not capable of exerting their hydrolytic activity after the period of germination had been completed. Secondly, the macroconidial population might be genetically diverse and selection might have occured. Depending on the conditions, different subsets of the population might germinate with varying sensitivity to the hydrolytic enzymes. Thirdly, during germination the fungus might be stimulated to secrete compounds inhibitory to the hydrolases or capable of degrading the hydrolases (Albersheim, 1974; Kuc 1963). Finally, the fungus might become adapt to the hydrolase mixture by modifying the cell wall structure of the growing hyphae.

In the present study we determined the tolerance of *F. solani* f.sp. *phaseoli* to a defined mixture of Chi-I* and Glu-I* at various phases of the germination process. Percentage germination of the macroconidia and the length of the germtubes was not influenced by the presence of the enzyme mixture. For all treatments, the germination percentage was over 95%

percent at 7 hrs p.i. and the average germtube length was more than 3 times the length of the macroconidium. Germtube tips were most sensitive when the length of the germtube was more than 3 times the length of the macroconidium. The pregermination volume was not responsible for decreased sensitivity to the hydrolase mixture since germlings were just as easily lysed by the hydrolase mixture after germination in excess of buffer as compared to normal pregermination conditions. Addition of sugar beet chitinase to *Cercospora beticola* spores resulted in a delay in germination and a slower initial growth rate (Nielsen *et al.*, 1994). In contrast, for *F. solani* f.sp. *phaseoli* no significant differences in either the percentage of germination or the length of the germtube were observed when germination was performed in the presence or absence of the enzyme mixture.

A higher concentration of C/G mixture needed to be added earlier on in the germination process, to achieve an equal amount of lysis of hyphal tips and growth inhibition of the fungal mycelium compared to the amount needed after 7 hours of pregermination. Clearly, shortening the pregermination time could compensate for the amount of enzyme mixture that needed to be added and *vice versa*: higher amounts of enzyme mixture could compensate for shorter pregermination times.

Two hypotheses to explain the phenomenon of adaptation were tested. One possibility could be that compounds are secreted by the germinating macroconidia that inhibit or destroy the activity of the hydrolase mixture. In our studies, the hydrolase mixture was incubated with or without germinating macroconidia for 7 hrs and then transferred to pregerminated macroconidia. The antifungal activity of both transferred solutions was identical, both with respect to the degree of lysis of hyphal tips as with respect to growth inhibition of the fungus, indicating that the hydrolases could be incubated with germinating *F. solani f.sp. phaseoli* for 7 hrs without significant loss of antifungal activity. Therefore it is concluded that no inactivation, inhibition or degradation of the hydrolases has occurred.

A second hypothesis would involve modification of the fungal cell wall during preincubation. To test this hypothesis, various amounts of the C/G mixture were added at the start of the germination period and lethal or semi-lethal quantities of the mixture were added after 7 hrs. Lysis of germtube tips and subsequent inhibition of fungal growth was significantly decreased when more than 0.5 µg had been added at 0 hrs p.i. When high amounts, 2.5 or 5 µg were applied at 0 hrs p.i., the fungus was completely insensitive to a second dose applied at 7 hrs p.i. This is surprising since the enzymes added at 0 hrs p.i. were still antifungal at 7 hrs p.i. as was concluded from the above described transfer experiments. In fact the fungus had become insensitive to as much as twice the normally lethal dose. The decreased sensitivity of *F. solani* f.sp. *phaseoli* as described, is therefore most likely caused by adaptation of the germinating hyphal wall. Ludwig and Boller (1990) noted that pretreatment of combinations of pea chitinase and ß-1,3-glucanase caused only a transient reduction in growth of various fungi,

including *Nectria haematococca* (*Fusarium solani*). They suggested that growth inhibition was due to tip lysis of the growing hyphae and that the fungus might have the capacity to adapt to the presence of the inhibitory proteins and to resume growth.

The underlying sensing and modifying mechanism of the fungus remains unknown. It needs to be clarified, whether the structure of the hyphal wall is indeed altered, making it more difficult for the hydrolases to degrade their respective substrates or whether the chemical composition of the cell wall is altered which would make the hydrolases less potent. In earlier studies with various *F. oxysporum* spp. it was shown that they contain more proteins in their cell walls than other fungi (Sivan and Chet, 1989). Kuo and Alexander (1967) showed that the resistance of the hyphal walls of *Aspergillus nidulans* to digestion by chitinase/ß-1,3-glucanase was directly correlated to the melanin content of the mycelium. *Aspergillus fumigatus* strains containing elevated levels of melanin showed reduced sensitivity to hydrolase attack (Luther and Lipke, 1980) Similar results were obtained with *Rhizoctonia solani* (Potgieter and Alexander, 1966). It remains to be shown whether this (temporary) adaptation is specific for *F. solani* f.sp. *phaseoli* or whether other fungi possess similar mechanisms. Recent data in our laboratory have shown that most fungi are far more sensitive to a total protein extract from TMV inoculated tobacco leaves if they are pregerminated (Sela-Buurlage *et al.*, Chapter 2).

It is not known whether any other chitinase or ß-1,3-glucanase or combinations thereof can elicit the adaptational response, such as the extracellular, class II, enzymes or other chitinases such as CBP20 (Ponstein *et al.*, 1994) or Chi-V (Melchers *et al.*, 1994). Additional research should focus on possible cross adaptation or sensitivity, i.e. does adaptation to one hydrolase cause increased or decreased sensitivity to a different hydrolase.

This is the first published report on the capability of a fungus to adapt to the presence of hydrolytic proteins. All data presented in this study have been obtained with an *in vitro* system. Additional studies are needed to determine whether the same phenomena holds for the *in planta* situation. However, Charest *et al.* (1993) showed by ultrastructural studies that several fungi alter their morphology in order to invade host tissue. In the strategy of engineering fungal resistance by creating transgenic plants, which constitutively express Chi-I and Glu-I, the obvious conclusion seems that the fungus migh overcome the introduced resistance. A reason why resistance might not be overcome easily lies in the fact that the macroconidia land on the host surface and have to germinate and penetrate the host tissue. Moreover, the germtube tip does not encounter these vacuolar proteins until penetration of the host cell is completed. Indeed Zhu and coworkers (1994) demonstrated resistance to *Cercospora nicotianae* in transgenic tobacco plants possessing both a rice basic chitinase and an alfalfa acidic glucanase. Similarly, transgenic tomato plants constitutively expressing both Chi-I and Glu-I showed enhanced resistance to *Fusarium oxysporum* (Jongedijk *et al.*, 1995; Van den Elzen, 1994). Most recently, field experiments with transgenic carrot expressing the same hydrolases demonstrated

multiple resistance to *Alternaris* spp., *Cercospora carotae* and powdery mildew. Finally, by engineering resistant transgenic plants expressing hydrolases, the use of tissue-specific or inducible promoters might be an alternative to withhold the fungus from possible adaptation. Therefore, the strategy of enhancing fungal resistance in plants by transformation of these crops with genes encoding antifungal proteins still remains very potent.

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Chapter 8

Tobacco Class I Chitinases are Processed by Proteases of *Fusarium solani* f.sp. *phaseoli*: Consequences for Antifungal Activity

ABSTRACT

Two tobacco class I chitinases, the PR-3 Chi-I and the PR-4 CBP20 and a class I PR-2 ß-1,3-glucanase (Glu-I) have potent antifungal activity *in vitro* towards *Fusarium solani* f.sp. *phaseoli* (Ponstein *et al.*, 1994; Sela-Buurlage *et al*, 1993). Various fungi, including *F. solani* f.sp. *phaseoli* were shown to become less sensitive to Chi-I and Glu-I, if macroconidia were allowed to germinate in their presence (Sela-Buurlage *et al*, Chapter 7). In this study we present evidence that germinating *F. solani* f.sp. *phaseoli* macroconidia not only adapt to Chi-I but also to CBP20. This process requires active enzymes since neither to denatured Chi-I nor denatured CBP20 could adaptation be observed. Very slight adaptation was observed to the PR-11 chitinase Chi-V. However to the class II PR-3a (PR-P) chitinase macroconidia were unable to adapt. Using SDS-PAGE and western analysis it was shown that after incubation with macroconidia the Chi-I and CBP20 proteins were proteolytically processed to smaller proteins. Both enzymes had a molecular mass which was around 5 kD smaller and had lost their chitin binding domain. Fungal protease(s) released during the first three hours of germination were shown to be responsible for this processing. Protease activity can be inhibited by EDTA, but not by PMSF or leupeptin, indicating that one or more metalloproteases are involved. Enzymatic activities of Chi-I and the 5 kD smaller Chi-I (Chi-I-ΔCBD) tested on the artificial substrate, CM-chitin, remained unchanged. N-terminal sequencing of Chi-I-ΔCBD revealed that cleavage of Chi-I had occurred between Pro_{67} and Thr_{68}. The synergistic activity of Chi-I-ΔCBD with Glu-I was 30-40 fold less antifungal to *F. solani* germlings compared to mature Chi-I and Glu-I. When high concentration of hydrolase mixtures were used, antifungal activity was similar for both Chi-I-ΔCBD and Chi-I. Whereas macroconidia adapt to Chi-I, only limited adaptation was observed when initial incubation was done with Chi-I-ΔCBD. This study underlines the important role for the chitin binding domain of plant chitinases for antifungal activity.

INTRODUCTION

In tobacco (*Nicotiana tabacum* cv. Samsun NN), pathogenesis-related (PR) proteins are *de novo* synthesized upon inoculation with tobacco mosaic virus. Many groups of PR-proteins have been identified and in previous studies it was shown that a number of PR-proteins possess antifungal activity. For example, *Fusarium solani* f.sp. *phaseoli* is sensitive to a number of these PR-proteins including chitinases and ß-1,3-glucanases causing lysis of germtube tips and growth inhibition (Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993). This is This is most probably due to the fact that chitin and glucan are major components of the cell wall of this fungus. However, only the class I, vacuolar and basic, isoforms (Chi-I, Glu-I) of these protein groups were antifungal (Sela-Buurlage *et al.*, 1993). Their extracellular, acidic counterparts, class II, possessed little or no antifungal activity. Synergy between the Chi-I and Glu-I hydrolases was demonstrated (Sela-Buurlage *et al.*, 1993).

In addition to the above mentioned PR-3 class I chitinase, a class I PR-4 protein, CBP20, was shown to act synergistically with Glu-I against *F. solani* f. sp. *phaseoli*, but more strikingly synergy was also observed with Chi-I (Ponstein *et al.*, 1994). Exact mode of action of both Chi-I and CBP20 is unknown but apparently in addition to their chitinolytic activity, the presence of a chitin-binding domain strongly increases antifungal activity. It is known that chitin-binding proteins without detectable chitinase activity such as hevein (Van Parijs *et al.*, 1991), stinging nettle lectin (Broekaert *et al.*, 1989) and some peptides from *Amaranthus caudatus* seed (Broekaert *et al.*, 1992) possess antifungal activity. Exceptions exist, however, such as wheat germ agglutinin that lack antifungal activity (Schlumbaum *et al.*, 1986; Chrispeels and Raikhel, 1991).

Previously we have shown that antifungal activity was most pronounced if macroconidia of *F. solani* f.sp. *phaseoli* were pregerminated before treatment with hydrolase mixtures. Moreover, germinating macroconidia were shown to adapt to the presence of these hydrolytic enzymes (Sela-Buurlage *et al.*, Chapter 7). In the present study we present evidence that metalloprotease(s), released during the early hours of germination, can remove the chitin-binding domain from both Chi-I and CBP20. We show that Chi-I-ΔCBD is far less capable of causing lysis of hyphal tips compared to Chi-I in the presence of Glu-I. In addition, germinating macroconidia of *Fusarium solani* f.sp. *phaseoli* can only slightly adapt to Chi-I-ΔCBD, indicating that the CBD plays an important role in antifungal activity of Chi-I and is at least partially responsible for the phenomenon of adaptation of germinating macroconidia to Chi-I. Consequences for antifungal activity are discussed.

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).

Fusarium solani f.sp. *phaseoli* was kindly provided by Dr. Th. Boller, Botanical Institute of the University of Basel, Basel, Switzerland. The fungus was maintained on V8 agar at 20°C in daylight. Macroconidia were harvested from 1- to 3-week-old sporulating plates in water. The macroconidia concentration was adjusted to 10,000 sp/ml.

Protein Purification and Analysis

Apoplastic fluid was isolated according to methods described by de Wit and Spikman (1982). Chi-I* and Glu-I* were isolated from apoplastic fluid from transgenic plants constitutively expressing one of the proteins targeted to the extracellular space (Melchers *et al.*, 1993; Sela-Buurlage *et al.*, (Chapter 6). Separation of the 32 and 34 kD isoforms of Chi-I was accomplished by gelfiltration (Sephadex SD75, Pharmacia). CBP20, PR-3a and Chi-V were isolated from TMV inoculated tobacco (Melchers *et al.*, 1994; Ponstein *et al.*, (1994), Legrand *et al.*,1987).

Protein concentrations were determined by the method of Bradford (1976) using BSA as the standard. Chitinase measurements were performed using CM-chitin as a substrate (Wirth and Wolf, 1990). Protein sequencing was performed by Eurosequence, Groningen.

Digestion of Chi-I and CBP20 by Protease(s)

Macroconidia (10^8) of *F. solani* f.sp. *phaseoli* were incubated in 300 ml water for 3.5 hrs on a rotary shaker at room temperature. Centrifugation at 1000*g* for 10 minutes and passage through a 0.22 µm filter was performed to remove macroconidia. The obtained cell free supernatant (SN) was frozen and lyophilized. After resuspension in 300 µl demi water, aliquots were stored at -20°C until further use. Hundred µl SN was incubated with 100 µl Chi-I, both the 32 kD isoform, the 34 kD isoform or CBP20 (all at 50 µg/ml) at room temperature for 24 hrs. As controls the chitinases were incubated without SN or with heat inactivated SN (10

minutes 100°C). Western analysis was performed as described in Ponstein *et al.* (1994). Polyclonal antibodies were raised against a) a class II chitinase PR-P (PR-3a) which recognizes the C-terminal domain of the class I chitinase, but not the chitin-binding domain, b) to hevein which recognizes the chitin-binding domain and c) to P2, the CBP20 homologue of tomato.

Chi-I-ΔCBD was produced by incubation of 500 µg Chi-I, at a concentration of 100 µg/ml with SN at a 1:1 (v/v) ratio. The resulting mixture of cleaved and uncleaved Chi-I was subjected to chitin affinity chromatography to remove uncleaved Chi-I according to methods described by Ponstein *et al.* (1994). Using western analysis with a concentration range of Chi-I, the remaining amount of Chi-I in the Chi-I-ΔCBD sample was determined.

All protease inhibitors were all dissolved at 10 times their final concentration in water. PMSF which is an inhibitor of serine proteases, was tested at a final concentration of 100 mg/ml (w/v), leupeptin, an inhibitor of peptidases at 40 µM, and EDTA which inhibits metalloproteases 5 mM (Scopes, 1987).

In Vitro Antifungal Assay

Antifungal assays were performed as described by Sela-Buurlage *et al.*, (1993) with *F.solani* f.sp. *phaseoli.* On a layer of potato dextrose agar macroconidia were allowed to germinate in the presence of 50 µl buffer, 50 mM potassium phosphate, pH 6, containing a concentration range of either 0 to 10 µg Chi-I, both the 32 kD and 34 kD isoform, CBP20, Chi-V or PR-P (class II PR-3). In case Chi-I is mentioned anywhere in this manuscript, the 32 kD isoform isolated from apoplastic fluid of transgenic tobacco plants overexpressing this protein extracellularly unless specified otherwise. Chi-I was also added after 10 minutes boiling. After incubation had proceeded for 7 hrs (7 hrs p.i.) the germlings were challenged with a concentration range of 0 to 2.5 µg Chi-I amended with 1% Glu-I (w/w) in 50 µl buffer (C/G mixture). Percentage germtube tips showing lysis was determined one hour later (8 hrs p.i.). Over the following 2-3 days growth inhibition was monitored and rated on a linear scale from 0 to 4, whereby 0 is no growth inhibition and 4 is 100% inhibition of growth.

RESULTS

Previously, it has been shown that Glu-I and Chi-I cause extensive lysis of germtube tips as well as growth inhibition of *Fusarium solani* (Sela-Buurlage *et al.*, 1993). The

extracellular, class II, proteins possessed little or no antifungal activity. Recently, another chitinase, CBP20 was identified in our lab (Ponstein *et al.*, 1994). In the N-terminal regions of both Chi-I and CBP20 a chitin binding domain (CBD) is located. CBP20 possesses synergistic antifungal activity with Glu-I but also with Chi-I. Another tobacco chitinase was determined to be antifungal in synergy with Glu-I (Melchers *et al.*, 1994). In these *in vitro* assays, hydrolase mixtures were added to germlings of *Fusarium solani* f.sp. *phaseoli*. These germlings were most sensitive when pregermination was continued for 6 to 7 hrs before treatment with the hydrolase mixtures. In contrast, when Chi-I/Glu-I mixtures were added to macroconidia no lysis of their germtube tips was ever observed (Sela-Buurlage *et al.*, Chapter 7). It was demonstrated that the germinating macroconidia adapted to Chi-I.

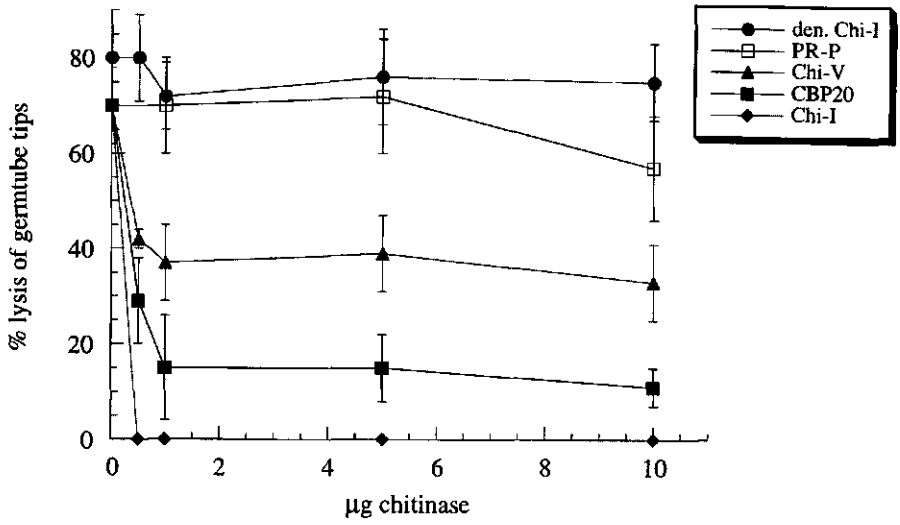


Figure 1. Chitinases added to *Fusarium solani* f.sp. *phaseoli* macroconidia influence lysis of germtube tube tips after addition at 7 hrs p.i. of 2.5 µg C/G mixture. Incubation of macroconidia with Chi-I, CBP20, Chi-V and PR-P was performed in a concentration range of 0 to 10 µg/well (=150 µl). Error bars indicate the standard deviation.

In the present study it has been investigated whether the germinating macroconidia could adapt to other chitinases and whether this would also cause the decrease in sensitivity to the C/G mixture. Results are shown in Figure 1 for percentage lysis. In Figure 2 results of growth inhibition upon adaptation to Chi-I and CBP20 are shown. When macroconidia were germinated in the presence of buffer, significant lysis of germtube tips as well as severe growth inhibition was observed upon challenge with 0.5 µg C/G mixture. Addition of 0.5 µg of Chi-I at 0 hrs p.i. lead to complete insensitivity of the germlings at 7 hrs p.i. to 2.5 µg C/G mixture since no lysis or growth inhibition was observed. If 1 µg of heat inactivated Chi-I was added, germlings

were as sensitive as the buffer controls (data not shown), indicating that the active hydrolase is necessary.

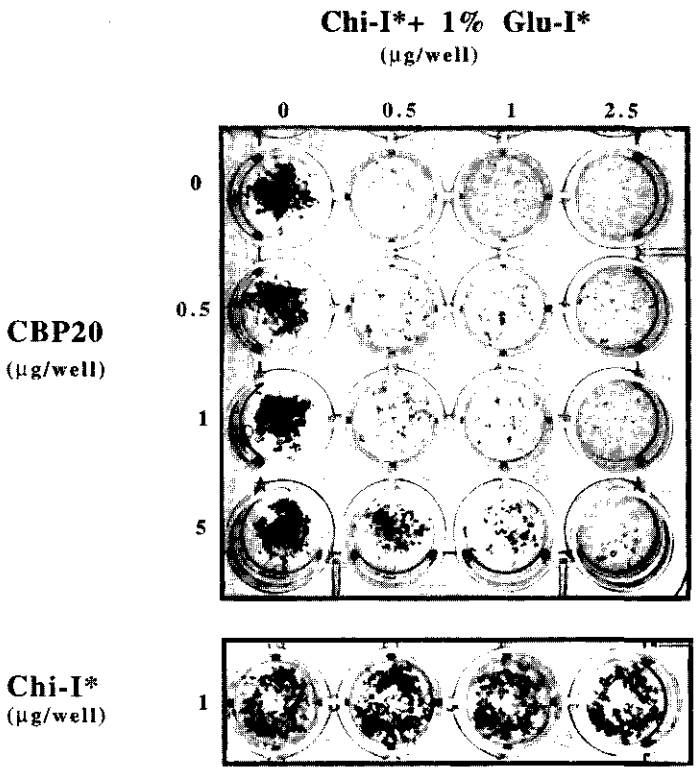


Figure 2. CBP20 added to *Fusarium solani* f.sp. *phaseoli* macroconidia influences growth inhibition after addition at 7 hrs p.i. of a C/G mixture (upper panel). A concentration range of 0 to 5 µg/well CBP20 was tested. At 7 hrs p.i. 0, 0.5, 1 or 2.5 µg of a C/G mixture was added per well. In the lower panel growth inhibition of *F. solani* is shown in case 1 µg Chi-I was added in stead of CBP20.

Addition of 0.5 µg of CBP20 at 0 hrs p.i. caused a two-fold decrease in the percentage lysis and growth inhibition compared to the buffer control after adding up to 2.5 µg C/G mixture at 7 hrs p.i.. Addition of 5 or 10 µg of CBP20 at 0 hrs p.i. could decrease the percentage lysis and growth inhibition even further (up to 3-4 fold), however, complete insensitivity was not observed. Similar results were obtained when the 34 kD Chi-I was used in stead of CBP20. Germinating macroconidia could adapt, however less efficiently than to the 32 kD isoform. Addition of up to 10 µg/well Chi-V caused a 2-fold decrease in percentage lysis and additionally, growth inhibition was decreased in case 0.5 or 1 µg C/G mixture was added at 7 hrs p.i. However, if 2.5 µg C/G was added at 7 hrs p.i. complete growth inhibition was

observed. The class II PR-3, PR-P, even when added up to 10 µg/well, was not capable of decreasing the sensitivity to the C/G mixture. None of the treatments at 0 hrs p.i.was able to cause an increase in sensitivity.

Protease Activity of *Fusarium solani* Macroconidia

How fungal germtubes adapt to Chi-I, CBP20 and Chi-V is unknown. As a first step, the germinating macroconidia were incubated with Chi-I for 7 hrs and subsequently analyzed on SDS-PAGE. As control Chi-I was incubated on PDA but without macroconidia. Two major bands of around 27 and 32 kD were visible after Coomassie staining in case the macroconidia were incubated with Chi-I (data not shown). The upper band also appeared in case Chi-I was incubated on PDA. Western analysis was subsequently performed and the upper band was identified as Chi-I by antiserum raised against PR-P capable of recognizing the C-terminal part of Chi-I, but not the chitin binding domain. The antiserum also crossreacted with the lower band (Chi-I-ΔCBD), indicating a possible processing of Chi-I by the germinating macroconidia. Western analysis was performed using antiserum specifically crossreacting with the chitin-binding domain (CBD) of Chi-I and only the upper band crossreacted . This indicated that the 5 kD CBD had been removed from the Chi-I protein although SDS-PAGE gels and Western blots did not reveal a small 5 kD band. The enzymatic activity of Chi-I, as determined on RBV-chitin, an artificial substrate, was unaltered after incubation with macroconidia (data not shown).

It has been shown previously that the amount of pregermination time required for the germlings to become sensitive to a C/G mixture was 3-4 hours (Sela-Buurlage *et al.*, Chapter 7). It was investigated whether the germinating *F. solani* f.sp. *phaseoli* macroconidia secreted proteases capable of cleaving Chi-I during the first hours of germination. After removal of the macroconidia the cell free solution (SN) was examined for the ability to hydrolase proteins. In case this SN was incubated with dried milk powder, the mixture had turned clear overnight indicating proteolytic breakdown (data not shown). Subsequently, the SN was analysed for the ability to hydrolase Chi-I. Incubation overnight at room temperature of the SN with Chi-I and subsequent analysis on SDS-PAGE and western blots demonstrated that (a) protease(s) was present capable of cleaving Chi-I. Results are presented in Fig 3. In each of the panels, lane 1 contains Chi-I incubated with SN, lane 2 contains only Chi-I and lane 3 contains Chi-I incubated with denatured SN. In panel I SDS-PAGE results are shown, in panel II and III results of the western analysis are presented. In panel II antiserum raised against hevein was used and in panel III antiserum raised against PR-P. The fact that in panel II all lanes contain one band of 32 kD indicated that the CBD was missing. Western analysis using antiserum against PR-P capable of

recognizing only the C-terminal part of Chi-I, but not the chitin binding domain demonstrated that the lower band of approximately 27 kD, Chi-I-ΔCBD observed in lane 1 of panel I is related to the 32 kD Chi-I. Clearly, these results with SN are the same as those observed when germinating macroconidia were incubated with Chi-I.

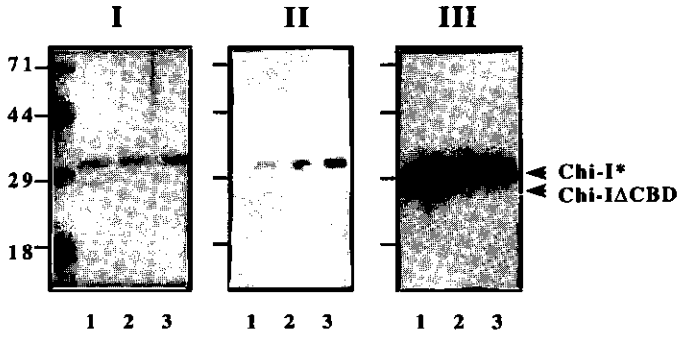


Figure 3. Proteases produced by germinating macroconidia of *F. solani* f.sp. *phaseoli* can degrade Chi-I. In each of the panels lane 1 contains Chi-I incubated with SN, lane 2 contains only Chi-I and lane 3 contains Chi-I incubated with denatured SN. In panel I SDS-PAGE results are shown, in panel II and III results of western analysis are presented. In panel II antiserum raised against hevein (1:1000) was used and in panel III antiserum raised against PR-P (1:5000).

Time course experiments indicated that after incubating Chi-I with SN for 2 hours at room temperature, trace amounts of Chi-I-ΔCBD could be detected (data not shown). Roughly half of the Chi-I was processed after 24 hours of incubation. Incubation for 48 hours did not further increase the amount of Chi-I-ΔCBD. Moreover, addition of a second dose of SN after 48 hours did not increase the amount of Chi-I-ΔCBD. The released 5 kD CBD could not be detected on SDS-PAGE.

Possibly, the cleavage of CBD from Chi-I plays a role in the adaptation of germinating macroconidia to Chi-I. Since both the 34 kD Chi-I and CBP20 are capable of decreasing sensitivity of *Fusarium solani* f.sp. *phaseoli* macroconidia to C/G and contain a CBD similar to Chi-I, also these proteins were incubated with the SN overnight at room temperature. On immunoblots incubated with antiserum recognizing either PR-P (in the case of the 34 kD Chi-I) or P2, the tomato homologue of CBP20 (in the case of CBP20) a very faint second band appeared below the predominant 34 kD Chi-I or CBP20 band, respectively (data not shown). As was the case with Chi-I also for 34 kD Chi-I and CBP20 no crossreaction of the lower band occurred with antiserum raised against hevein. After 48 hours of incubation with SN, ca. 50%

of the 34 kD Chi-I was processed. Chi-V, Glu-I and PR-P were not processed by the SN (data not shown). Incubation of CBP20 with SN for 48 hours never yielded more than 10-20% processed protein. This observation is in agreement with the results described above where adaptation to Chi-I was far more pronounced than adaptation to CBP20. Therefore, we focussed our research on adaptation to Chi-I. Since the 32 kD Chi-I is more abundant than the 34 kD isoform, the 32 kD Chi-I was used in the remainder of these studies.

N-terminal Sequencing of the Chi-I-ΔCBD

Western analysis had indicated that two bands originated from Chi-I: The upper band had a molecular weight of 32 kD, namely the mature Chi-I. At approximately 27 kD a lower band was observed which lacked the, N-terminal CBD. Both bands were cut out of the gel and sequenced.

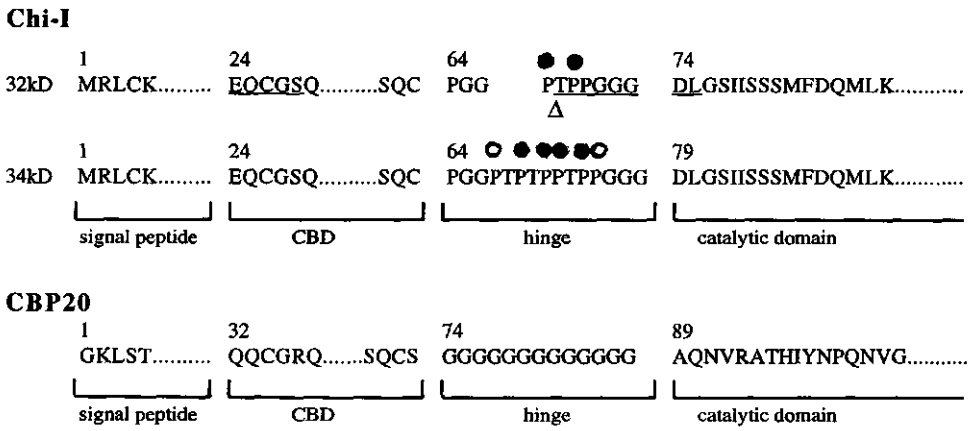


Figure 4. Alignment of N-terminal amino acid sequences of 32 kD and 34 kD Chi-I and CBP20. The sequenced parts of are underlined. Δ indicates the processing site of fungal proteases resulting in Chi-I-ΔCBD. Various domains are indicated. Prolyl hydroxylation is indicated: (●) indicates complete hydroxylation, (○) indicates variable hydroxylation.

Results are shown in Fig 4 where the 32 kD Chi-I, the 34 kD Chi-I and CBP20 are aligned. The first 5 amino acid residues of the N-terminus of the upper band of the 32 kD Chi-I were confirmed to represent the N-terminal region of the Chi-I protein, namely EQCGS. Subsequently, the N-terminus of the lower band, Chi-I-ΔCBD, was determined to be TPPGGGDL, indicating that the proteolytic removal of the CBD had taken place between Pro_{67} and Thr_{68} in the so-called hinge region of the 32 kD Chi-I. Since no other proteins could be

detected in the gel, it was concluded that processing was very specific. Since the hinge region of the 32 kD Chi-I and the hinge region of the 34 kD Chi-I contain the same processing site it can be assumed that removal of the CBD from the 34 kD Chi-I has occured at the same site. The hinge region of CBP20 lacks this processing site and it remains to be decided where processing has occured in this protein.

Protease Inhibitors and their Effect on Cleavage of Chi-I

As mentioned the CBD was removed from Chi-I after incubation with fungal proteases and therefore various protease inhibitors were tested to determine the nature of the responsible protease(s). They included PMSF, which is an inhibitor of serine proteases, leupeptin, an inhibitor of peptidases and EDTA, which inhibits metalloproteases (Scopes , 1987). Results of these experiments are shown in Fig 5. Chi-I was incubated with SN either in the presence (+) or absence (-) of the various inhibitors and western analysis was used to determine whether processing had occurred. In case the inhibitor was able to prevent processing of Chi-I, the lower band (Chi-I-ΔCBD) should not appear. EDTA was capable of inhibiting cleavage of Chi-I (lanes 5 and 6), whereas PMSF (lanes 3 and 4) and leupeptin (lanes 7 and 8) could not. These results suggest that (a) metalloprotease(s) was (were) likely responsible for processing of Chi-I.

Role of Processing of Chi-I in the Adaptation Process

Experiments were performed with EDTA to determine whether processing of Chi-I was essential in the phenomenon of adaptation of the germinating macroconidia to Chi-I. Macroconidia of *F. solani* f.sp. *phaseoli* were germinated in the presence of 5 mM EDTA and 1μg Chi-I. Controls included incubation with a) buffer, b) 5 mM EDTA or c) 1 μg Chi-I. Incubation was continued for 7 hours after which germlings were challenged with a concentration series of 0, 0.5, 1 or 2.5 μg C/G mixture. Percentage lysis of germtube tips and growth inhibition was monitored. EDTA alone, when added at this concentration, did not cause any (toxic) effect and the germlings showed the same level of sensitivity as the buffer controls. As expected, 1 μg Chi-I at 0 hrs p.i. caused the germlings to be insensitive to 2.5 μg C/G at 7 hrs p.i.. Also in case incubation was performed with 1 μg Chi-I and 5 mM EDTA, germlings were equally insensitive to 2.5 μg C/G mixture added at 7 hrs p.i. Western analysis showed that EDTA had indeed inhibited the proteolytic processing of Chi-I since no Chi-I-ΔCBD was detected.

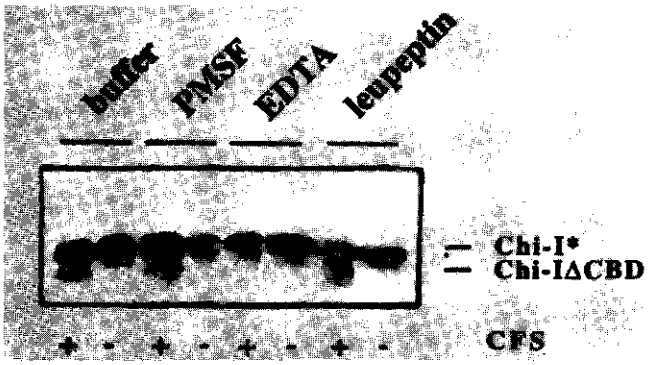


Figure 5. Processing of Chi-I by proteases from germinating macroconidia of *F. solani* f.sp. *phaseoli* (SN) can be inhibited. Results of western analysis are presented whereby antiserum raised against PR-P (1:5000). Lanes 1), 3), 5) and 7) contain Chi-I incubated with SN (+) and lanes 2), 4), 6) and 8) Chi-I incubated without SN (-). Lanes 1) and 2) are incubated with buffer (50 mM potassium phosphate buffer, pH 6); lanes 3) and 4) with 100 mg/mL PMSF in buffer; lanes 5) and 6) with 5 mM EDTA in buffer; lanes 7) and 8) with 40 μM leupeptin. Chi-I and the processed form, Chi-IΔCBD, are indicated.

Adaptation of *F. solani* f.sp. *phaseoli* Macroconidia to Chi-I-ΔCBD

The results described above seemed to indicate that the proteolytic removal of the CBD from Chi-I was not the underlying mechanism of adaptation of the macroconidia to Chi-I. Possibly some trace processing had occurred, which had lead to adaptation, however, which could not be detected on western blots. Final proof of the involvement of CBD in the adaptation process was obtained by experiments with purified Chi-I-ΔCBD. Chi-I-ΔCBD was produced by *in vitro* cleavage of Chi-I by SN. Any uncleaved Chi-I was removed using chitin affinity chromatography. Using Western analysis with Chi-I as a reference it was determined that after chitin affinity chromatography the amount of Chi-I in the mixture of Chi-I-ΔCBD and Chi-I had dropped from 50% to 0.6% (data not shown).

Firstly, germlings of *F. solani* f.sp. *phaseoli* were tested for their sensitivity to Chi-I-ΔCBD compared to mature Chi-I in mixing experiments with Glu-I. After 7 hours of pregermination of the macroconidia Chi-I-ΔCBD was added and one hour after addition, percentage lysis of hyphal tips was counted. Results are presented in Table 1. When 0.1 μg or higher of both Chi-I and Glu-I were added to the germlings almost 100% lysis of hyphal tips was observed. Similar results were observed when Chi-I-ΔCBD was used in stead of Chi-I. When 0.5 μg Chi-I was mixed with 5 ng Glu-I still over 70% lysis was observed. When the same amount of Chi-ΔCBD was mixed with 5 ng Glu-I almost no lysis was observed (2%).

Table 1. Antifungal activity of Chi-I-ΔCBD in the presence of Glu-I on germlings of *F. solani* f.sp. *phaseoli* is diminished compared to Chi-I. Average percentage lysis of germtube tips is listed as well as the standard deviation.

		Glu-I (ng/well)					
		0	5	10	50	100	500
Chi-I (ng/well)	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	5	0 ± 0	0 ± 0	2 ± 2	12 ± 9	21 ± 11	31 ± 13
	10	0 ± 0	0 ± 0	6 ± 4	19 ± 12	27 ± 19	46 ± 22
	50	0 ± 0	31 ± 15	46 ± 15	49 ± 8	68 ± 2	75 ± 6
	100	0 ± 0	45 ± 13	68 ± 19	89 ± 8	90 ± 7	95 ± 4
	500	0 ± 0	76 ± 3	91 ± 6	96 ± 3	96 ± 4	99 ± 1
Chi-IΔCBD (ng/well)	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	10	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 2	29 ± 15
	50	0 ± 0	1 ± 1	1 ± 2	13 ± 19	31 ± 16	72 ± 6
	100	0 ± 0	0 ± 0	5 ± 9	23 ± 22	42 ± 21	71 ± 19
	500	0 ± 0	1 ± 1	17 ± 16	51 ± 27	74 ± 19	94 ± 5

Similarly, if 0.5 μg Glu-I was mixed with 5 ng Chi-I, 30% lysis was observed. Again if the same amount of Glu-I was mixed with 5 ng Chi-I-ΔCBD no lysis was observed. These results indicate that at limiting amounts of Chi-I-ΔCBD the capacity of Chi-I-ΔCBD to lyse germtube tips was severely decreased, up to as much as 30-40 fold compared to Chi-I. Growth inhibition was scored after 2 days. For Chi-I, results are presented in Fig 2 of Chapter 6. Here results are presented for Chi-I-ΔCBD in Fig. 6. When 0.1 μg or higher of both Chi-I and Glu-I was added, 100% inhibition was observed. In case Chi-I-ΔCBD was substituted for Chi-I, a significant decrease in growth inhibition was observed.

In addition, the antifungal activity of Chi-I-ΔCBD compared to Chi-I on macroconidia was analysed. Therefore, macroconidia were germinated in a concentration range of 0 to 0.5 μg of either Chi-I-ΔCBD or Chi-I for 7 hours. No differences in percentage germination or speed of growth was observed. Also, no lysis of hyphal tips nor growth inhibition was observed in any of the treatments (data not shown).

Subsequently, it was analysed whether macroconidia could adapt to Chi-I-ΔCBD. Therefore the macroconidia were germinated in a concentration range of 0 to 0.5 μg of either Chi-I-ΔCBD or Chi-I and at 7 hrs p.i. 5 μg C/G mixture was added. Percentage lysis of germtube tips was scored at 8 hrs p.i. and growth inhibition after 2 days. Results are shown in Fig. 7 in the upper part for percentage lysis and in the lower part for growth inhibition. When no protein had been added to the macroconidia at 0 hrs p.i., ca. 90% of the germtube tips was lysed

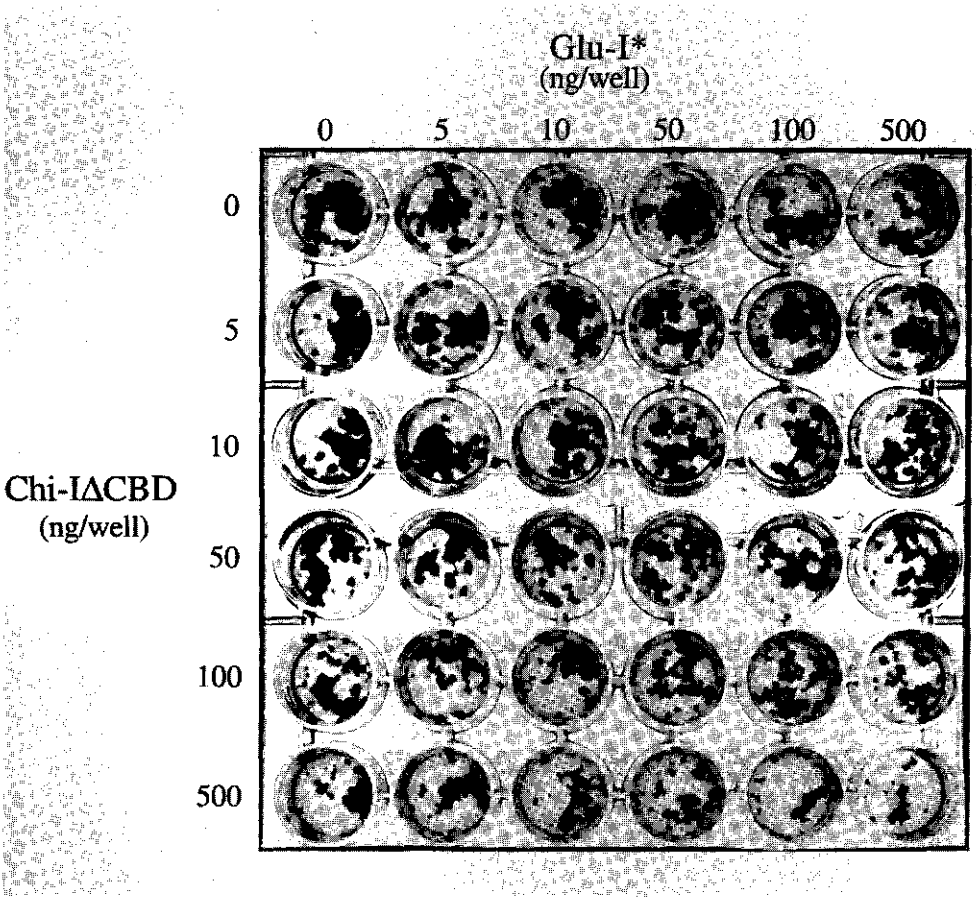

Glu-I*
(ng/well)
0
5
10
50
100
500
Chi-IΔCBD
(ng/well)
0
5
10
50
100
500

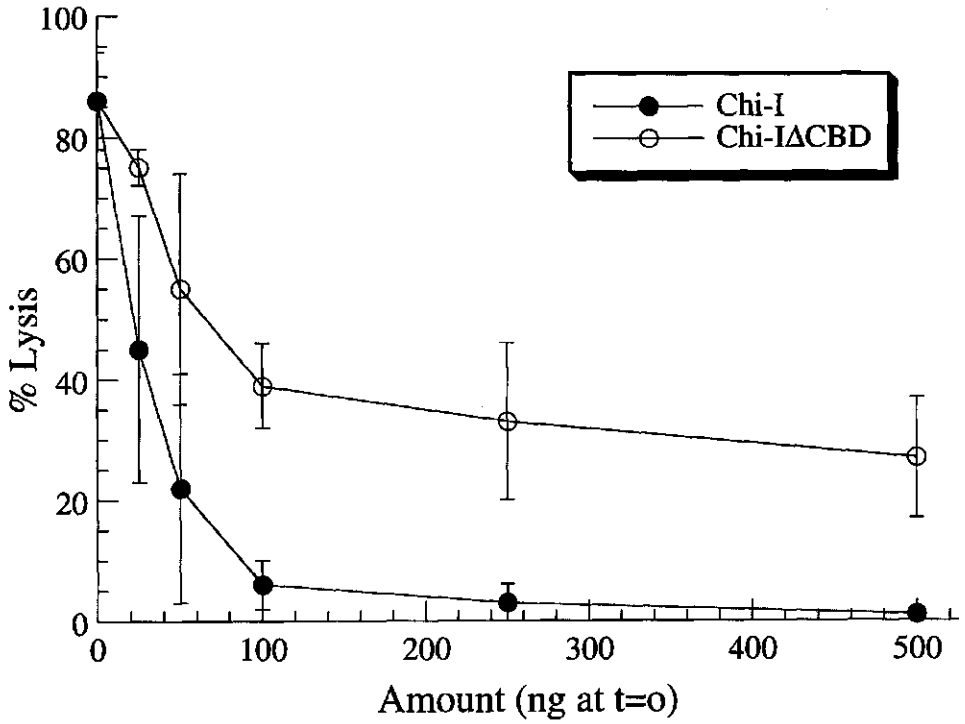
Figure 6. Antifungal activity of Chi-I-ΔCBD in the presence of Glu-I on germlings of *F. solani* f.sp. *phaseoli* is diminished compared to Chi-I. Effect on growth inhibition after 2 days is shown.

after addition of 5 µg C/G mixture. Similarly, inhibition of fungal growth after 2 days was over 90%. In case 25 ng Chi-I was added at 0 hrs p.i., only 45 % lysis was observed as well as intermediate growth inhibition after 2 days. When 100 ng Chi-I was added at 0 hrs p.i., no lysis and no growth inhibition was observed after the addition of 5 µg C/G mixture at 7 hrs p.i.. If 25 ng of Chi-I-ΔCBD was added, over 80% of the germtube tips were lysed and significant growth inhibition was observed. When 100 ng of Chi-I-ΔCBD was added at 0 hrs p.i., still 40% lysis of germtube tips was observed as well as severe growth inhibition. Even if 500 ng Chi-I-ΔCBD was added at 0 hrs p.i., still 30% lysis of the germtube tips and severe growth inhibition were observed, indicating that complete adaptation was not reached.

DISCUSSION

Class I chitinases (Chi-I) and class I ß-1,3-glucanases (Glu-I), purified from tobacco (*Nicotiana tabacum* cv. Samsun NN) exhibit synergistic antifungal activity *in vitro* on germlings of *Fusarium solani* f.sp. *phaseoli*, resulting in lysis of germtube tips and inhibition of mycelial growth (Sela-Buurlage *et al.*, 1993). By increasing the pregermination time of macroconidia of *F. solani* f.sp. *phaseoli* up to 7 hrs, the sensitivity of the emerging germtube to the hydrolases was shown to rise (Sela-Buurlage *et al.*, Chapter 7). At shorter pregermination times a higher concentration of hydrolases was needed to obtain maximum lysis of germlings. If the enzymes were added before the macroconidia had germinated, no lysis of germtube tips was observed. Germination percentage and speed of germtube growth was equal, irrespective of the presence of antifungal hydrolases. These earlier results indicated that the *F. solani* f.sp. *phaseoli* macroconidia adapt to Chi-I. Ludwig and Boller (1990) noted that pretreatment of combinations of pea chitinase and ß-1,3-glucanase caused only a transient reduction in growth of various fungi, including *Nectria haematococca* (*Fusarium solani*). They suggested that growth inhibition was due to tip lysis of the growing hyphae and that the fungus might adapt to the hydrolases and resume growth.

In the present study we demonstrate that not only Chi-I, but also another tobacco chitinase CBP20 (Ponstein *et al.*, 1994) induces adaptation of macroconidia of *F. solani* f.sp. *phaseoli*. To other tobacco chitinases such as the class II PR-3, PR-P (Sela-Buurlage *et al.*, 1993), and Chi-V (Melchers *et al.*, 1994) the macroconidia did not adapt. These results indicate



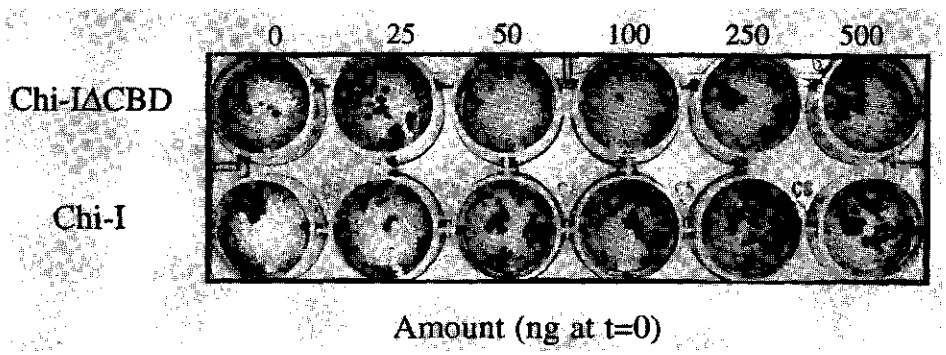


Figure 7. Chi-I (-•-) or Chi-IΔCBD (-o-) added to macroconidia of *F. solani* f.sp. influences lysis of germtube tips (upper part) and growth inhibition (lower part) after addition of 5 µg C/G mixture added at 7 hrs p.i. Error bars indicate standard deviation.

that the adaptation response is very specific for class I chitinases with a chitin binding domain, as is the case for Chi-I and CBP20.

In this study we wanted to study the possible underlying mechanisms for this phenomenon in more detail. We showed previously that after transferring C/G mixtures which had been incubated with germinating macroconidia to germlings, observed lysis of germtube tips was as unchanged indicating that antifungal activity of proteins was intact (Sela-Buurlage *et al.*, Chapter 7). We demonstrated earlier that the macroconidia lose the capability to adapt after 4-5 hours of pregermination (Sela-Buurlage *et al.*, Chapter 7). Therefore we studied the first 3 hours of germination in water to determine whether any proteolytic or inhibitory compounds were produced. After removal of the germinating macroconidia, the resulting supernatant (SN) was incubated overnight with Chi-I. It was demonstrated using western analysis that the mature Chi-I protein was processed and that specifically the N-terminal chitin-binding domain of 5 kD was removed, to yield a protein of 27 kD. N-terminal sequencing confirmed that the processed Chi-I protein of 27 kD indeed lacked the N-terminal chitin-binding domain. Processing by the fungal proteases occurs in the 32 kD Chi-I between Pro_{67} and Thr_{68}. Like the 32 kD isoform Chi-I, also the 34 kD isoform and CBP20 could be cleaved. Since similar motifs are present in the 34 kD Chi-I it can be anticipated that cleavage might occur at the same site. Apart from many glycine residues many prolyl residues are present in the hinge region. It has been shown by Sticher *et al.* (1993) that the prolyl residues which are located exclusively in this region can be hydroxylated. They observed that in the 32 kD isoform 2 prolyl residues, Pro_{67} and Pro_{69} are completely hydroxylated. In the 34 kD isoform 4 prolyl residues, Pro_{64}, Pro_{66}, Pro_{67} and Pro_{69} are completely hydroxylated and 2 prolyl residues Pro_{62} and Pro_{70} show variable hydroxylation. Possibly prolyl hydroxylation affects the processing sites, since extended incubation with excess SN never yielded more than 50% processed Chi-I. The hinge region of CBP20 consists of only glycine residues and thus lacks the processing site found in the Chi-I isoforms. It remains to be decided where processing of CBP20 has occurred. Denatured Chi-I, PR-P and Chi-V were also incubated with SN, but no additional bands were observed. By incubating Chi-I with SN in the presence of various protease inhibitors specific we tentatively identified the active component(s) in the SN as metalloproteases. Fric and Wolf (1994) demonstrated that from conidia of *Erysiphe graminis* high exoprotease activity could easily be released. They determined this activity to be located on the conidial surface and to be released into the surrounding medium in the course of germination. Endoprotease activity was also present but appeared membrane-bound. Concomitantly, many plants respond to pathogen attack with *de novo* synthesis of various (PR) proteins both locally at the point of attack, but also systemically in the upper parts of the plant. Some of these proteins have been determined to be protease inhibitors. (Balandin *et al.*, 1995; Ryan, 1990).

Although it was shown that proteins with a chitin binding domain such as hevein (Van

Parijs *et al.*, 1991), stinging nettle lectin (Broekaert *et al.*, 1989) and certain peptides from *Amaranthus caudatus* seed (Broekaert *et al.*, 1992) have antifungal activity, wheat germ agglutinin, which consist of four chitin binding domains, lacks antifungal activity (Raikhel and Wilkins, 1987; Chrispeels and Raikhel, 1991). Also Iseli *et al.* (1993) claimed that the chitin binding domain of Chi-I did affect binding to chitin, but did not affect antifungal nor catalytic activity. Here however, we demonstrate that although like Iseli *et al.* (1993) the Chi-I-ΔCBD protein has the same catalytic activity, antifungal activity was decreased 30-40 fold compared to the intact Chi-I. This seems to indicate that the chitin binding domain plays a significant role in the antifungal activity of Chi-I (and possibly also CBP20). At first sight these results seem contradicting as the C/G mixture does not loose antifungal activity during 7 hrs of germination as described in Chapter 7. However, Glu-I present in this mixture might compensate for the decrease in antifungal activity of Chi-I-ΔCBD on germlings.

Here we propose that the chitin binding domain also plays a role in the adaptation response. Macroconidia were germinated in the presence of Chi-I-ΔCBD and after 7 hours, they were challenged with a normally lethal dose of the C/G mixture. The adaptation of macroconidia to this protein lacking the CBD was severely diminished and complete insensitivity, as could be induced by Chi-I, was not observed. Clearly the cleavage of the chitin binding domain from Chi-I plays a major role in adaptation of *F. solani* f.sp. *phaseoli* macroconidia to Chi-I.

At this point it can not be excluded that additional factors contribute to this phenomenon. The fact that Chi-V which lacks a CBD can cause limited adaptation supports this, indicating that the chitinolytic domain might also play a role. Possibly the presence of hydrolytic enzymes is sensed and cell wall synthesis is subsequently altered leading to a decreased sensitivity. Another option might be the production of inhibitor proteins rendering the chitinolytic enzymes ineffective (Albersheim, 1974; Kuc 1963). As the enzymes used in this study were chitinases it is possible that small chitin fragments are released from the macroconidial wall or the germtube during chitinolytic action. It can be foreseen that such chitin fragments released by Chi-I in the early hours of germination can act as inhibitors. However, our earlier studies demonstrated (Sela-Buurlage *et al.*, Chapter 7) that a C/G mixture had retained its antifungal activity after incubation on germinating macroconidia for 7 hours. Alternatively, such chitin oligomers might interact with the germinating macroconidia and activate the adaptation response. Lipo-chitin oligosaccharides from various rhizobial bacteria act as signal molecules in the development of root nodules (Spaink and Lugtenberg, 1994). Similarly, it is known that chitin fragments of only specific size, that is tetramers and pentamers, can elicit specific alkalinization responses in tomato cell cultures (Felix *et al.*, 1993). These tetramers and pentamers were tested on *F. solani* f. sp. *phaseoli* macroconidia up to 100.000 fold the amount effective on the tomato cell suspensions, but no adaptation could be induced (data not shown). At this point it cannot be excluded that additional mechanisms for adaptation exist. For example, tomato cell suspensions

responded with rapid alkalinization of their growth medium to spore exudates of *Cladosporium fulvum*. It was found that not chitin fragments but ergosterol, the main sterol of most higher fungi, was responsible for the observed response (Granado *et al.*, 1995). Maybe also for *F. solani* f. sp. *phaseoli* studied here, maybe the chitinases capable of inducing adaptation can release components other than chitin from the macroconidial wall.

All the data in the present study have been obtained with an *in vitro* system. Additional studies need to be performed to prove that the same phenomena hold for the *in planta* situation. However, some speculations can be made as to how these observations relate to the *in vivo* situation. In the strategy to engineer fungal resistance by creating transgenic plants, which constitutively express these two hydrolytic enzymes, Chi-I and Glu-I, it might be concluded that the fungus might easily overcome the action of the introduced proteins. However, the macroconidia initially interact with the host surface and have to germinate and penetrate the host tissue before they become pathogenic. In transgenic plants where these vacuolar proteins are constitutively expressed the fungus does not encounter the proteins until penetration of the host cell is accomplished. Indeed tomato plants constitutively expressing both the Chi-I and the Glu-I proteins in the vacuole show enhanced resistance to *Fusarium oxysporum* (Jongedijk *et al.*, 1995; Van den Elzen, 1994). Moreover, field trials involving transgenic carrot overexpressing the same proteins proved that these plants contain enhanced levels of resistance to various pathogens (Melchers *et al.*, 1995). Also Zhu and coworkers (1994) demonstrated elevated levels of resistance to *Cercospora nicotianae* in transgenic tobacco plants expressing both a rice basic chitinase and an alfalfa acidic glucanase. By engineering resistant transgenic plants expressing hydrolases, the use of tissue specific or inducible promoters might be an option to circumvent the fungus from adaptation. Therefore, the strategy of enhancing fungal resistance in plants by transformation with genes encoding antifungal proteins still remains very potent.

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Chapter 9

Summary and Concluding Remarks

SUMMARY AND CONCLUDING REMARKS

As described in Chapter 1, many mechanisms exists in host plants to resist pathogen attack. In various laboratories attempts are made to use some of the host's natural defense mechanisms in genetic engineering to produce transgenic plants with enhanced levels of resistance. At MOGEN one of the strategies employed involves constitutive overexpression in transgenic plants of one or more proteins with antifungal activity. Obviously the identification of antifungal proteins is a prerequisite. An *in vitro* assay was set up to monitor protein extract(s) for the presence of antifungal protein(s). Many fungi could be assayed in such a way for their sensitivity to a series of protein extracts.

It is known in tobacco that resistance against pathogen attack is induced, the so-called SAR (see Chapter 1). Amongst many responses observed during SAR, is the *de novo* synthesis of pathogenesis-related, PR-proteins. The nature of many of these PR-proteins is presently unknown, but for some of these proteins enzymatic activity has been determined: chitinases and glucanases (Legrand *et al.*, 1987; Kauffmann *et al.*, 1987). Their substrates chitin and glucan are the major components of the fungal cell wall (Wessels and Sietsma, 1981). Mauch and coworkers (1988) showed that crude protein extracts of bean pods infected with *Fusarium solani* f.sp. *phaseoli* and chitinases and glucanases purified from such extracts were antifungal towards a number of fungi. The system we focused on as a source for antifungal proteins were leaves of tobacco (*Nicotiana tabacum* cv. Samsun NN) inoculated with tobacco mosaic virus (TMV). Chapter 2 offers an overview of the *in vitro* antifungal activity of induced protein extracts from tobacco leaves, inoculated with TMV. The extract was prepared in such a way that the presence of PR-proteins was favored. From such a crude extract, proteins active against a given fungus could be purified. Calibration of the protein extracts for the known PR-proteins was performed. Over fifty percent of the proteins present in the crude extract consists of PR-proteins. The protein extracts prepared from tobacco leaves inoculated with TMV were tested *in vitro* on thirty fungi. Over 80% of the fungi was sensitive to this tobacco extract, the remaining 20% was not. Many explanations for the observed insensitivity might exist. It could be that no antifungal proteins to a given fungus are present or that the relative amounts of the active protein(s) are too low. Another possibility might be production or release of specific inhibitors or proteases by the insensitive fungi. A difference in cell wall structure or growth dynamics might diminish sensitivity to a protein extract. Screening fungi, in the case of the sporulating ones, at more than one phase of their life cycle, gave further detail on growth phase dependent antifungal activity of certain protein(s). Most strikingly, pregerminated spores of almost all fungi were far more sensitive to the protein extract than non-pregerminated spores.

In this thesis some examples are given of antifungal proteins that have been identified in our lab, focusing on chitinases and ß-1,3-glucanases. Chapter 4 the describes the purification

and identification of some tobacco chitinases and ß-1,3-glucanases and their antifungal activity *in vitro*. *Fusarium solani* f.sp. *phaseoli* was used to monitor the antifungal effects of purified proteins. It was demonstrated that only the class I, vacuolar isoforms of the PR-2 and PR-3 proteins exhibited antifungal activity. Their extracellular counterparts possessed little or no activity. Also it was shown that the Chi-I and Glu-I proteins acted extremely synergistically. In chapter 3 is described how corresponding genes, and the gene encoding another antifungal protein AP24 (Woloshuk *et al.*, 1991), were identified. Through introduction of an additional stop codon, the vacuolar targeting signals were removed, resulting in the targeting of mature Chi-I*, Glu-I* and AP24* to the extracellular space. As described in chapter 6, by purification of the Chi-I* and Glu-I* from apoplastic fluids from transgenic plants expressing single genes, it could be shown that the individual proteins when applied to *Fusarium solani* exhibited little or no antifungal effect. Rather, antifungal activity of Chi-I and Glu-I solely depended on the synergy of these proteins. When either of the hydrolases was amended with as little as one percent of the other, severe lysis of germtube tips and growth inhibition of the fungus was observed. This indicates that biochemical techniques, required for the purification of chitinases and ß-1,3-glucanases from plants, are not sufficient to ensure 100% purity. Overexpression of proteins in transgenic plants yields improved purification methods. It is only through the use of such genetically engineered plants that proof could be obtained on the antifungal nature of Chi-I and Glu-I proteins.

Another example of how the *in vitro* assaying system can aid in identification of antifungal proteins is shown in chapter 5 where CBP20, the class I PR4-protein is isolated. It contains a chitin-binding domain (CBD) like the class I PR-3 protein, Chi-I, and also exhibited chitinase activity. It was shown that CBP20 possessed antifungal activity towards *Fusarium solani* f.sp. *phaseoli* and acted synergistically with Glu-I. It is assumed that the CBD of CBP20 is involved in antifungal activity by allowing the enzyme to adhere to its substrate. Since both CBP20 and Chi-I have a CBD and are chitinases and presumably attack the same target substrate in the hyphal cell wall, the observed synergistic antifungal activity of these two enzymes was unexpected. This underlines the oversimplification by grouping enzymes on the basis of (one of) their function(s).

Fusarium solani f.sp. *phaseoli* was chosen as a model fungus to study in more detail the underlying mechanism for the decrease in sensitivity of macroconidia compared to germlings to a total protein extract observed in chapter 2. The same decrease in sensitivity was observed in case mixtures of Chi-I and Glu-I were tested. By 7 hrs of germination, germlings of this fungus are fully sensitive to a Chi-I/Glu-I (C/G) mixture. In chapter 7 results are presented where macroconidia of Fusarium *solani* f.sp. *phaseoli* are incubated with a C/G mixture. Germtubes emerging from these treated macroconidia have become insensitive, adapted, to normally lethal amounts of the same C/G mixture added after 7 hrs. It was found that the level of adaptation

correlates with the amount of C/G mixture added to the macroconidia. Also, macroconidia could only adapt if treated in the first 3 to 4 hrs of germination. It was shown that the C/G mixture remained antifungal during the germination time of 7 hrs thus excluding the possibility of degradation of inactivation by the germinating macroconidia.

Finally, in chapter 8, various chitinases were assayed for their ability to induce the adaptation mechanism of *Fusarium solani* f.sp. *phaseoli*. Clearly, Chi-I, even without Glu-I, was the most potent protein in induction of adaptation. CBP20 could act as an inducer although less effective than Chi-I. Another chitinase Chi-V, with antifungal activity in synergy with Glu-I towards amongst others *Fusarium solani* f.sp. *phaseoli* (Melchers *et al.*, 1994) was even less capable of inducing adaptation. Finally, PR-P could not induce adaptation.

Since Chi-I was most effective in induction of adaptation, studies were continued with this hydrolase. From 4-hour old *Fusarium solani* f.sp. *phaseoli* germlings, a metalloprotease containing fraction could be isolated which could specifically process Chi-I. It was determined that the CBD was removed from the Chi-I protein, resulting in a processed form, Chi-I-ΔCBD. The role of the CBD in antifungal activity of a Chi-I was further investigated. Its antifungal activity on germlings was assessed. It was shown that this mutant protein when applied to *Fusarium solani* germlings was 30-40 fold less antifungal than Chi-I when mixed with Glu-I. These results indicate that the CBD does in fact boost the antifungal activity of Chi-I on germlings.

In our studies it could be shown that the protease fraction could also process the 34 kD Chi-I. Since the same processing site as in the 32 kD isoform is present in the hinge domain of this protein, this was anticipated. Apart from many glycine residues many prolyl residues are present in the hinge region. It has been shown previously that the prolyl residues which are located exclusively in this region can be hydroxylated (Sticher *et al.*, 1993). Possibly prolyl hydroxylation affects the processing sites, since extended incubation with excess SN never yielded more than around 50% processing of the Chi-I. Processing of CBP20 by the protease fraction was also observed but far less efficiently than 34 kD Chi-I. The hinge region of CBP20 is very different to the one present in both Chi-I isoforms, since it consists of only glycine residues and thus lacks the processing site. It remains to be decided where processing of CBP20 has occurred. Possibly, the fact that removal of CBD from CBP20 occurs less efficiently might explain the observed synergistic antifungal activity between Chi-I and CBP20 on germlings.

The ability of CBD in inducing the adaptation response of macroconidia was studied. The presence of mature Chi-I with its CBD caused macroconidia to adapt to a higher extent compared to the processed form, Chi-I-ΔCBD. We hypothesize that CBD proteolytically released from Chi-I, adheres to the emerging germtube tip and protects it from subsequent chitinolytic attack since other Chi-I molecules can bind less efficiently. Possibly, when pregermination is allowed to continue beyond 3 to 4 hours, either the proteases have been

diluted or degraded or the growth of the germtube progresses so rapidly that protection does not occur efficiently. However this hypothesis does not explain the adaptation induced by Chi-V, which lacks a CBD. Presumably, also the chitinase activity itself plays a role. This would also explain the fact that Chi-I-ΔCBD can still induce adaptation comparable to Chi-V. Possibly, chitin fragments released from the germinating macroconidia act as inhibitors of Chi-I or as elicitors in a signalling pathway. Chitin oligomers of specific size, capable of eliciting responses in cell suspensions of tomato (Felix *et al.*, 1993) could however not induce the adaptation response (our unpublished data). It must be stated that these chitin fragments were obtained from crab shell and possibly differ in size and/or nature than those possible released from the macroconidia.

At first sight the results, which showed that Chi-I-ΔCBD was less antifungal on germlings, seem to contradict the fact that during 7 hrs of germination the C/G mixture does not lose antifungal activity as described in chapter 7. However, the fact that Glu-I, and thus the synergy, is present in this mixture probably masks the fact Chi-I-ΔCBD is less antifungal on germlings.

In this thesis it is shown that the *in vitro* assay allows for the identification of potent antifungal proteins and it is also a useful tool to study the effect of a fungus on the antifungal proteins. Once genes corresponding to such antifungal proteins have been identified, transgenic plants can be created. Reisolation of the proteins from transgenic plants and *in vitro* assays are necessary to determine whether the antifungal activity is identical to the initially observed effect. Also optimal combinations of antifungal proteins can be determined in case a multigene strategy is employed, involving the simultaneous overexpression of two or more antifungal proteins. It has been shown, that the simultaneous expression of a chitinase and a ß-1,3-glucanase in transgenic tobacco, tomato and carrot can enhance resistance to a number of pathogens (Zhu *et al.*, 1994; Van den Elzen *et al.*, 1994; our unpublished results).

If it appears that removal of the CBD also occurs *in vivo* it might be an option to use tissue specific or pathogen-inducible promoters. Thus, constitutive expression is prevented and pathogens will have less chance to adapt to the chitinases. Adequate measures in the engineering of transgenic plants to circumvent the options a pathogen might have to overcome the introduced transgenes will become available, making this route of introducing fungal resistance in agricultural crops a very promising one.

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SAMENVATTING

Vele landbouwgewassen lopen grote schade op door schimmelinfekties. Veredeling van planten met behulp van transgene strategieën is een veelbelovende methode voor introduktie van schimmelresistentie. Planten bezitten vele mechanismen om zich te verdedigen tegen pathogenen. Een aantal van dergelijke verdedigingsmechanismen kan geëxploiteerd worden bij het maken van transgene planten met verhoogde resistentie tegen pathogenen. In hoofdstuk 1 is een overzicht gegeven van de diverse strategieën die gevolgd (kunnen) worden. Een van die strategieën behelst de constitutieve expressie van een of meerdere eiwitten met antischimmel aktiviteit. Een eerste vereiste voor de toepassing van een dergelijke strategie is de identifikatie van antischimmel eiwitten. Van tabak is bekend dat systemische resistentie tegen pathogenen geïnduceerd wordt na inoculatie met tabaksmozaïekvirus (TMV). In dit proefschrift is TMV-geïnduceerde tabak gebruikt als bron voor isolatie en karakterisering van antischimmel eiwitten. Eén van de vele reakties die tijdens systemische resistentie in deze planten wordt waargenomen, is *de novo* synthese van pathogeen-gerelateerde, PR-eiwitten. Een aantal van deze PR-eiwitten zijn chitinases en ß-1,3-glucanases. Hun substraten, chitine en ß-1,3-glucaan, zijn belangrijke bestanddelen van de celwand van vele schimmels. Een extrakt van TMV-geïnduceerde tabak werd verrijkt voor PR-eiwitten (hoofdstuk 2). Meer dan vijftig procent van de eiwitten in een dergelijk extrakt bleken bekende PR-eiwitten te zijn. Een *in vitro* toets werd gebruikt om dertig schimmels te testen op hun gevoeligheid voor het tabaksextrakt. Het extrakt werd toegediend aan zowel ongekiemde als gekiemde sporen. Meer dan tachtig procent van de schimmels bleek gevoelig voor het extrakt. Sporen van nagenoeg alle schimmels waren na kieming veel gevoeliger voor het extrakt dan ongekiemde sporen.

In hoofdstuk 4 wordt de zuivering van enkele chitinases en ß-1,3-glucanases uit tabak en hun antischimmel aktiviteit *in vitro* beschreven. *Fusarium solani* f.sp. *phaseoli* werd gebruikt als modelschimmel. Alleen de vacuolaire, basische, isovormen van de geteste chitinases (Chi-I) en ß-1,3-glucanases (Glu-I) bleken antischimmel aktiviteit te bezitten. Lysis van kiembuizen alsmede groeiremming van het mycelium werd waargenomen. De intercellulaire, zure, homologen bleken nauwelijks of geen antischimmel aktiviteit te bezitten. Tevens werd gevonden dat Chi-I en Glu-I synergistisch werkten. De genen die coderen voor Chi-I en Glu-I alsmede voor een ander antischimmel eiwit, AP24, werden gekloneerd (hoofdstuk 3). Door modificaties aan te brengen in deze genen werden de transgene eiwitten, Chi-I*, Glu-I* en AP24*, naar de intercellulaire ruimte getransporteerd. Zuivering van Chi-I* of Glu-I* uit de intercellulaire wasvloeistof van transgene planten was eenvoudig daar behalve het transgene eiwit nauwelijks andere basische eiwitten aanwezig bleken te zijn. Eventueel endogeen geïnduceerde Chi-I of Glu-I eiwitten waren alleen vacuolair aanwezig en werden niet teruggevonden in de intercellulaire wasvloeistof. De transgene eiwitten werden gezuiverd om te bewijzen dat

transport naar de intercellulaire ruimte de aktiviteit van Chi-I* en Glu-I* niet beïnvloed had. Deze gezuiverde eiwitten bezaten echter in vergelijking tot Chi-I en Glu-I, die uit TMV-geïnduceerde tabak geïsoleerd waren, nauwelijks of geen antischimmel aktiviteit. Uit nader onderzoek bleek dat de voorheen geteste Chi-I en Glu-I nooit compleet zuiver waren geweest en dat de waargenomen antischimmel aktiviteit toegeschreven kon worden aan de synergistische werking tussen Chi-I en Glu-I. Door aan een van de hydrolases (Chi-I* of Glu-I*) slechts één procent van de andere hydrolases (Glu-I* of Chi-I*) toe te voegen, werd wel een hoog percentage lysis van kiembuizen alsmede groeiremming van het mycelium waargenomen.

Naast Chi-I is nog een tweede chitinase met een chitine bindend domein (CBD) geisoleerd uit tabak, genaamd CBP20 (hoofdstuk 5). Er is aangetoond dat CBP20 antischimmel aktiviteit bezit en dat het synergistisch werkt met zowel Glu-I als Chi-I.

In hoofdstuk 2 was veelvuldig waargenomen dat tabaksextrakten minder remmend werkten op ongekiemde sporen dan op gekiemde sporen. Deze verminderde gevoeligheid van ongekiemde sporen werd ook waargenomen wanneer Chi-I en Glu-I mengsels werden gebruikt. Na zeven uur vóórkieming waren kiembuizen volledig gevoelig voor mengsels van Chi-I met één procent (w/w) Glu-I (C/G). In hoofdstuk 7 zijn experimenten beschreven waarbij macroconidia van *Fusarium solani* f.sp. *phaseoli* werden geincubeerd met C/G mengsels. Op deze manier gekiemde macroconidia bleken ongevoelig voor C/G mengsels die normaliter antischimmel aktiviteit bezaten. Deze ongevoeligheid berust waarschijnlijk op adaptatie. Het nivo van adaptatie bleek gecorreleerd aan de hoeveelheid C/G mengsel dat aan de macroconidia werd toegediend. Adaptatie vond alleen plaats gedurende de eerste drie tot vier uur van incubatie.

In hoofdstuk 8 zijn verschillende chitinases getest op hun vermogen om adaptatie te induceren bij *Fusarium solani* f.sp. *phaseoli.* Chi-I bleek, zelfs in afwezigheid van Glu-I, de beste inducer van adaptatie te zijn. CBP20 bleek ook adaptatie te induceren, maar veel minder efficient dan Chi-I. Een ander tabakschitinase met antischimmel aktiviteit, Chi-V, was ook in staat te induceren, echter in veel geringere mate dan CBP20. Een intercellulair chitinase, PR-P, bleek niet in staat adaptatie te induceren. Aangezien Chi-I de beste inducer was, werd dit hydrolase gebruikt in de verdere studies. Incubatiemedium van gekiemde macroconidia bleek een metalloprotease te bevatten dat het Chi-I eiwit kon afbreken tot een kleiner eiwit. Het CBD bleek te worden afgesplitst, waardoor Chi-I-ΔCBD ontstond. Ook uit de 34 kD isovorm van Chi-I en uit CBP20 kon door het metalloprotease het CBD afgesplitst worden.

De rol van het CBD voor antischimmel aktiviteit van Chi-I is verder bestudeerd. De antischimmel aktiviteit van Chi-I-ΔCBD op gekiemde macroconidia en het vermogen van Chi-I-ΔCBD om in macroconidia adaptatie te induceren werd bepaald. Op gekiemde macroconidia bleek Chi-I-ΔCBD dertig tot veertig keer minder synergistisch met Glu-I te werken dan het intacte Chi-I. Uit deze resultaten blijkt dat het CBD de antischimmel aktiviteit van Chi-I duidelijk verhoogt. Vervolgens werd aangetoond dat Chi-I met een CBD adaptatie beter induceert dan Chi-I-ΔCBD. Een mogelijke verklaring zou kunnen zijn dat het afgesplitste CBD door binding

aan chitine de antischimmel aktiviteit van Chi-I competitief remt. Deze verklaring is echter niet toereikend om het fenomeen adaptatie volledig te verklaren daar een zekere mate van adaptatie geïnduceerd wordt door Chi-I-ΔCBD en ook door Chi-V, die beiden geen CBD bevatten. Mogelijk speelt bij adaptatie toch ook het chitinase domein een rol.

Het is nog niet duidelijk of de waargenomen adaptatie van *Fusarium solani* f.sp. *phaseoli* ook bij andere schimmels optreedt. Indien de adaptatie die *in vitro* wordt waargenomen ook *in vivo* plaatsvindt, zou dit een bedreiging kunnen zijn voor de introductie van resistentie in transgene planten door overexpressie van Chi-I. Adaptatie blijkt echter alleen in de eerste uren van kieming te kunnen optreden wanneer de meeste schimmels zich nog niet in de plant bevinden. Mocht het boven beschreven fenomeen van afbraak van Chi-I *in vivo* toch plaatsvinden, dan kunnen weefsel-specifieke of pathogeen-induceerbare promoters gebruikt worden, waardoor pathogenen minder kans hebben om te adapteren. Mits toereikende maatregelen worden genomen die de eventuele induktie van adaptatie voorkomen, zal het gebuik van transgene planten met een verhoogd nivo van antischimmel eiwitten een belangrijke en duurzame bijdrage kunnen leveren aan de molekulaire resistentieveredeling.

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NAWOORD

Dan eindelijk het proefschrift af! Eigenlijk ben ik al jaren aan het promoveren zonder dat ik me er bewust van was. Na een valse start als Ph. D. student in Amerika aan 'The University of Californis at Berkeley' m*et als* grootste tegenvaller dat de ingenieurstitel aan die universiteit ni*et als* M.Sc werd gezien was ik zeer blij met een 'analisten' baan bij MOGEN. Onder de bezielende leiding van Charles en Ben werd ik gauw wegwijs gemaakt in het lopende onderzoek. Met het vertrek van Charles nam ik mijn kans waar en ging op zijn stoel zitten als *in vitro* specialist m*et als* enige taak het identificeren van antischimmel eiwitten. No problem, you'd think. Langzaamaan begon het echter wel te dagen dat als ik ooit hoger opwilde en een spannendere carriere wou opbouwen de doctorstitel essentieel bleek. Dit niet in de laatste plaats mat het oog op een baan in het buitenland. Ongeveer omstreeks dezelfde tijd vertrok Ben naar de Universiteit en was het besluit daar. Of mee naar Amsterdam of promoveren bij MOGEN. Gesteund door mede-collega's in de vorm van Andre en Leo heb ik voor het laatste gekozen. Als promotor werd Professor de Wit bereid gevonden en op dat moment was ik met een promotieonderzoek bezig. Maar wanneer was ik nou eigenlijk begonnen? Eigenlijk min of meer op de dag dat ik mijn voet binnen de deur zette.

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The outside cover is the work of my friend, the graphic designer Vardit Daphne who volunteered to do this work (she might not have if she had known beforehand how much work it was?).

Another international name that must be acknowledged here is Professor Thomas Boller who is responsible for the original ideas behind much of the chitinase and glucanase knowledge available today and who willingly participated in some of this work also.

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CURRICULUM VITAE

Marianne Beatrix Sela-Buurlage werd op 7 maart 1962 geboren te Amersfoort. In 1980 behaalde zij het diploma gymnasium ß aan het Johan van Oldenbarnevelt gymnasium te Amersfoort. Vervolgens ging zij studeren aan de Landbouwuniversiteit te Wageningen, waar zij in 1987 het ingenieursexamen Plantenveredeling behaalde, met als hoofdvakken Plantenveredeling en Fytopathologie en als bijvak Plantenfysiologie. Na het examen vertrok zij naar de Verenigde Staten en is werkzaam geweest op de Department of Plant Pathology van de University of California in eerst Berkeley en vervolgens Riverside. Sinds Februari 1990 werkt ze bij het plantenbiotechnologie bedrijf MOGEN te Leiden, waar zij het onderzoek heeft verricht wat uiteindelijk resulteerde in dit proefschrift met Prof P.J.G.M de Wit als promotor en Dr. L.S. Melchers als copromotor. In Mei 1990 trouwde zij met Uri, in Februari 1992 werd Lior geboren en in Juni 1996 Yarden. Per November begint zij een als post-doc bij Prof. R. Fluhr aan het Weizmann Institute in Rehovot, Israel.