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UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

A STUDY OF THE INTERACTION BETWEEN ALFALFA (*MEDICAGO SATIVA*) AND ONE OF ITS FUNGAL PATHOGENS, *COLLETOTRICHUM TRIFOLII*

A dissertation

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

By

ISABELLE IRÈNE SALLES

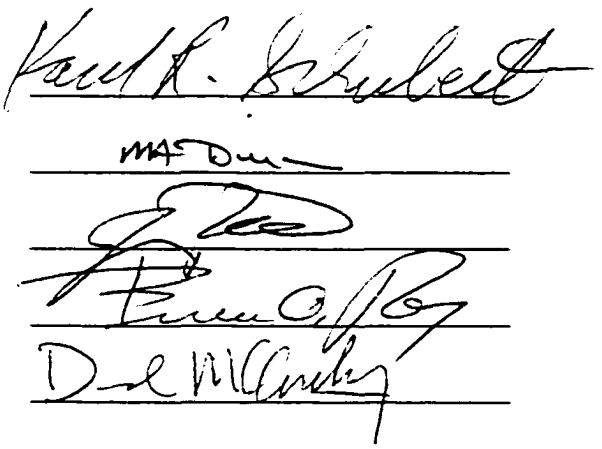
Norman, Oklahoma

2001

A STUDY OF THE INTERACTION BETWEEN ALFALFA (*MEDICAGO SATIVA)* AND ONE OF ITS FUNGAL PATHOGEN, *COLLETOTRICHUM TRIFOLII*

A dissertation APPROVED FOR THE
DEPARTEMENT OF MICROBIOLOGY

BY



This dissertation is dedicated to my parents.

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To the memory of Jean and Rémy Salles who left us too early.

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LIST OF ABBREVIATIONS

A287: absorbance at 287 nm
ATP: adenosine triphosphate
BSA: bovine serum albumin
C4H: cinnamic acid 4-hydroxylase
CaMV: cauliflower mosaic virus
CATB: hexadecyl trimethylammonium bromide
cDNA: complementary DNA
CHI: chalcone isomerase
CHR: chalcone reductase
CHS: chalcone synthase
CTP: cytosine triphosphate
Da: dalton
DEPC: diethylpyrocarbonate
DNase: deoxyribonuclease
DTT: dithiothreitol
EDTA: ethylenediamine tetra-acetic acid
EtBr: ethidium bromide
FW: fresh weight
HPLC: high-performance liquid chromatography
IEF: isoelectric focusing electrophoresis
IFR: isoflavone reductase
IFS: isoflavone synthase
IOMT: isoflavone-*O*-methyltransferase
KDa: kilodalton
MOPS: morpholinopropane sulfonic acid
mRNA: messenger RNA
MW: molecular weight
OD: optical density
PAGE: polyacrylamide gel electrophoresis
PAL: phenylalanine ammonia-lyase
PCR: polymerase chain reaction
PDA: potato dextrose agar
pI: isoelectric point
RNase: ribonuclease
rRNA: ribosomal RNA
SDS: sodium dodecyl sulfate
SSC: saline sodium citrate
TAE: Tris-acetate-EDTA electrophoresis buffer
TE: tris EDTA
TEMED: N,N,N',N'-tetramethylethylenediamine
UV: ultraviolet

ABSTRACT

Plant pathogens such as *Colletotrichum trifolii* have devastating effects on crop species. A rapid and effective activation of plant defenses is necessary for the establishment of resistance to the pathogen. The induction of several defense responses in alfalfa (*Medicago sativa*) after infection by a virulent and avirulent race of *C. trifolii* was examined. A phytoalexin response was differentially induced in resistant and susceptible leaves. Free medicarpin and conjugates of medicarpin (glycosylated medicarpin) and formononetin (formononetin-7-*O*-glucoside) started to accumulate 24 and 48 hours after inoculation in resistant leaves, respectively, as opposed to 48 and 72 hours after inoculation in susceptible leaves. A more rapid induction of the phytoalexin response in resistant leaves compared to susceptible leaves was further demonstrated by the earlier accumulation of transcripts of genes involved in the biosynthesis of medicarpin, including *PAL*, *CHR* and *IFR*, 4 hours after inoculation.

The alfalfa/*C. trifolii* interaction follows a gene-for-gene interaction for race-cultivar specificity where in general, products of host resistance genes providing resistance to the fungus are interacting with direct or indirect products of avirulence genes (specific-elicitors) from the pathogen.

The differential response for phytoalexin accumulation in alfalfa after *C. trifolii* infection, along with the nature of the interaction suggests the involvement of race-specific elicitors; however it was not possible to demonstrate their existence.

Glucanases are lytic enzymes that hydrolyse β-1,3-glucans present in high amounts in fungal cell walls, and are produced by the plant after pathogen attack. Glucanases can directly affect fungi by digesting their cell walls, or indirectly by releasing elicitors that will activate plant defenses. The second potential role was investigated by constitutively expressing the *aglu1* cDNA that encodes an alfalfa glucanase normally present in alfalfa leaves only after fungal infection, and study the effect of *aglu1* expression on the phytoalexin response during infection by *C. trifolii.* The constitutive expression of the *aglu1* transgene did not increase the phytoalexin response, therefore suggesting that Aglu1 glucanase may not be involved in releasing elicitors from the fungal cell walls, at least for the fungus used in this study, *C. trifolii.*

Genetic engineering has proven to be a powerful tool in plant disease control. Transgenic alfalfa plants transformed previously with a glucanase and/or a rice chitinase under the constitutive control of the CaMV 35S promoter were analyzed for their level of expression of glucanase and

chitinase activities. No difference in chitinase activity could be detected. Differences in glucanase activity could be detected in plants transformed with the glucanase cDNA although the transgene was no longer constitutively expressed, suggesting that some other mechanisms increasing the glucanase activity level were occurring along with silencing of the transgene. Trangenic plants re-transformed with the *aglu1* cDNA were evaluated for resistance against *C. trifolii.* Overexpressing the *aglu1* transgene in alfalfa did not reduce the symptoms following pathogen attack. However, transgenic plants expressing the transgene produced other glucanase isoforms that may play a role in plant defense perhaps against other fungi. This needs to be further investigated.

CHAPTER 1

INTRODUCTION

1.1. Overview

Plants are equipped with constitutive and inducible defensive systems to kill pathogens or to limit their growth within the plant. As a result, most plants are resistant to most potential pathogens. Within potential susceptible plant species, some cultivars carry resistance genes that will confer resistance against a specific pathogen with a corresponding avirulence gene (Ebel and Mithöfer, 1998). This is the gene-for-gene mechanism first described with flax and the flax rust pathogen *Melampsora lini* by Flor (1942). A gene-for-gene interaction appears to arise from the recognition between products of pathogen avirulence genes and products of plant resistance genes that lead to plant resistance by the activation of a multicomponent defense response (Dixon and Lamb, 1990). In an incompatible interaction, host-pathogen recognition leading to subsequent downstream responses enables the plant to resist attack by the pathogen, whereas in a compatible interaction, the recognition process does not occur and the pathogen is able to invade the plant (Keen, 1990).

One of the distinctive features of an incompatible interaction is the hypersensitive response (HR) (Lamb *et al.*, 1989). It appears to involve a programmed cell death in which cells at the infection site and its vicinity collapse and eventually turn brown (necrosis). The pathogen is deprived of nutrients and can not proliferate. Other components of the plant defense response are the rapid production of reactive oxygen species intimately associated with the HR, the accumulation of antimicrobial phytoalexins, hydrolytic enzymes such as glucanases and chitinases, pathogenesis-related proteins (PR proteins) and the production of cell wall strengthening compounds such as lignin and hydroxyproline rich proteins (Dixon *et al.*, 1994). Plant defenses can be triggered non-specifically by abiotic and biotic elicitors (general elicitors), and specifically by the direct or indirect products of avirulence genes (specific elicitors) (Johal *et al.*, 1995). Figure 1.1 summarizes the key events involved during plant/pathogen interactions that will be discussed in more detail in this chapter.

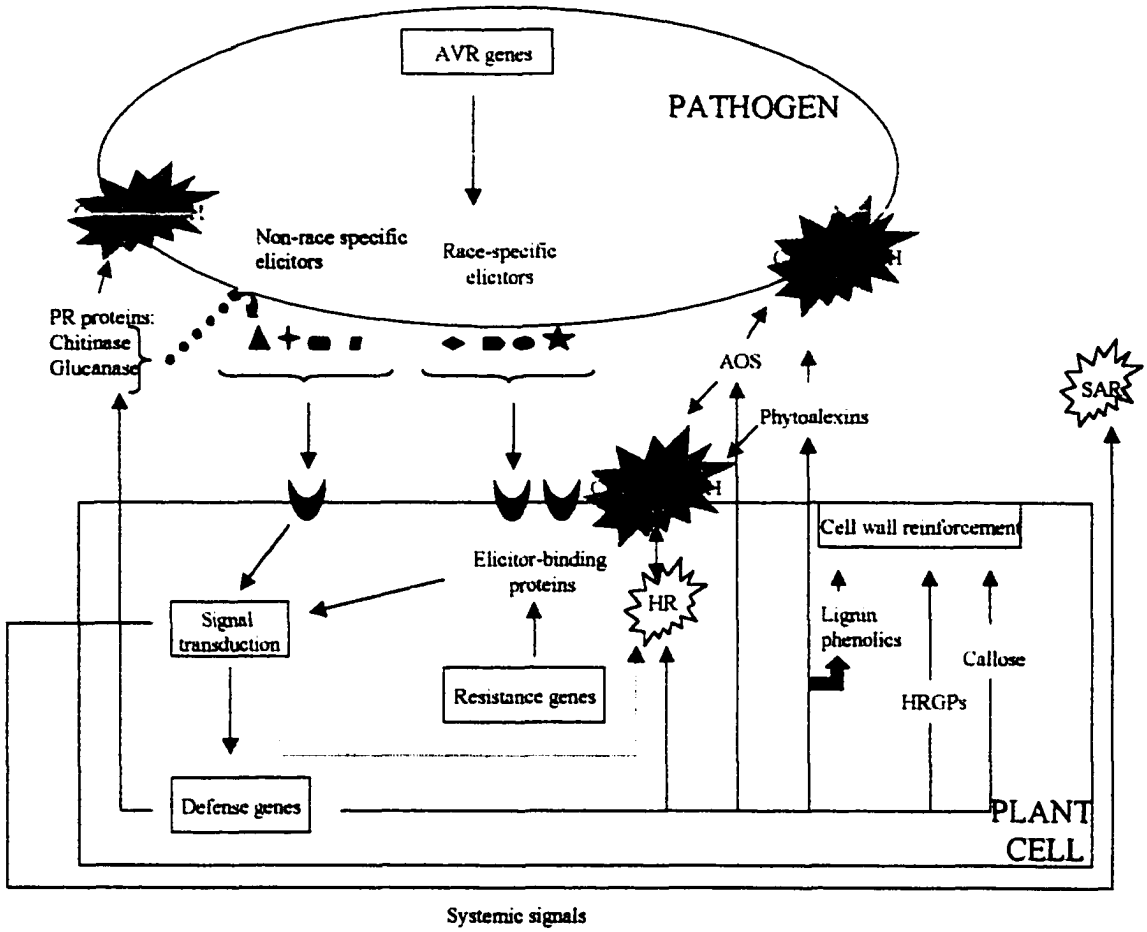


Figure 1.1: Signals and host defenses in plant/pathogen interactions. AVR: avirulence; PR: pathogenesis-related; AOS: active oxygen species; SAR: systemic acquired resistance; HRGPs: hydroxy-rich-glycoproteins.

1.2. The hypersensitive response

Plants have evolved an array of different defensive responses following pathogen attack. Most incompatible interactions, in which specific pathogen-related recognition components (direct or indirect products of avirulence genes) are recognized by products of resistance genes in the plant, are accompanied by the triggering of a battery of plant defenses leading to the hypersensitive response (HR). The HR involves a localized cell death at the infection site, restricting the spread of the pathogen (Chasan, 1994). Plant defenses are also induced in compatible interactions, but the timing and the intensity of the response are respectively faster and higher in resistant plants (Yang *et al.*, 1997).

Defenses associated with the HR will be discussed in the following sections of this chapter. They are not only induced at the infection site but also in neighboring cells, and are thought to limit the spread of the pathogen. Signals emanating from cells expressing a HR appear to induce defense gene transcription in adjacent cells (Dangl *et al.*, 1996). Jacobek and Lindgren (1993) showed that a bacterial mutant unable to trigger a HR in bean was still able to induce the transcription of several defense genes. They hypothesized that there are two distinct signal transduction pathways for the

activation of the HR and for activation of other plant defenses such as phytoalexin synthesis.

It has long been hypothesized that the cell death occurring during the HR response is the most important factor limiting the growth of the pathogen. However, it has been very difficult to assess the importance of cell death in the absence of a way to block other components of the HR during a resistance response (Greenberg, 1997). Gorg *et al.* (1993) found that cell death in barley homozygous for the resistance gene *mlg* occurred concomitantly with the cessation of fungal development whereas this was not the case for heterozygous plants. Powdery mildew infection did not develop in homozygous plants and only 20 % of the heterozygous plants were infected. It was suggested that because the arrest of the fungal infection occurred before cell death in heterozygous plants, cell death was not a requisite step in plant resistance.

It is now generally accepted that plant HR is a form of a programmed cell death. Consequently, researchers have recently given considerable attention to comparative mechanistic studies relating plant and animal plant cell death (Heath, 2000). Mitsuhara *et al.* (1999) reported that the overexpression of mammalian and nematode suppressor genes of programmed cell death in tobacco plants resulted in a reduction of HR in

response to a viral infection. On the other hand, the expression of a mammalian cell death-inducing gene in tobacco triggers cell death and the accumulation of a pathogenesis-related protein, PR1. The hypothesis that a cell death program involving phosphorylation/dephosphorylation processes is activated in the plant is supported by the fact that this cell death is blocked by the addition of a protein phosphatase inhibitor, which also inhibits cell death induced by viral infection (Lacomme and Santa Cruz, 1999).

1.3. Pathogenesis-related proteins

Pathogenesis-related (PR) proteins were first discovered in the early 1970's in tobacco leaves developing a HR to tobacco mosaic virus (TMV). Since then, the presence of PR proteins has been reported for a number of plant species infected by various pathogens (Stintzi *et al.*, 1993). PR proteins are defined as "plant proteins that are induced in pathological or related situations" (Van Loon *et al.*, 1994). They are induced after bacterial, viral or fungal infection (Van Loon, 1985), and also after application of chemicals that mimic the effect of pathogen infection or other stresses (Bol *et al.*, 1990).

Most PR proteins share the following characteristics: they are stable and remain soluble at low pH, they are relatively resistant to proteolytic

enzymes, and they are usually monomers with relatively low molecular weight (8-50 kDa). Because of these physicochemical properties, PR proteins are able to survive in the environments to which they are targeted: the vacuole, the cell wall and the apoplast (Stintzi *et al.*, 1993). Some PR genes induced after pathogen infection in one part of the plant (*e.g.*, leaves) may be expressed constitutively in other parts (*e.g.*, roots) or in cultured cells (Kitajima and Sato, 1999).

PR proteins have been associated with local resistance (HR), as mentioned earlier and with systemic acquired resistance (SAR) occurring at a point distant from the infection area (Stintzi *et al.* 1993). After a plant develops a HR to a necrotizing pathogen, uninoculated parts of the plant show an increased resistance upon challenge by the same or other unrelated pathogens. Smaller lesions are indicators of resistance (Lawton *et al.*1993).

PR proteins were originally classified into five different classes in tobacco (Stintzi *et al.*, 1993). Van Loon *et al.*, (1994) extended the classification to eleven families (Table 1.1). The previously used system for PR nomenclature and classification was exclusively based on electrophoretic mobilities. Now, with the appearance of more informative techniques, especially protein sequencing, six families of PR proteins were added to the original five (Van Loon *et al.*, 1994).

The original five classes of PR proteins are now known to have anti-fungal activities but the biochemical mechanisms of action of many of these proteins are not fully understood except for those of PR-2 and PR–3. These two classes of PR-proteins correspond to β-1,3-glucanases and chitinases, respectively. These two hydrolytic enzymes degrade fungal cell walls (Kitajima and Sato, 1999). In the next section, the primary structure and role in plant defense of glucanases and chitinases will be reviewed.

Table 1.1: Recognized and proposed families of pathogenesis-related proteins (Van Loon *et al.*, 1994).

Family	"Type member"	Properties	Reference
PR-1	Tobacco PR-1a	antifungal	Antoniw *et al.* (1980)
PR-2	Tobacco PR-2	β-1,3-glucanase	Antoniw *et al.* (1980)
PR-3	Tobacco P, Q	chitinase	Van Loon (1982)
PR-4	Tobacco R	antifungal	Van Loon (1982)
PR-5	Tobacco S	antifungal	Van Loon (1982)
PR-6	Tomato inhibitor I	proteinase-inhibitor	Green and Ryan (1972)
PR-7	Tomato P6g	endoproteinase	Vera and Conejero (1988)
PR-8	Cucumber chitinase	chitinase	Métraux *et al.* (1988)
PR-9	Tomato "lignin-forming peroxidase"	peroxidase	Lagrimini *et al.* (1987)
PR-10	Parsley "PR1"	"ribonuclease-like"	Somssich *et al.* (1986)
PR-11	Tobacco class V chitinase	chitinase	Melchers *et al.* (1994)

1.4. Glucanases and chitinases

1.4.1. Introduction

β-1,3-glucanases and chitinases are widely distributed in plant species. They hydrolyze polymers of β-1,3-glucan and β-1,4-N-acetylglucosamine, respectively. These polymers are major components of the cell walls of higher fungi (Boller, 1985). β-1,3-Glucans are found in plant cell walls as callose (Stone, 1984), whereas chitin has not to date been found in plants (Meins *et al.*, 1992). Multiple physiological and morphological functions have been proposed for glucanases, including roles in microsporogenesis (Worrall *et al.*, 1992), pollen tube growth (Roggen and Stanley, 1969), fertilization (Ori *et al.*, 1990), cell division (Waterkeyn *et al.*, 1967), degradation of seed glucans during germination (Vogeli-Lange *et al.*, 1994), and the removal of phloem callose (Clarke and Stone, 1962). Because there is no known substrate for chitinases in plants, and chitin is present in most fungal cell walls, the role of chitinases has been associated almost exclusively with plant defense (Dixon *et al.*, 1996).

The major interest in glucanases and chitinases is based on the hypothesis that they play an important role in plant defense. They are constitutively expressed in most plant tissues at low levels (Kombrink *et al.*, 1988; De Carvalho *et al.*, 1992). Expression of genes incoding these proteins

is induced upon bacterial, viral, or fungal infection, and by elicitors or ethylene treatment at the both mRNA and protein levels (Boller, 1985; Vogeli-Lange *et al.*, 1988; Ward *et al.*, 1991). Chitinases and glucanases have been shown to have antifungal activities *in vitro* alone or in combination (Mauch *et al.*, 1988b). Some chitinases have lysozyme activity and, therefore, they can also digest bacterial cell walls (Boller *et al.*, 1983).

Many β-1,3-glucanases and chitinases have been purified and their genes have been cloned from plant sources. Genomic and cDNA clones have been extensively characterized from different plant species such as tobacco, barley, rice, potato, soybean, alfalfa, pea, *Arabidopsis*, bean, etc. Southern blot analysis has shown that β-1,3-glucanases and chitinases are encoded by small gene families. Sequences of members from the same family are highly conserved (For reviews see Meins *et al.*, 1992; Graham and Sticklen 1994). They are usually monomers of molecular mass varying from 20 to 40 kDa. The majority of β-1,3-glucanases are endo-hydrolases, releasing 2 to 6 glucose units from β-1,3-glucans including laminarin (a commercially available substrate composed mostly of a mixed polymer of β-1,3-glucans with some branched β-1,6-glucans). Chitinases are also mostly endochitinases producing chito-oligosaccharides of 2 to 6 N-acetylglucosamine units (Stintzi *et al.*, 1993).

Several isoforms of chitinases and glucanases can be found in the same plant. Basic and acidic enzymes are named according to their isoelectric point (pI) (Meins *et al.*, 1992). Acidic chitinases and glucanases were found in the intercellular washing fluids after bacterial or viral infection, or following treatment of plants with ethylene or salicylate (Parent and Asselin, 1984; van den Bulke *et al.*, 1989). In contrast, basic enzymes are found intracellularly in the vacuole (Boller and Vogeli, 1984; Mauch and Staëhelin, 1989).

1.4.2. Primary structure

1.4.2.1. β-1,3-glucanases

Glucanases have been divided into 3 classes based on a comparison with the amino acid sequences of tobacco glucanases (Payne *et al.*, 1990). The first class consists of at least 4 basic proteins that are synthesized as preproproteins. They have an N-terminal hydrophobic leader sequence (signal peptide) targeting the protein to the lumen of the endoplasmic reticulum (ER). They also have an N-glycosylated C-terminal extension required for vacuolar localization that is removed by processing to give the mature enzyme (Shinshi *et al.*, 1988). The maturation process of class II and III glucanases is far less clear. Class II glucanases include acidic isoforms

such as tobacco PR-2, -N, -O, GL153, GL161, Ci30, and two stylar isoforms Sp41a and Sp41b. PR-Q' belongs to the third class. PR 2, N-, O, and Q' lack the C-terminal extension, and class II stylar isoforms, GL161, and GL 153 have an extension of 4, 8, and 13 residues, respectively, although the removal of these extensions by processing has not yet been shown (Meins *et al.*, 1992).

Class I and II glucanases share an identity in primary structure of approximately 50 % (Linthorst *et al.*, 1990). The class III acidic glucanase PR-Q' shows 54 to 59 % identity with the class II isoforms and 55 % identity with class I isoforms. Class I and II differ at a minimum of 48 % of the positions in their amino acid sequences. However, the identity is much higher between members of the same class. For example, more than 90 % homology exists between class II PR-P and PR-Q (Payne *et al.*, 1990). The primary structures of glucanases are illustrated in Figure 1.2.

There are differences in specific activities between β-1,3-glucanases of the same plant species for a given substrate, and in substrate specificity between β-1,3-glucanases from different plant species (Stintzi *et al.*, 1993).

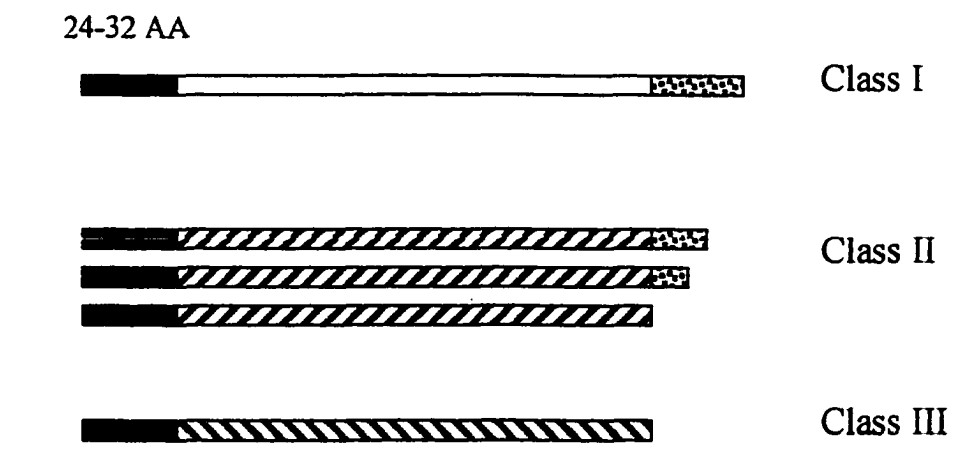


Figure 1.2: Primary structures of plant glucanases.

1.4.2.2. Chitinases

Three classes of plant chitinases were originally proposed based on their primary structures (Shinshi *et al.*, 1988). Class I consists of basic isoforms with four major domains: a leader sequence, an N-terminal cysteine-rich domain of approximately 40 amino acids, a highly conserved main structure and a C-terminal extension (Sahai and Manocha, 1993). Class I chitinases are synthesized as preproproteins (Meins *et al.*, 1992). Neuhaus *et al.* (1991b) demonstrated that the C-terminal extension of tobacco class I chitinase A was necessary to target the protein into the vacuole and sufficient to target a class III cucumber chitinase, normally secreted chitinase to the vacuole. Another characteristic of this class is the hypervariable hinge region localized between the cysteine-rich domain and the main domain. The hinge region usually consists of about 8 to 11 amino acid residues rich in glycine and proline (Meins *et al.*, 1992). Class II chitinases are usually acidic proteins and are localized extracellularly. Their catalytic domain possesses a strong homology with the class I catalytic domain. The major difference between the two classes is the absence of the cysteine-rich region in class II (Graham and Sticklen, 1994). Class III chitinases have no sequence similarity with the enzymes of the other two classes. Some class III enzymes are found in the vacuole, while others are in

the extracellular compartment (Meins *et al.*, 1992). Chitinases with lysozyme activities are found in this class (Métraux *et al.*, 1989). Collinge *et al.*, (1993) proposed a fourth class that includes the basic sugar beet chitinase, the basic rapeseed chitinase, and the acidic bean PR4 chitinase. These chitinases contain a cysteine-rich domain, a hinge region and a catalytic domain similar to that of class I chitinases. However, there are four deletions in their amino acid sequence, one in the cysteine-rich domain and three within the catalytic domain, which reduce the length of the mature protein by approximately 50 amino acids.

The sequence identities between class I and IV are relatively low, about 41 to 47% (Collinge *et al.*, 1993) and 60 to 65 % between class I and II (Beintema, 1994). Like glucanases, the identity between isoforms from the same class is higher: 69 % identity between the individual chitinases of class I, and 59 to 63 % identity between the individual chitinases of class IV (Collinge *et al.*, 1993; Beintema, 1994). The different classes of chitinases are depicted in Figure 1.3.

1.4.3. Role of glucanases and chitinases in plant defense

The possible role of β-1,3-glucanases and chitinases in plant defense has been extensively studied over the years. Evidence for their involvement

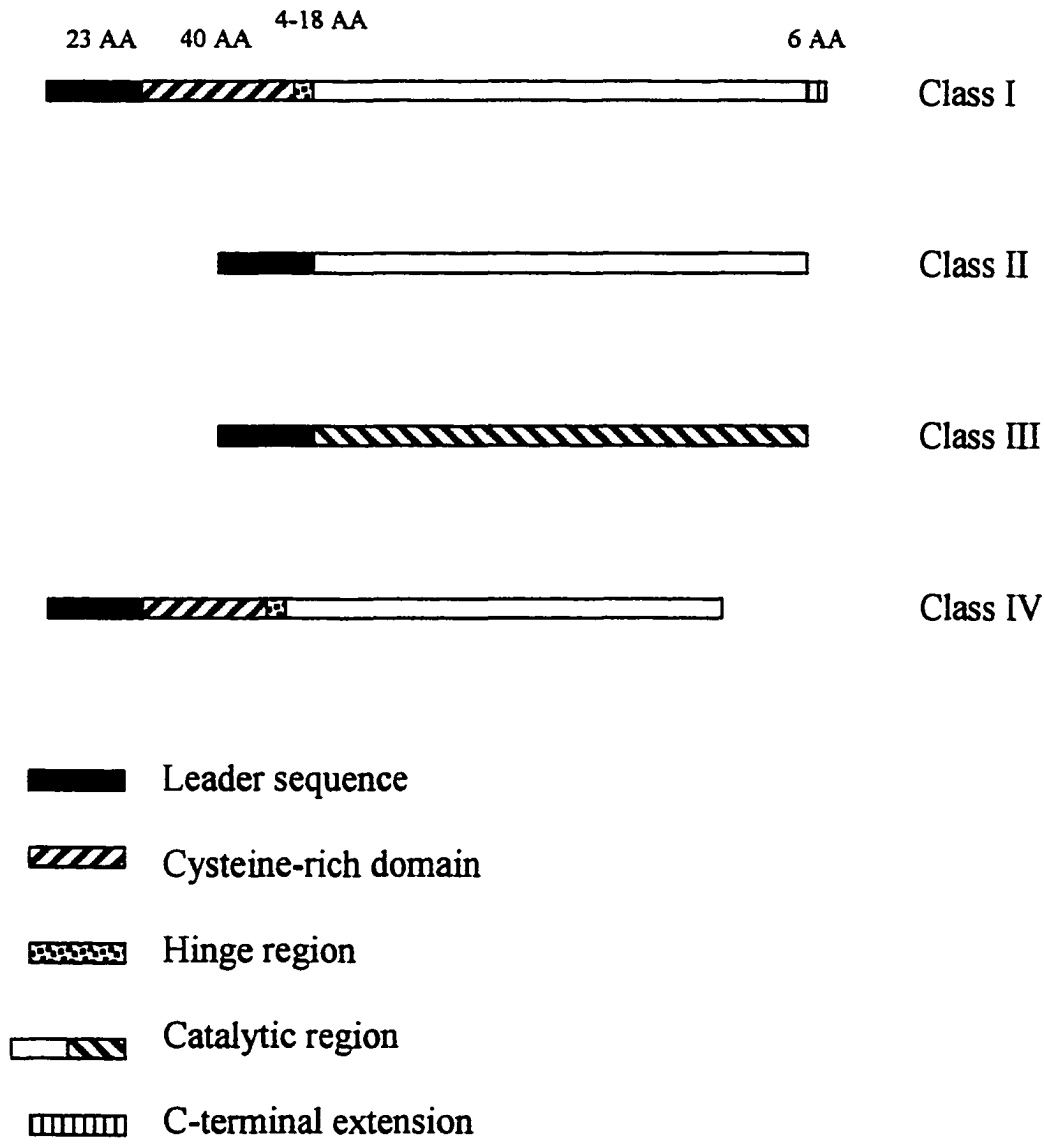


Figure 1.3: Primary structure of plant chitinases.

in plant defense came from the observation that both enzymes are often induced both locally and systemically after pathogen infection as part of the HR and SAR, respectively (Meins and Ahl, 1989; Lawton *et al.*, 1993). Both enzymes are induced in tobacco after fungal (Meins and Ahl, 1989) or TMV infection (Legrand *et al.*, 1987; Kauffmann *et al.*, 1987). They are also induced in potato (Kombrick *et al.*, 1988), pea (Mauch *et al.*, 1984), cucumber (Boller and Métraux, 1988), muskmelon (Roby *et al.*, 1987), tomato (Joosten and de Wit, 1989b), and soybean (Yi and Hwang, 1996) after fungal infection. Other treatments such as exposure to elicitors or ethylene have also been reported to induce expression of glucanases and chitinases (Vogeli *et al.*, 1988; Roby *et al.*, 1987).

Further indirect evidence for the role of glucanases and chitinases in plant defense arose from *in vitro* studies of their potential anti-fungal properties. It had been suggested that glucanases and chitinases play a direct role in defense by digesting fungal cell walls containing glucan and chitin polymers. Mauch *et al.* (1988a; 1988b) demonstrated the direct fungicidal activity of chitinases and glucanases. A combination of the two enzymes from pea pods inhibited synergistically the growth of 15 out of 18 fungi tested. The inhibition was apparently caused by the lysis of hyphal tips. Chitin and β-1,3-glucans are synthesized in the apex of the growing hyphae

of fungi. Beyond the apex, glucan and chitin form fibres that may be overlaid by polysaccharide and protein layers. Consequently, glucans and chitin in these regions are not accessible to β-1,3-glucanases and chitinases (Wessels, 1986, 1988). The simultaneous action of the two enzymes may be required for an effective lysis of the fungal cell wall as pea glucanase or chitinase alone did not inhibit fungal growth (Collinge *et al.*, 1993). Several other reports document the anti-fungal properties of β-1,3-glucanases and chitinases *in vitro* (Sela-Buurlage *et al.*, 1993; Ji and Kuc, 1996; Roberts and Selitrennikoff, 1986; Leah *et al.*, 1991).

Another piece of indirect evidence to demonstrate the potential role of β-1,3-glucanases and chitinases in plant defense came from studies of the tomato/*Cladosporium fulvum* interaction. The two enzymes were induced earlier and at a higher level in resistant cultivars than in susceptible cultivars (Joosten and DeWit, 1989). Moreover, van Kan *et al.* (1992) showed that an extracellular acidic glucanase accumulated to similar levels in both compatible and incompatible interactions, but the accumulation occurred much faster for the incompatible interaction. The pattern of expression of an intracellular basic glucanase was different from the one described above. The intracellular glucanase was induced rapidly in both interactions, but declined with time only in the incompatible interaction. The difference in the

pattern of expression between the two glucanases was discussed by Van Kan *et al.*, (1992). Because the fungus was growing intercellularly they argued that basic glucanases could not harm the fungus except when the cell lyses, whereas acidic glucanases could be directly in contact with the fungus.

The function of β-1,3-glucanases in plant defense has been investigated more directly with antisense transformation in common tobacco and *Nicotiana sylvestris* (Beffa and Meins, 1996). *N. sylvestris* plants were transformed with the class I tobacco glucanase gene *gla* in reverse orientation under the control of the CaMV35S promoter. Plants homozygous for this construct showed a severe reduction in levels of class I β-1,3-glucanases. There was no induction of this enzyme after *Cercospora nicotianae* infection. Unexpectedly, there was no increased susceptibility to the fungus in transformed plants containing the antisense construct when compared to untransformed plants (Neuhaus *et al.*, 1992). Beffa *et al.* (1993) demonstrated that plants compensate for class I glucanase deficiency by producing an "ersatz" glucanase and have a decreased susceptibility to viral infection. The fact that the plants compensated for glucanase deficiency specifically during infection may indicate the importance of glucanases in plant defense.

Direct evidence that both glucanases and chitinases interact with fungal cell walls *in vivo* comes from several immunocytochemical studies. It was demonstrated that the enzymes accumulate around fungal cell walls *in planta* and are simultaneously produced at the same sites, suggesting the synergistic antifungal effect observed *in vitro* is likely to occur *in vivo* (Benhamou *et al.*, 1990, 1993; Mauch and Staëhelin, 1989). From these immunocytochemical studies, Mauch and Staëhelin (1989) proposed a model in which extracellular enzymes can act directly by digesting fungal cell walls or indirectly by releasing elicitors which, in turn, will induce other plant defenses, whereas vacuole-localized enzymes are part of a last line of defense when the attacked host cells lyse.

1.5. Active oxygen species (AOS)

One of the most rapid events in plant/pathogen interactions is the production of active oxygen species (AOS) by the plant, the so-called oxidative burst (Medhy, 1994). The release of AOS can be seen in both compatible and incompatible interactions in the initial phase of the infection. However, the oxidative burst is maintained longer in plants exhibiting a resistant response to the pathogen and is initiated at the onset of the HR (Baker and Orlandi 1995).

There is accumulating evidence that indicates that AOS play an important role in plant defense. The predominant species of AOS detected in plant-pathogen interactions are $O_2^{\cdot}$, H_2O_2, and $OH^{\bullet}$ (Wojtaszek, 1997). In plants, superoxide exists in equilibrium with its conjugate acid $^{\bullet}HO_2$ (hydroperoxyl radical). $^{\bullet}HO_2$ and $O_2^{\cdot}$ undergo dismutation to produce H_2O_2. H_2O_2 can oxidize transition metals such as Fe^{2+} with $O_2^{\cdot}$ acting as the initial reducing agent (Lamb and Dixon, 1997; Baker and Orlandi, 1995). AOS are toxic to healthy plant cells if they accumulate. Therefore, plant cells have antioxidant mechanisms. Superoxide dismutase is generally viewed as an important antioxidant. H_2O_2 is removed from the cell by catalase and various peroxidases including ascorbate and glutathione peroxidases (Baker and Orlandi, 1995).

Direct antimicrobial activity of AOS has been demonstrated for bacterial (Keppler *et al.*, 1989) and fungal pathogens (Peng and Kúc, 1992). Recent work established that oxygen intermediates are key mediators in cell death during the HR. This hypothesis is supported by studies showing that addition of exogenous H_2O_2 to soybean cells induces cell death and genes encoding cellular protectants like glutathione-*S*-transferase or glutathione peroxidase. Cell death could be inhibited by adding an inhibitor of the NADPH oxidase (diphenylene iodonium: DPI). This also suppressed the

oxidative burst (Levine *et al.*, 1994). In addition, Mittler *et al.* (1996) showed that cell death but not the activation of some plant defenses is inhibited in tobacco cells infected with TMV at low oxygen pressure. Other evidence for the role of AOS in cell death was suggested by Mittler *et al.* (1998). During viral-induced cell death in tobacco, the expression of ascorbate peroxidase, a key detoxifying enzyme, was post-transcriptionally suppressed, likely causing a reduction in the capability of cells to scavenge H_2O_2, therefore allowing the rapid induction of the cell death. However, some reports are questioning the role of AOS in mediating cell death (Baker and Orlandi, 1995; Dorey *et al.*, 1999).

The oxidative burst also contributes to cell wall strengthening during pathogen attack. Bradley *et al.*, (1992) showed that there was an oxidative cross-linking of two cell wall proteins in elicited bean or soybean cells. This response occurred within two minutes and increased the resistance of the cell wall to the action of fungal wall degrading enzymes.

A correlation between the production of AOS and phytoalexins was first suggested by Doke (1983) when he showed that fungal elicitors of phytoalexins also increased the production of $O_2^{\cdot-}$. Apostol *et al.* (1989) showed that exogenous H_2O_2 stimulates phytoalexin production. A more recent study showed that even if H_2O_2 and harpin (bacterial elicitor) are able

to induce cell death, they have different effects on defense gene expression in *Arabidopsis* suspension cultures. H_2O_2 induced the expression of genes encoding enzymes such as phenylalanine ammonia-lyase (PAL) and glutathione S-tranferase (GST, an anti-oxidant enzyme) but not anthranilate synthase (ASA1), an enzyme required for the biosynthesis of the *Arabidopsis* phytoalexin camalexin. Harpin on the other hand induced expression of *asa1* and *gst* genes, and this could be induced independently of H_2O_2 (Desikan *et al.*, 1998). From these last results it seems that the AOS and phytoalexins are induced in parallel by the harpin via two independent signalling pathways.

Finally, reactive oxygen species have been shown to be involved in systemic acquired resistance (SAR). The primary oxidative burst at the infection site induces systemic signals that lead to microbursts at a distant site, accompanied by micro-HRs (Alvarez *et al.*, 1998).

1.6. Phytoalexins

Phytoalexins are low molecular weight antimicrobial compounds synthesized from precursors that accumulate in plants in response to fungal pathogens, elicitors or other biotic or abiotic stresses (Dixon *et al.*, 1983). Müller and Börger (1940) demonstrated that potato tubers resistant to an

incompatible race of *Phytophthora infestans* were resistant to subsequent challenge by a compatible race of the fungus. They speculated that, in response to infection, potato tubers were producing specific substances (phytoalexins) that inhibited further growth of the fungus.

Phytoalexins have a great diversity in their structure (isoflavonoids, terpenoids, stilbenes, polyacetylenes, dihydrophenanthrene) and their biosynthesis (Figure 1.4; Ebel, 1986). It is interesting to note that within the same or taxonomically related plant species there is considerable conservation in the chemical structure of phytoalexins (Hammerchmidt, 1999). For example, legumes usually produce isoflavonoids and solanaceous plants tend to produce sesquiterpenes. Due to this conservation, phytoalexins are used as chemotaxonomic markers (Dixon, 1986). A given plant specie may produce a small number of closely related phytoalexins, although there are exceptions to this; the acetylenic phytoalexin wyerone is produced by the legume *Vicia faba,* which also produces the isoflavonoid medicarpin. In addition, the same phytoalexin may be found in different, but closely related species as it is the case with medicarpin (Dixon *et al.*,1983). Within different cultivars of a given specie there may be differences in the amount of phytoalexins that each cultivar can produce, and this could reflect the nature of the invading pathogen (Bailey, 1987).

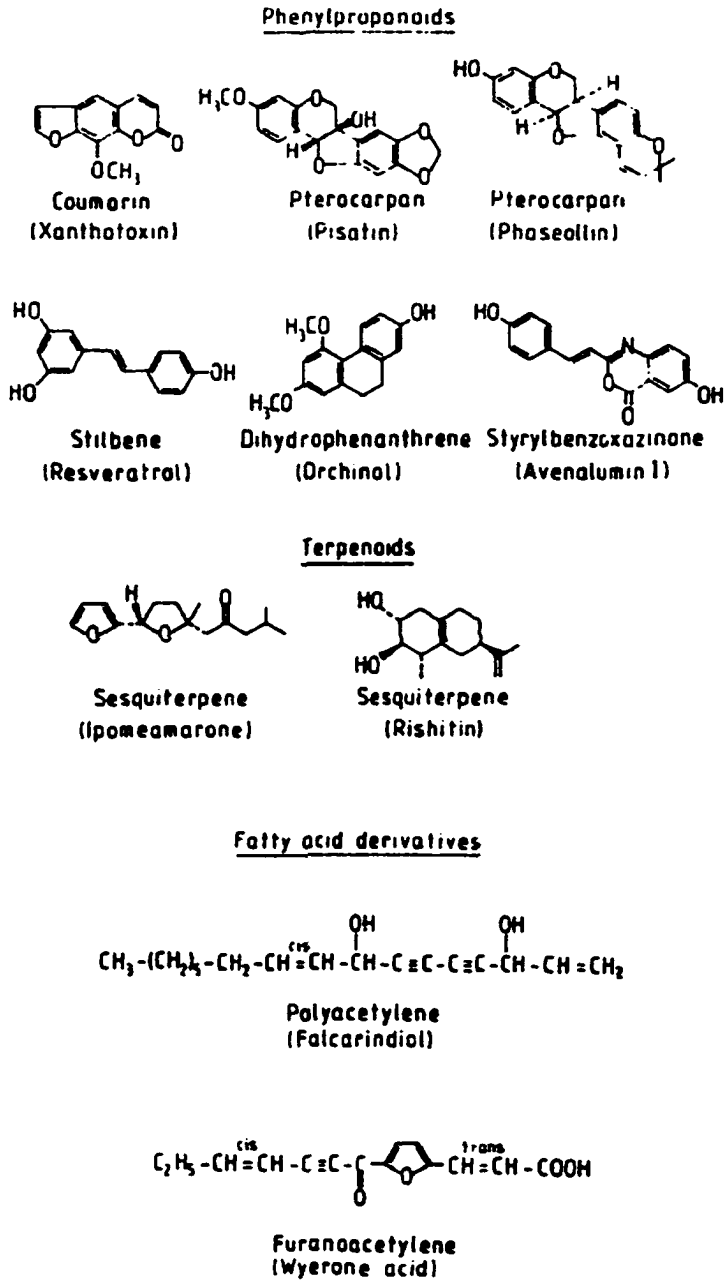


Figure 1.4: Structures of a number of selected phytoalexins (Ebel, 1986).

1.6.1. Biosynthesis of medicarpin

This section will focus on medicarpin, the major phytoalexin in alfalfa. Medicarpin is synthesized from L-phenylalanine via the isoflavonoid pathway as illustrated in Figure 1.5. Enzymes involved in the biosynthesis of medicarpin have been characterized and their corresponding genes cloned (Dixon *et al.*, 1992; He *et al.*, 1998).

It has been shown that medicarpin and related compounds rapidly accumulate in cell suspension cultures of alfalfa (*Medicago sativa* L.) after treatment with a crude elicitor from *Colletotrichum lindemuthianum* (Dalkin *et al.*, 1990; Kessmann *et al.*, 1990a). The elicitation of medicarpin in alfalfa is preceded by increases in the activities of all eleven enzymes required for its biosynthesis (Dalkin *et al.*, 1990; Dixon *et al.*, 1995). Ni *et al.* (1996) demonstrated that there was a transcriptional activation of several genes involved in the phenylpropanoid pathway after elicitation. Genes encoding L-phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and chalcone reductase (CHR) were most rapidly activated (10-20 min).

Three isoflavonoid conjugates have been reported from alfalfa cell cultures: medicarpin-3-*O*-glucoside-6"-*O*-malonate (MGM), formononetin-7-*O*-glucoside (FG) and formononetin-7-*O*-glucoside-6"-*O*-malonate (FGM) (Kessmann *et al.*, 1990b; Tiller *et al.* 1994). Medicarpin accumulation in

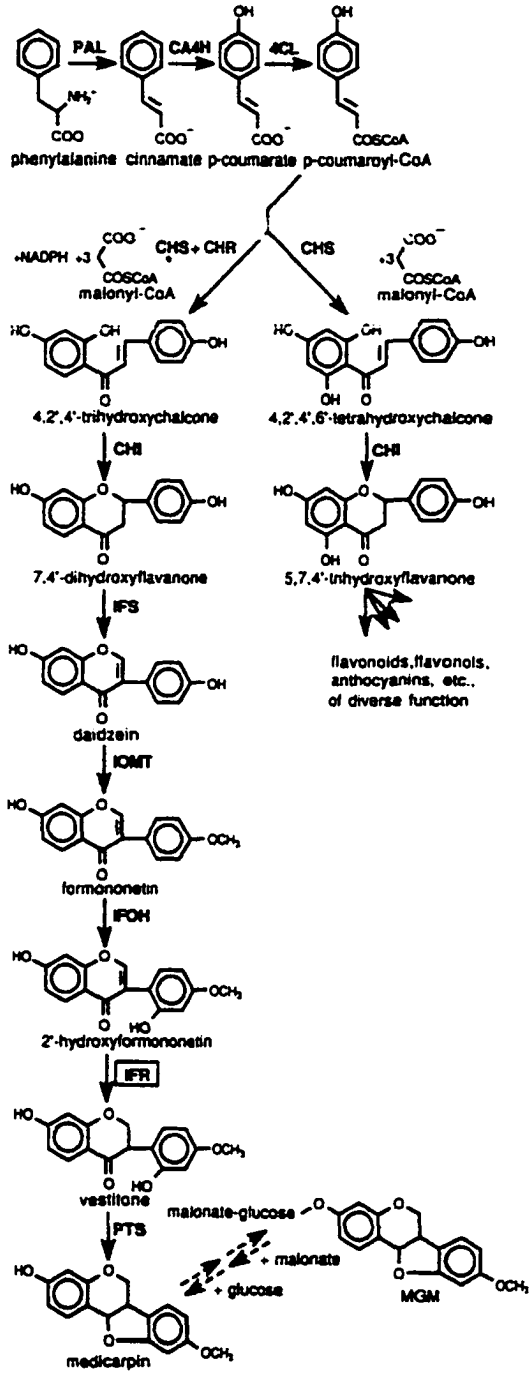


Figure 1.5 : Biosynthetic pathway leading to (-) medicarpin in alfalfa. PAL, L-phenylalanine ammonia lyase; CA4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; IFS, isoflavone synthase; IOMT, isoflavone-*O*-methyltransferase; IFOH, isoflavone 2'-hydroxylase; IFR, isoflavone reductase; PTS, pterocarpan synthase; MGM, medicarpin-3-*O*-glucoside-6"-*O*-malonate (Oommen *et al.*, 1994).

alfalfa cell cultures was followed by an increase in the levels of one of its conjugates, MGM. Labeling studies with [^{14}C]phenylalanine indicated that medicarpin was the primary product, although a significant part of newly synthesized medicarpin was conjugated. Treatments of cells with [^{14}C]phenylalanine, elicitor and a inhibitor of PAL suggested that phytoalexin could be released from a preformed conjugate under these conditions (Kessmann, *et al.*, 1990b).

1.6.2. Role of phytoalexins in plant defense

Phytoalexins are induced in plants in response to microbial pathogen infection and have been considered as an important host defense mechanism (Keen, 1981). Phytoalexins have been associated with resistance in many gene-for-gene systems (Hammerschmidt, 1999). One example of this was given by Long *et al.* (1985) who showed a strong correlation between the accumulation of the isoflavonoid phytoalexin glyceollin in soybean and resistance to *Pseudomonas syringae* pv. *glycinea* (Psg). There was a linear inverse relationship between the production of glyceollin and the growth of bacterial cells. On the other hand, suppression of the accumulation of glyceollin by more than 90% with an inhibitor of PAL resulted in the loss of resistance to *Phytophthora megasperma* (Moesta and Grisebach, 1982).

Indirect evidence supporting the role of phytoalexins in plant defense is their potent antifungal activity. Blount *et al.* (1992) showed that medicarpin inhibited the growth of *Phoma medicaginis, Phytophthora megasperma* and *Nectria haematoccoa.* Other indirect evidence supporting the notion that phytoalexins are involved in the resistance of plants to pathogens is that phytoalexins accumulate to inhibitory concentrations at the site of pathogen development. Using a radioimmunoassay specific for the soybean phytoalexin glyceollin, Hahn *et al.* (1985) showed that the timing and localization of glyceollin accumulation correlated well with the restricted (incompatible interaction) or the unlimited growth of the pathogen (compatible interaction).

Phytoalexins as well as other inducible plant defenses are associated with the HR (Dixon *et al.*, 1983). Glyceollin accumulates to 10 % of the dry weight of soybean tissues developing an HR to *Phytophthora megasperma* f.sp. *megasperma* within 24 to 28 hours after inoculation (Keen and Horsh, 1972).

Interactions where the pathogen is able to detoxify phytoalexins have provided insight into the contribution of phytoalexins in plant defense. Research by van Etten *et al.* (1989) showed that virulence of *Nectria hematococca* to peas depended upon the ability of the pathogen to detoxify

pisatin. The detoxification was due to the demethylation of pisatin by a fungal pisatin demethylase. Genetic analysis showed that pisatin detoxification was necessary for pathogenicity. Disruption of *pda*, the gene coding for pisatin demethylase, or transformation of a *pda*- strain with a pisatin demethylase gene resulted in a small decrease or increase, respectively, in virulence (Wasmann and Van Etten, 1996; Ciufetti and Van Etten, 1996). These results suggest that pisatin detoxification may not be the only factor determining pathogenicity.

Generation of phytoalexin-deficient mutants has helped to confirm the proposed role of phytoalexins in disease resistance in *Arabidopsis* reviewed by Hammerschmidt (1999). Results from overproducing or blocking the production of phytoalexins in transgenic plants have likewise been informative (Dixon *et al.*, 1996). For example, a stilbene synthase gene from grapevine was introduced into tobacco and provided resistance to *Botrytis cinerea* (Hain *et al.*, 1993). The same gene introduced into tomato and rice provided resistance to *Phytophthora infestans* and *Magnaporthe grisea,* respectively (Thomzik *et al.*, 1997; Stark-Lorenzen *et al.*, 1997). More recent studies in alfalfa gave equally encouraging results. The transformation of alfalfa with a grapevine stilbene synthase gene led to increased resistance to *Phoma medicaginis* (Hipskind and Paiva, 2000).

Alfalfa plants overexpressing the alfalfa isoflavone *O*-methyltransferase (*IOMT*) gene encoding an enzyme involved in the biosynthesis of medicarpin displayed resistance to *P. medicaginis* (He and Dixon, 2000).

Finally, phytoalexins are presumed to be one the plant defenses induced by elicitor molecules released from the pathogen or host during an infection. Furthermore, phytoalexin accumulation provides a system to characterize elicitors of plant resistance as will be described below.

1.7. Cell wall strengthening

In the early phase of a pathogen attack, plants respond by synthesizing different cell wall components such as callose, lignin, wall-bound phenolics, and hydroxyproline-rich glycoproteins to act as a physical barrier to the pathogen (Lamb *et al.*, 1989).

1.7.1. Callose

Callose is a polymer of β-1,3-glucans and is synthesized by callose synthase, which has been purified from several plant species (Vidhyasekaran, 1997). Callose is a minor component of healthy plant tissues but is present in larger amount when the plant is infected by pathogens (Vidhyasekaran, 1997). Benhamou (1995) showed that

parenchyma cells in eggplant stem tissues inoculated with *Verticillium alboatrum* were producing callose to stop the fungus at the attempted infection sites. The deposition of β-1,3-glucans may not only act as a physical barrier to block fungal development in the host tissue, but also could generate elicitors as suggested by Benhamou (1992).

1.7.2. Lignin

Lignin is a major plant cell-wall polymer composed of varying relative amounts of 4-coumaryl, coniferyl and sinapyl alcohols. Monomers of lignin are synthesized from phenylalanine and the first three steps leading to 4-coumaroyl-CoA are common to a large numbers of other phenylpropanoid products such as flavonoids and isoflavonoid phytoalexins. Two of the several enzymes involved in the formation of lignin, the cinnamoyl-CoA reductase (CCR) and the cinnamyl-alcohol dehydrogenase (CAD) have been extensively studied (Vidhyasekaran, 1997).

Many plants respond to pathogen attack by expression of genes involved in the biosynthesis of lignin and other phenolic compounds, with resultant deposition of these compounds in the cell wall (Vidhyasekaran, 1997). Grand *et al.* (1987) showed that CAD activity was rapidly induced after elicitor treatment in bean cell cultures. A new pathogen defense-related

dehydrogenase (ELI3) having a high amino acid sequence similarity with lignin-related CAD although displaying different substrate specificity was identified by Logemann *et al.*, (1997). The observation that ELI3 was both induced in elicited-parsley cell cultures and infected-parsley leaves, but not in lignifying tissues strongly suggests its specific role in pathogen defense other than lignin biosynthesis (Logemann *et al.*, 1997).

1.7.3. Hydroxyproline-rich glycoproteins (HRGPs)

HRGPs constitute an important component of the plant cell wall. The primary role of HRGPs is to provide structural support to the cell wall and allow its extension. They also contribute to plant defense by strengthening the wall upon pathogen attack (Showalter, 1993) and are involved in HR and SAR (Dixon and Lamb, 1990).

A type of HRGP called extensin immobilizes certain plant pathogens. This agglutination may result from the interaction of extensin that has a net positive charge with negatively charged surfaces of plant pathogens (Leach *et al.*, 1982).

The synthesis of HRGPs is developmentally regulated (Keller and Lamb, 1989) and induced in response to mechanical wounding, elicitors, pathogen attack or ethylene treatment (Vidhyasekaran, 1997; Boudart *et al.*,

1995, García-Muniz *et al.*, 1998). García-Muniz *et al.* (1998) showed that maize *HRGP* mRNAs accumulated after fungal elicitor treatment, and that the level of induction correlated with the concentration of the elicitor. Ethylene but not methyl jasmonate or salicylic acid appeared to be involved in the transduction pathway of *HRGP* gene activation in reponse to the fungal elicitor.

Following pathogen attack, HRGPs undergo glycosylation and insolubilization by cross-linking that may lead to a more impenetrable cell wall to the pathogen (Vidhyasekaran, 1997). The insolubilization or immobilization of HRGPs was demonstrated to be driven by hydrogen peroxide. The immobilization of HRGPs was suggested to act as an anchor for deposition of phenolic compounds (Wojtaszek *et al.*, 1995).

1.8. Elicitors of plant defenses

Originally the term elicitor was applied to agents able to induce phytoalexin production in plant tissues. Now the term has been used to describe agents that induce any defense response (Dixon *et al.*, 1994). Elicitors can be divided into two categories: race-specific and general elicitors. Race-specific elicitors are believed to be direct or indirect products of pathogen avirulence genes, whereas general elicitors are released from

pathogen or plant cell walls during early phases of the infection (Ebel and Scheel, 1997). Elicitors are diversified in their nature and may be proteins, oligosaccharides, glycoproteins or fatty acids.

One model for the action of elicitors is that they act as ligands recognized by receptors localized on the plant plasma membrane (Ebel and Cosio, 1994). The first resistance gene involved in a gene-for-gene interaction to be cloned was *Pto,* a tomato gene conferring resistance to strains of *Pseudomonas syringae* pv. *tomato* containing the *avrPto* avirulence gene. The *Pto* protein sequence resembles a cytoplasmic serine/threonine kinase. In this case, the *avrPto* elicitor may interact directly with the Pto kinase in the cytoplasm, or Pto may be involved downstream of a receptor that binds to the elicitor (Martin *et al.,* 1993).

Once elicitors are recognized by plant receptors, a signal transduction pathway is then initiated, triggering a multicomponent defense response including the accumulation of phytoalexins (Ebel and Mithöfer, 1998). A variety of elicitors from both plant and pathogen origin can induce the accumulation of phytoalexins as will be illustrated in this section.

1.8.1. Race –specific elicitors

Race-specific elicitors are often products of avirulence genes from the pathogen, and are able to induce a HR on plant cultivars carrying the corresponding resistance gene. Examples include the products of the avirulence genes *avr4* and *avr9* from the tomato pathogen *Cladosporium fulvum*, and *nip1* from the barley pathogen *Rhynchosporium secalis* (Van Kan *et al.*, 1991; Knogge *et al.*, 1994).

Van den Ackerveken *et al.* (1992) demonstrated the specificity of Avr9 by transforming a virulent race of *C. fulvum* with the *avr9* gene. The transformants became avirulent on tomato carrying the resistance gene *cf9*. The *avr9* gene codes for a 63 amino acid (aa) peptide that is processed into a 28 aa peptide in the plant. Both plant and fungal proteases are responsible for the processing of the precursor Avr9 into the race-specific elicitor (Van den Ackerveken *et al.*, 1993). The race specific elicitor Avr4 is also synthesized as a preproprotein and cleaved into the mature elicitor by plant and fungal proteases (Von Heijne, 1986; Van den Ackerveken *et al.*, 1993). A single base-pair change in the *avr4* gene is responsible for virulence in races that previously failed to attack tomato carrying the *cf4* resistance gene (Joosten *et al.*, 1994).

Nip1 is a phytotoxin protein with a molecular mass <10 kDa secreted by *R. secalis*. The race-specificity of Nip1 was demonstrated by Rohe *et al.*, (1995). Co-inoculating a virulent race with Nip1 on barley carrying the *rrs1* resistance gene lead to an incompatible interaction. Moreover, a virulent race transformed with *nip1* failed to infect sensitive barley plants. Similar to the *avr4/cf4* interaction, one single nucleotide change in the *nip1* gene was detected in a virulent race of the pathogen, and thus was responsible for the loss of elicitor activity (Rohe *et al.*, 1995).

Other fungal race-specific elicitors have been described from *Uromyces vignae*, a pathogen of cowpea. The elicitor peptides induced a HR in resistant cultivars (D'Silva and Heath, 1997).

Avirulence genes have also been characterized in bacterial pathogens. These include the *avrD* gene from *Pseudomonas syringae* pv. *tomato*. The elicitors produced are thought to be low-molecular weight extracellular products, called syringolides 1 and 2. Syringolides are C-glycosyl lipids that are not direct products of *avrD* but synthesis of which is directed by this gene (Ebel and Mithöfer, 1998). When the *avrD* gene is transformed into virulent *P. s.* pv. *glycinea* , soybean leaves infected with the bacteria develop a HR upon inoculation (Keen *et al.*, 1990). Purified syringolides elicit the HR in soybean cell suspensions or soybean leaves that are expressing the

resistance gene *Rpg4,* and will also induce glyceollin production (Keen and Buzzell, 1991; Hammerschmidt, 1999). A 34 kDa soybean protein binding to the syringolide elicitors has been characterized and may be a receptor mediating the signaling by these elicitors (Ji *et al.*, 1998).

1.8.2. General elicitors

Among the elicitors not associated with race-specific resistance are carbohydrates and proteins from cell walls of fungi, and oligogalacturonides from plant cell walls. Oligosaccharide elicitors can be divided into 4 classes: oligoglucan, oligochitin, oligochitosan and oligogalacturonide (Côté and Hahn, 1994).

The interaction between soybean (*Glycine max*) and the fungal oomycete pathogen *Phytophthora megasperma* f.sp. *glycinea* is one of the best-known plant/pathogen systems for studying the release of elicitors leading to phytoalexin production. Glucans released from *Phytophthora megasperma sojae* cell walls by different treatments [such as autoclaving cell walls and incubating them with crude extracts or purified glucanase from soybean cotyledons] induced a phytoalexin response in soybean cotyledon bioassays (Ayers *et al.*, 1976; Yoshikawa *et al.*, 1981; Keen *et al.*, 1983). The elicitor was characterized as a hepta β-glucoside, a component of

mycelial cell walls of *P. megasperma* (Sharp *et al.*, 1984). However, this structure was unlikely to be present *in vivo*. Later, Okinaka *et al.* (1995) proposed a primary structure for the elicitor consisting of a β-1,6-linked glucan backbone chain of variable length with side chains composed of one or two molecules of β-1,3-linked glucose. The factor responsible for the release of the glucan elicitor from the *P. megasperma* cell walls was an endo-β-glucanase from soybean tissue (Keen and Yoshikawa, 1983).

Chitin has been demonstrated to elicit phytoalexin production in rice cell cultures and lignification in wheat (Ren and West, 1992; Barber *et al.*, 1989). Chitosan (de-acetylated chitin) has broad-spectrum elicitor activity. It induces phytoalexins in pea (Hadwiger *et al.*, 1994; Walker-Simmons *et al.*, 1983), callose deposition in soybean and parsley (Köhle *et al.*, 1984; Conrath *et al.*, 1989) and proteinase inhibitors in tomato (Walker-Simmons *et al.*, 1983).

The last class of oligosaccharides with elicitor activity is the oligogalacturonides. Homogalacturonan formed of α-1,4-D-galactosyluronic acid residues is a major component of plant cell walls. Oligogalacturonides result from the depolymerisation of homogalacturonan. The number of galactosyluronic acid residues required for eliciting defense responses varies between 9 and 15. Among the defense responses stimulated by

oligogalacturonides are the production of phytoalexins, pathogenesis-related (PR) proteins, proteinase inhibitors, lignin, and hydroxyproline-rich glycoproteins (Côté and Hahn, 1994; Boudart *et al.*, 1995).

Oligogalacturonides are released from plant cell walls mostly by hydrolytic enzymes from the pathogen. The polygalacturonase from *Rhizopus nigricans* induced casbene synthase, an enzyme leading to the synthesis of the diterpene phytoalexin casbene, in castor bean (Lee and West, 1981). Davis *et al.* (1984) demonstrated that a pectin lyase from *Erwinia carotovora* elicited phytoalexins in soybean tissues. An endopolygalacturonase of *Colletotrichum lindemuthianum* induced defense responses more rapidly in resistant bean cultivars than in sensitive ones (Lafitte *et al.*, 1993). Boudart *et al.* (1998) demonstrated that the endopolygalacturonase of *Colletotrichum lindemuthianum* released pectic fragments from resistant bean cell walls that were then able to induce an increase in PR proteins in both resistant and susceptible plants. Pectic fragments released from cell walls of a susceptible bean cultivar had no effect on the defense response of resistant or susceptible bean plants. Further evidence is needed to confirm the hypothesis that the determination of resistance or susceptibility in the bean/*C. lindemuthianum* interaction depends on pectic fragments released from the plant cell walls.

A number of glycoproteins have been reported to have elicitor activity. A 46kDa protein from *Phytophthora nicotianae* can induce the production of phytoalexins in tobacco (Farmer and Helgeson, 1987). A 42kDa glycoprotein was isolated from *P. megasperma glycinea* and was capable of inducing several defense responses in cultured parsley cells (Nurberger *et al.*, 1994). Baillieul *et al.* (1995) identified a 32kDa glycoprotein from culture filtrates of *P. megasperma.* Infiltration of a few nanograms of the protein into tobacco leaves caused the development of an HR and an accumulation of transcripts of genes encoding enzymes of the phenylpropanoid pathway. Additional glycoproteins with elicitor activity have been characterized in other fungal species such as *Colletotrichum lindemuthianum* and *Puccinia graminis* f.sp. *tritici* (Coleman *et al.*, 1992; Kogel *et al.*, 1988). Glycopeptides from yeast have been reported to induce ethylene biosynthesis and the activity of PAL in cultured tomato cells (Basse *et al.*, 1992). A yeast elicitor also induced the production of the phytoalexin medicarpin in alfalfa cell cultures (Kessmann *et al.*, 1990b; Dalkin *et al.*, 1990; Tiller *et al.*, 1994).

An elicitor named harpin was isolated from *Erwinia amylorova* and was able to induce a HR on non-host plants. The gene encoding harpin (*hrpN*) is located in the *hrp* gene cluster of the bacteria (Wei *et al.*, 1992).

To cause disease on its host, the bacterial pathogen must express genes encoding for enzymes harming the host, but also genes of the *hrp* cluster. The *hrp* genes are responsible for the hypersensitive reaction and pathogenicity in host plants. *Hrp-* mutants showed reduced virulence and were not able to induce an HR on either resistant host plants or non host plants that usually respond with an HR (Chasan, 1994).

Finally, elicitins are a group of small proteins (around 10 kDa) secreted by *Phytophthora* species. Two elicitins from *P. capsici* and *P.cryptogea* trigger a HR in non-host tobacco plants (Ricci *et al.*, 1989). Cryptogein from *P. cryptogea* induced plant defenses such as production of ethylene and accumulation of phytoalexins in non-host tobacco plants (Milat *et al.*, 1991). In the *Phytophthora parasitica*/tobacco interaction the amount of the elicitin produced was negatively correlated with the degree of virulence of the fungus. A high production of elicitins confers a decrease in virulence and vice versa (Kamoun *et al.*, 1994).

1.9. Strategies for enhancing crop resistance against fungal pathogens

Microbial diseases remain a serious factor limiting crop productivity. Pesticide treatments used to decrease disease losses are tremendously costly

and harmful to the environment (Lamb *et al.*, 1992). Moreover, chemicals become less efficient due to the development of resistance in pathogens. Therefore, there is an interest in the development of alternative strategies to provide plants with defense mechanisms against microbial diseases. One such strategy is to transfer into plants genes encoding enzymes, such as glucanases and chitinases, having inhibitory effects on fungi (Cornelissen and Melchers, 1993).

Because chitinases and glucanases have antifungal properties, one strategy consists of expressing them constitutively in the plant. The first report of this approach was the transformation of a bean vacuolar chitinase under the control of the CaMV 35S constitutive promoter in tobacco and canola plants. Transgenic plants expressing the transgene showed a decreased susceptibility to *Rhizoctonia solani* (Broglie *et al.*, 1991). Benhamou *et al.* (1993) demonstrated later that *R. solani* hyphae appeared severely damaged in these transgenic canola plants compared to wild type. The damage seemed to correlate with an increase in chitin breakdown suggesting that the bean chitinase transgene was responsible at least in part, for the enhanced resistance of canola plants to *R. solani.* Another example of a transformed plant harboring an enhanced resistance to a fungal pathogen is

rice overexpressing a rice chitinase gene that provided resistance against *R. solani* (Lin *et al.*, 1995).

A constitutively expressed soybean glucanase cDNA was introduced into tobacco plants by Yoshikawa *et al.* (1993). Transgenic tobacco plants expressing the transgene displayed enhanced resistance to a large group of fungi. Because the soybean glucanase was previously shown to release active glucan elicitors from fungal cell walls (Keen and Yoshikawa, 1983), it was argued that at least a part of the increased resistance was due to the release of elicitors that then triggered secondary responses. The release of elicitors was presumed to lead to a rapid induction of plant defenses such as phytoalexin production, providing the enhanced protection observed in the transgenic plants, although that was not clearly demonstrated.

Interestingly, over-expressing chitinases or glucanases can sometimes result in the inhibition of the transgene or the inducible homologous gene expression. For example, the transformation of a class I (basic) tobacco chitinase under the control of the CaMV 35S promoter into *Nicotiana sylvestris* plants did not increase resistance to *Cercospora nicotianae*. Levels of chitinase in transformed plants were sometimes lower than in non-transformed plants suggesting that the transgene inhibited homologous host gene expression (Neuhaus *et al.*, 1991a). This phenomenon is called gene

silencing and has also been reported for over-expression of glucanase genes in plants. A glucanase gene (*gn1*) from *Nicotiana plumbaginifolia* was introduced into *Nicotiana tabacum.* Plants hemizygous for the transgene indeed expressed a high level of glucanase activity, whereas, in homozygous plants, no protein could be detected. It was postulated that the dose of GN1 in the transgenic plants could trigger the silencing phenomenon (de Carvalho *et al.*, 1992).

Because glucanases and chitinases seem to have synergistic antifungal effects, there have been several attempts to engineer increased resistance in plants by introducing the genes encoding these enzymes in tandem under the regulation of a constitutive promoter. For instance, protection against *Cercospora nicotianae* was obtained by overexpressing a rice chitinase gene (*rch10*) and an alfalfa glucanase gene (*aglu1*) in tobacco plants. Plants heterozygous or homozygous for the transgenes gave higher protection against the fungus than plants transformed with either transgene alone or non-transformed plants (Zhu *et al.*, 1994). The same constructs introduced either alone or in tandem into alfalfa gave different results. Transgenic alfalfa plants constitutively expressing Aglu1 glucanase exhibited resistance to *Phytophthora megasperma* but not *Stemphylium alfalfae, Colletotrichum trifolii* or *Phoma medicaginis.* No reduced symptoms were observed with

any of the fungi tested for alfalfa plants transformed with chitinase or both glucanase and chitinase transgenes (Masoud *et al.*, 1996). Jach *et al.* (1995) generated transgenic tobacco plants by introducing genes encoding a class II barley chitinase and a glucanase in tandem under the control of the CaMV 35S promoter. The transgenic plants challenged by *Rhizoctonia solani* displayed significantly enhanced protection against the fungus compared to non-transformed plants or plants carrying either transgene alone. A combination of the class II chitinase and a barley ribosome inactivating protein gave similar enhanced protection.

Engineering plants for enhanced resistance to fungal pathogens has resulted in some successes and some failures. The major limitation in this approach is the narrow range of resistance displayed by the transgenic plants: most plants are resistant to one pathogen. One explanation for that is the complexity of the plant/fungus interaction and finding the right combination of genes that will be efficient in that particular interaction.

Very encouraging results in broadening the resistance of transgenic plants against fungi were obtained by introducing a gene encoding an endochitinase from the fungus *Trichoderma harzianum* under the control of the CaMV 35S promoter (Lorito *et al.*, 1998). Tobacco and potato plants transformed with the transgene were resistant to 3 foliar fungi, *Alternaria*

alternata, *A. solani*, *Botrytis cinerea* and one soilborne fungus, *Rhizoctonia solani* (Lorito *et al.*, 1998). These results are in accordance with the stronger and wider anti-fungal activity that *Trichoderma* chitinase displays compared to its counterparts in plants (Lorito *et al.*, 1993). Thus, *Trichoderma* seems to be an interesting source of genes that can be used to genetically engineer plant resistance to fungal pathogens (Lorito *et al.*, 1998).

Another strategy to control plant diseases was developed by Keller *et al.* (1999). A pathogen-inducible tobacco promoter from the *hsr203J* gene fused with a gene encoding a highly active elicitor from *Phytophthora cryptogea* (cryptogein) was introduced into tobacco. The transgene was expressed when tobacco plants were infected by virulent *P. parasitica* var. *nicotianae* but not in non-infected plants. The expression of the transgene correlated with the induction of several defense genes and a HR. The transgenic plants were also resistant to several other fungi unrelated to *Phytophthora* ssp. (Keller *et al.*, 1999).

1.10. Objectives

I was interested in studying plant defenses in the forage legume alfalfa using a gene-for-gene interaction with *Colletotrichum trifolii*, the causal agent of anthracnose disease, and investigating the possible involvement of

elicitors in determining the outcome of this plant/pathogen interaction. The phytoalexin response provided a good system for the study of differential gene expression in alfalfa as all the genes encoding enzymes involved in the biosynthesis of medicarpin have been isolated, and because it can be induced after fungal infection or elicitor treatment (Dixon *et al.*, 1992; Kessmann *et al.*, 1990a, Tiller *et al.*,1994). In addition, a previous study by O'Neill and Saunders (1994) indicated that the synthesis of phytoalexins is one of the plant defenses taking place during the infection by *C. trifolii.* Finally, a variety of elicitors (race specific or non-race specific) induce phytoalexins in plants, as illustrated in section 8 of this chapter. Understanding the initial step where alfalfa is sensitized to the pathogen is important to elucidate elicitor-inducible signal transduction pathways and provide new possibility for genetic engineering in alfalfa.

The second aim of this study was to gain an insight into the role of glucanases in plant defense and most particularly their role in releasing elicitors from fungal cell walls, which has been previously demonstrated *in vitro* (Mauch and Staëhelin, 1989; Keen and Yoshikawa, 1983). Yoshikawa *et al.* (1993) transformed tobacco plants with a soybean β-1,3-endoglucanase that released elicitors *in vitro* from *Phytophthora megasperma* cell walls (Keen and Yoshikawa, 1983) and observed enhanced resistance against *P.*

parasitica var nicotianae. It was argued that the glucanase produced in the transgenic plants was able to release elicitors from the cell walls, thus leading to the production of phytoalexins, although this was not demonstrated.

The release of potential elicitors by glucanases was addressed by overexpressing a glucanase in alfalfa and studying its ability to induce a more rapid production of phytoalexins during the course of infection by *C. trifolii.* In parallel, the effect of overexpressing a glucanase and/or chitinase in alfalfa on other defense gene expression was examined on available transgenic alfalfa plants (Masoud *et al.,* 1996) and on new glucanase overexpressing lines. The new transgenic alfalfa plants were also tested for enhanced resistance to *C. trifolii.*

To summarize, the objectives of this dissertation were:

(1) To examine the interaction between the fungal pathogen, *Colletotrichum trifolii,* and the forage legume, alfalfa (*Medicago sativa*), and to describe potential differential accumulation patterns of three plant defenses between resistant and susceptible alfalfa cultivars (Chapter 2),

(2) To assess primary evidence for the involvement of elicitors in the interaction (Chapter 3),

(3) To investigate the potential role of glucanases in plant defense against fungal pathogens (Chapters 4 and 5).

CHAPTER 2

INDUCTION OF PLANT DEFENSES IN ALFALFA (*MEDICAGO SATIVA*) BY THE FUNGAL PATHOGEN *COLLETOTRICHUM TRIFOLII*

Anthracnose is a major disease caused by *Colletotrichum trifolii* that limits growth and forage yield in alfalfa (*Medicago sativa).* Symptoms vary from a few small irregularly shaped blackened areas that are visible on resistant stems to large oval- to diamond-shaped lesions that develop on susceptible stems (Stuteville and Erwin, 1990). The penetration of host tissue by *C. trifolii* starts by conidial germination followed by germ tube formation. Appressoria form at the tip of the germ tube. Only in the susceptible tissues do the appressoria differentiate into infective hyphae that penetrate the cell wall and ramify within the plant tissue (Dickman *et al.*, 1995).

The first anthracnose resistant cultivar, Arc, was developed by multiple cycles of phenotypic selection for resistance (Barnes *et al.*, 1969; Elgin and Ostazeski, 1982). In 1978, a second race of *C. trifolii* appeared, causing severe damage even in the resistant cultivar Arc. The alfalfa/*C. trifolii* interaction follows a gene-for-gene interaction. Different single

dominant genes (*an1* and *an2*) carry resistance to race 1 and 2, respectively. The cultivar Arc has the race 1 resistance gene *an1* and lacks the race 2 resistance gene *an2*, while the cultivar Saranac (Sar) lacks both of the genes. The cultivar Saranac AR (SarAR) carries both of the genes and is resistant to both races (Elgin and Ostazeski, 1985). Although the biochemical mechanisms for the race/cultivar specificity in this interaction remain unknown, evidence suggest that the synthesis of medicarpin, the major phytoalexin in alfalfa, is one of the important plant defenses taking place within the cultivar Arc (Baker *et al.*, 1989; O'Neill, 1996). In other studies, medicarpin has been implicated in the development of resistance in alfalfa against several pathogens (Blount *et al.*, 1992; Higgins, 1972; Olah and Sherwood, 1971; Vaziri *et al.*, 1981).

In addition to local resistance, alfalfa exhibits an acquired immunity during which tissues are protected from an attack by a virulent isolate after exposure to an avirulent isolate. O'Neill *et al.*, (1989) determined that the degree of protection from race 2 *C. trifolii* was dependent upon the spore density of race 1 *C.trifolii* inoculum. Later studies from O'Neill (1996) demonstrated that an accumulation of medicarpin occurred in protected alfalfa tissues (preinoculated by race 1) when challenged by race 2.

In this chapter I describe three different plant defense responses in leaves of the three different alfalfa cultivars challenged by race 1 or race 2 of *C. trifolii*: the accumulation of the phytoalexin medicarpin, the increase in β-1,3-glucanase activity and the increase in chitinase activity. Their possible roles in the outcome of the plant responses to the fungal pathogen are discussed.

MATERIALS AND METHODS

Fungal inoculation. Race 1 and race 2 of *Colletotrichum trifolii* were cultured on potato dextrose agar at 24°C. Spore suspensions were prepared by scraping spores from the agar plates flooded with sterile distilled water. The solution was filtered through Miracloth to remove mycelial fragments. Spore concentrations for all inoculations were adjusted to 10^6 spores/ml.

For each inoculation, stems supporting three trifoliate leaves were cut with scissors and placed into petri dishes containing a filter paper wetted with sterile deionized water. When indicated, leaves were wounded with a pinwheel (✱—) on their undersurface. Immediately after, they were inoculated with the appropriate spore suspension, or dipped into sterile deionized water (controls). Cuttings were dipped into spore suspension or

water for 2 min. Plates were sealed with parafilm and incubated at room temperature.

Alfalfa cultivars. Seeds of *Medicago sativa* cultivars Arc, Saranac and SaranacAR were provided by the United States department of Agriculture GRIN-Western Regional Plant Introduction Station (http://www.grin/usda.gov). Alfalfa is an autotetraploid and cross-pollinated. Thus, seeds issued from one plant are heterogeneous and therefore, not all the seeds from cultivars Arc or SarAR carry anthracnose resistance genes. Plants were grown in the greenhouse and about 10 to 15 plants were selected for resistance to the 2 races of *C. trifolii* (see next section). One plant of each cultivar was then multiplied through vegetative cuttings in order to increase the material available.

Disease evaluation. Resistance or sensitivity was evaluated 7 days after inoculation. Leaves were excised and cleared by boiling them in 100 % ethanol for 1 minute. Staining of fungal structures was obtained by dipping leaves for one minute in lactophenol cotton blue (Becton Dickinson Cat. No. 4361188). Lactophenol cotton blue is a blue stain for fungal elements allowing easier visualization and examination by microscopy. Leaves were

then examined by light microscopy. Disease severity was estimated by the presence or absence of fungal structures (acervuli mostly, setae and production of conidia) in the different cultivars.

Preparation of protein extracts from alfalfa leaves. Plants tissues (≈ 2 g) were frozen in liquid nitrogen, ground with a mortar and pestle and then resuspended in 3 ml of 100 mM sodium acetate, pH 5.0. Samples were centrifuged at 10,000 g for 15 min. The supernatants were dialyzed (3,500 Da MW cut off dialysis tubing, Spectrum, Fisher Cat. No. 086705B) overnight against 10 mM sodium acetate, pH 5.0, with one buffer change. Samples were concentrated using a centricon 10 concentrator (Amicon Cat. No. 4205). Protein concentrations were determined by the Bradford method with bovine serum albumin as a standard. Extracts were stored at 4°C until used for colorimetric assays and electrophoresis.

Glucanase colorimetric assay. Glucanase activity was measured according to the procedure provided by Sigma for the enzymatic assay of laminarinase (Sigma Cat. No. L5272) except the volumes used for the assay were reduced. The following reagents were pipetted into 1.5 ml Eppendorf tubes: 100 µl of laminarin solution (2.5% [w/v] of laminarin [Sigma Cat. No.

L9634] in water), 300 µl of 0.1 M sodium acetate buffer, pH 5.0, and 100 µl of sample to be tested. A negative control was carried out for each sample by replacing 100 µl of laminarin by 100 µl of deionized water. The blank sample contained 100 µl of deionized water, 100 µl of laminarin and 300 µl of buffer. All the samples were incubated for 1 h at 37°C. At the end of the incubation, a volume (900 µl) of deionized water was mixed with 1 ml of copper solution (Appendix 1), then 100 µl of each reaction were added. Samples for establishing a standard curve were made by serial dilutions of glucose. The water and the copper solutions were mixed first, then glucose was added. All samples were quickly vortexed and incubated for 10 min in a boiling water bath. After the samples had cooled (10 min), 1 ml of arsenic-molybdate solution (Appendix 1) was added to the samples. The tubes were shaken until foaming stopped and diluted to obtain accurate readings of OD at 540 nm for test, blank and standard samples.

Determination of glucanase activity:

$$\text{Units/ml enzyme} = \frac{(\mu g \text{ of glucose})^{1}\,(0.5)^{2}}{(60)^{3}\,(0.1)^{4}\,(0.1)^{5}}$$

[1]:µg of glucose equivalents was determined by using the standard curve
[2]:Total volume of the assay in ml
[3]:Time of assay in min
[4]:Volume of enzyme or plant sample used in the assay in ml

[5]:Volume of the assay used in colorimetric determination in ml
One Unit = liberation of 1 μg of reducing sugar (measured as glucose) from laminarin per minute at pH 5.0 at 37°C.

$$\text{nmol glucose released/mg protein} = \frac{\text{Units/ml enzyme /180 *1000}}{\text{Protein concentration of test sample}}$$

Chitinase colorimetric assay. Chitinase activity was measured according to Boller and Mauch (1988) with some modifications. The following were pipetted in order into 1.5 ml Eppendorf tubes: 0.4 ml of chitin (Sigma Cat. No. C8908) solution (1.25% [w/v] in 10 mM potassium phosphate, pH 7.0), 50 μl of sterile deionized water and 50 μl of the sample to be assayed. A negative control was carried out by replacing the chitin solution by the same volume of buffer. In addition, two different blank samples were prepared. One of them contained 0.4 ml of chitin solution with 100 μl of water, the second one 0.4 ml of buffer with 100 μl water. A positive control (chitinase, Sigma Cat. No.C6137) was also carried through the procedure. The tubes were incubated for 3 h at 37°C. The reaction was stopped by centrifugation (5,000 g, 5 min) and 175 μl of each supernatant was transferred into a clean tube. Chitooligosacharides were further hydrolyzed by adding 25 μl of N-acetyl-glucosaminidase (diluted 10-fold with water, NAGase, Sigma Cat. No. A3189) to each tube. After 2h at 37°C, 35 μl of freshly made 1M

sodium borate was added to the reaction. Standard curves were prepared with different dilutions of N-acetyl-*D*-glucosamine (NAG, Sigma Cat. No. A8625) to which sodium borate was also added. The samples were incubated for 3 min in a boiling water bath and then rapidly cooled in ice. After the addition of 1 ml of DMAB reagent (Appendix 1), the samples were incubated for 20 min at 37°C. The absorbance at 595 nm was measured using a Beckman 7500 diode array spectrophotometer and chitinase activity was determined as explained below.

Determination of chitinase activity:

$$\text{Units/ ml enzyme} = \frac{(\text{nmol NAG released})^{1} \quad (0.5)^{2}}{(180)^{3}\,(0.05)^{4}\,(0.175)^{5}}$$

[1]:nmol of NAG was determined by using the standard curve
[2]:Total volume of the assay in ml
[3]:Time of assay in min
[4]:Volume of enzyme or plant sample used in the assay in ml
[5]:Volume of the assay used in colorimetric determination in ml
One Unit = liberation of 1 nmol of NAG from chitin per minute at pH 7 at 37°C.

$$\text{Units/ mg protein} = \frac{\text{Units/ml enzyme}}{\text{Protein concentration of test sample}}$$

Native Polyacrylamide Gel Electrophoresis (PAGE). Polyacrylamide separating gels (15%) were prepared by mixing 11.5 ml of 30% (w/v)

acrylamide (30:0.8 acrylamide/bis), 2.8 ml of 3 M Tris-HCl (pH 8.9) for anodic PAGE or 2.8 ml of 3 M acetic acid-KOH (pH 4.3) for cathodic PAGE, and 8 ml of water. Ammonium persulfate (APS) (10% w/v) (225 µl for anodic PAGE and 500 µl for cathodic PAGE) and TEMED (5 µl) were added to the solution after degassing for 10 min. Stacking gels were made by addition of 1.3 ml of 30% (w/v) acrylamide, 2.5 ml of 0.5 M Tris-HCl (pH 6.8) for anodic PAGE or 2.5 ml of 0.5 M acetic acid-KOH (pH 6.7) for cathodic PAGE, 100 µl 10% APS and 4 µl of TEMED. Samples (150 µg of total proteins per lane) contained bromophenol blue or methylene blue for anodic or cathodic PAGE, respectively. Gels were electrophoresed at room temperature for 3 h at a constant voltage of 100 V.

Detection of chitinase isoforms. After electrophoresis, native polyacrylamide gels were incubated in 0.1 M sodium acetate (pH 5.0) for 5 min. They were then overlaid on a 1% agarose gel (attached to a glass plate) containing 0.04% (v/v) of glycol chitin and 0.1 M sodium acetate (pH 5.0). The apparatus was wrapped with parafilm. Bubbles that may have formed were eliminated by rolling a pipette over the polyacrylamide gel. Gels were incubated for 1h at 37°C. Acrylamide gels were removed and agarose gels were then incubated for 5 min at room temperature in freshly made 0.01%

(w/v) fluorescent brightener (Sigma Cat. No. F3543) in 500 mM Tris-HCl (pH 8.9). The brightener solution was removed and gels were destained in distilled water overnight in the refrigerator. Chitinase isozymes could be visualized as cleared bands under UV light.

Chitinase isoforms were designated with a C for chitinase, a for acidic, b for basic, and numbered sequentially from the lowest protein mobility (1) to the highest protein mobility.

Glycol chitin synthesis. The preparation of glycol chitin was carried out according to Trudel and Asselin (1989) and Molano et al. (1977). The procedure is described elsewhere in Chapter 4 (chitinase radiometric assay). Only two changes were made: the acetic anhydride used here was not tritiated and the final step of the procedure differed. Instead of being filtered, the chitin was centrifuged at 10,000 g for 15 min at 4°C. Then the pellet was resuspended in 1 volume of methanol, homogenized in a mortar and pestle, and centrifuged as described above. The pellet was resuspended in 0.02% sodium azide to give a final 1% (w/v) stock solution.

Detection of glucanase isoforms. After electrophoresis, acrylamide gels were incubated in 0.05 M sodium acetate (pH 5.0) for 5 min, and were then

incubated for 45 min at 37°C in a laminarin solution. The laminarin (1 g) was dissolved in a beaker placed in a boiling waterbath with 75 ml of water and 75 ml of 0.05 M sodium acetate (pH 5.0). Gels were then incubated in methanol: water: acetic acid (5:5:2;v/v/v) for 5 min and washed in water. Staining of glucanase isoforms was obtained by microwaving the gels for 3 min in a solution containing 0.3 g of 2,3,5-triphenyltetrazolium chloride (Sigma Cat. No. T8877) in 200 ml of 1 M NaOH.

Glucanase isoforms were designated with a G for glucanase, a for acidic, b for basic, and numbered sequentially from the lowest protein mobility (1) to the highest protein mobility.

Analysis of isoflavonoids. Medicarpin and other isoflavonoids were extracted from alfalfa leaves and quantitated by HPLC as described previously by Edwards and Dixon (1991) and Kessmann *et al.* (1990).

Leaves (0.5 g to 1 g of fresh weight [FW]) were weighed and ground in liquid nitrogen in a mortar and pestle. Cold acetone (10 ml/g FW) was added to the powder and the extract was incubated overnight at 4°C with shaking. The crude extracts were centrifuged at 3,000 g for 10 min and the pellets were resuspended in cold acetone (5 ml/g FW) and centrifuged as above. The supernatants were pooled, dried under nitrogen, and resuspended

in 100% methanol (1 ml/g FW). Samples (20 µl) were applied to a Metasil ODS C18 reverse phase column (250 × 4.6 mm, 5 µm packing, Metachem), and eluted at 0.8 ml/min with a linear gradient of acetonitrile increasing from 20% to 54% in 1% phosphoric acid over 45 min. The column was then washed for 10 min with 100% acetonitrile and reequilibrated with 20% acetonitrile for 10 min. The eluant was monitored for UV absorbance at 287 nm and 310 nm. Identification and quantification of free medicarpin was achieved by running samples of authentic medicarpin and diode array analysis of UV spectra. To determine if the medicarpin conjugates were glycosylated, samples were digested by almond β-glucosidase (Sigma Cat. No. 49290). Samples were first dried under nitrogen and resuspended in 3 ml of 0.15 M citrate-phosphate buffer, pH 5.2 (Appendix 1). Digestion with 2 mg/ml β-glucosidase was carried out overnight at 37°C with gentle shaking. Isoflavonoids were extracted with ethyl acetate (1 v/v) twice. After centrifugation (3 min at 10,000 g), the upper phase was pooled and dried under nitrogen. Samples were resuspended in 120 µl of 100% methanol prior to HPLC analysis as above.

RNA gel blot analysis.

1.*Total RNA extraction.*

To avoid RNAse contamination, glassware was baked in an oven at 200 °C overnight, gloves were always used and pipette tips were autoclaved several times. Total RNAs were isolated from 300 mg of frozen tissue of alfalfa leaves (Arc) using TRI REAGENT (Molecular Research Center Inc. Cat. No. TR118). Tissues were ground in liquid nitrogen with a mortar and pestle, and homogenized in 2 ml of TRI REAGENT. Homogenates were incubated for 5 min at room temperature and centrifuged at 10,000 g for 10 min at 4°C. Resulting supernatants were transferred to a fresh tube and 0.4 ml of chloroform was added. Samples were shaken for a few seconds, held at room temperature for 2 to 15 min, and centrifuged at 10,000 g for 15 min at 4°C. The mixtures separated into a lower red phenol-chloroform phase, an interphase and a colorless aqueous phase. RNA remained mostly in the upper phase whereas the DNA and proteins were in the interphase and lower phase. RNAs were precipitated by mixing the aqueous phase with 0.5 ml of isopropanol and 0.5 ml of 3 M sodium acetate (DEPC-treated). Solutions were stored at room temperature for 5 to 10 min and centrifuged at 12,000g for 8 min. RNA pellets were washed with 75% ethanol (1 ml) and subsequently centrifuged at 7,500 g for 5 min at room temperature. RNA

pellets were air dried for about one hour and resuspended in 50-100 µl of DEPC-treated water. To facilitate the solubilization of RNAs, solutions were incubated 5-15 min at 55-60°C in a waterbath.

2. *Formaldehyde agarose gel electrophoresis.*

Agarose (1.2 g) was added to 100 ml of 1X MOPS (Appendix 1). Agarose was dissolved by heat in a microwave and melted agarose was cooled to 65°C before addition of 1.8 ml of 37% (v/v) (12.3 M) formaldehyde. The mixture was poured into a gel box previously treated with DEPC-treated water. RNA loading buffer (6X) (Appendix 1) was added to the total RNA (10 µg in 20 µl DEPC-treated water) to a final concentration of 1X. Samples were incubated 3-5 min in a boiling water bath, chilled on ice and loaded onto the gel after addition of 0.5 µl of ethidium bromide per sample. A RNA ladder (0.24-9.5 Kb RNA ladder, GibcoBRL Cat. No.15620-016) was also loaded onto the gel. Electrophoresis was carried out at 70 V for 2-3 h.

3.RNA transfer to nylon membranes.

Total RNAs were transferred to Nylon membranes (Hybond N+, Amersham) with the turboblotter from Schleicher & Schuell. After completion of the electrophoresis, RNA gels were rinsed twice in deionized water, and twice in DEPC-treated water prior to the transfer. Membranes were wetted in distilled water then soaked in 20 X SSC (Appendix 1) transfer buffer for 5

min. The transfer apparatus were assembled according to the manufacturer's instructions, and transfers were carried out overnight. Membranes were then washed in 2 X SSC for 5 min. RNAs were fixed to the membranes by baking them at 80 °C for 2 h under vacuum or were cross-linked by exposing the blot to UV light of 254-312 nm for 5 min.

4.*RNA hybridization*

Membranes were wetted in 6X SSC buffer for 2 min, and sealed into a plastic bag with 20 ml of prehybridization buffer (Appendix 1). Prehybridizations were carried out at 42°C for at least 4 h. Prehybridization buffer was poured off and replaced with hybridization buffer (Appendix 1) containing a labeled cDNA probe (Ready to go DNA labelling Beads [dCTP], Amersham Pharmacia Biotech Cat. No. 27924001). The alfalfa cDNAs (*PAL, C4H, CHS, CHR, CHI, IFS, IOMT, IFR* and *Aglu1*) used in this experiment were provided by Dr. RA Dixon's laboratory (Samuel Roberts Noble Foundation). Hybridizations were carried out overnight at 42°C. The following day, membranes were washed one time at 55°C in 2 X SSC buffer containing 0.5% SDS, one time at 55°C in 2 X SSC /0.1% SDS buffer, and one time at room temperature in 1 X SSC/0.1% SDS buffer. One additional wash was performed at 55°C in 1 X SSC/0.1% SDS buffer if

needed. Membranes were then placed into cassettes with X-ray film (RX Fuji medical X-ray film, Fisher Cat. No. 04441115) at -80°C for at least 48 h. Films were developed and fixed with Kodak GBX solutions.

RESULTS

1. Evaluation of disease symptoms caused by Colletotrichum trifolii in alfalfa.

Isolates from race 1 and race 2 of *C. trifolii* exhibited morphological differences on potato dextrose agar media (Figure 2.1). The edges of the race 1 cultures were uneven compared to the race 2 cultures. Moreover, the number of spores collected from the agar plates was higher for race 2 compared to race 1 for the same number of plates. However, the germination of spores from each inoculum was nearly 100%.

Previous anthracnose disease resistance evaluations showed that 65-75 % of Arc seedlings were resistant to race 1 of *C. trifolii,* 59% of SarAR seedlings were resistant to race 1 and 53% of SarAR seedlings were resistant to race 2 (Elgin and Ostazeski, 1982). Therefore it was necessary to select appropriate individual plants for vegetative propagation prior to investigating the potential induction of plant defenses during a compatible and an incompatible interaction.

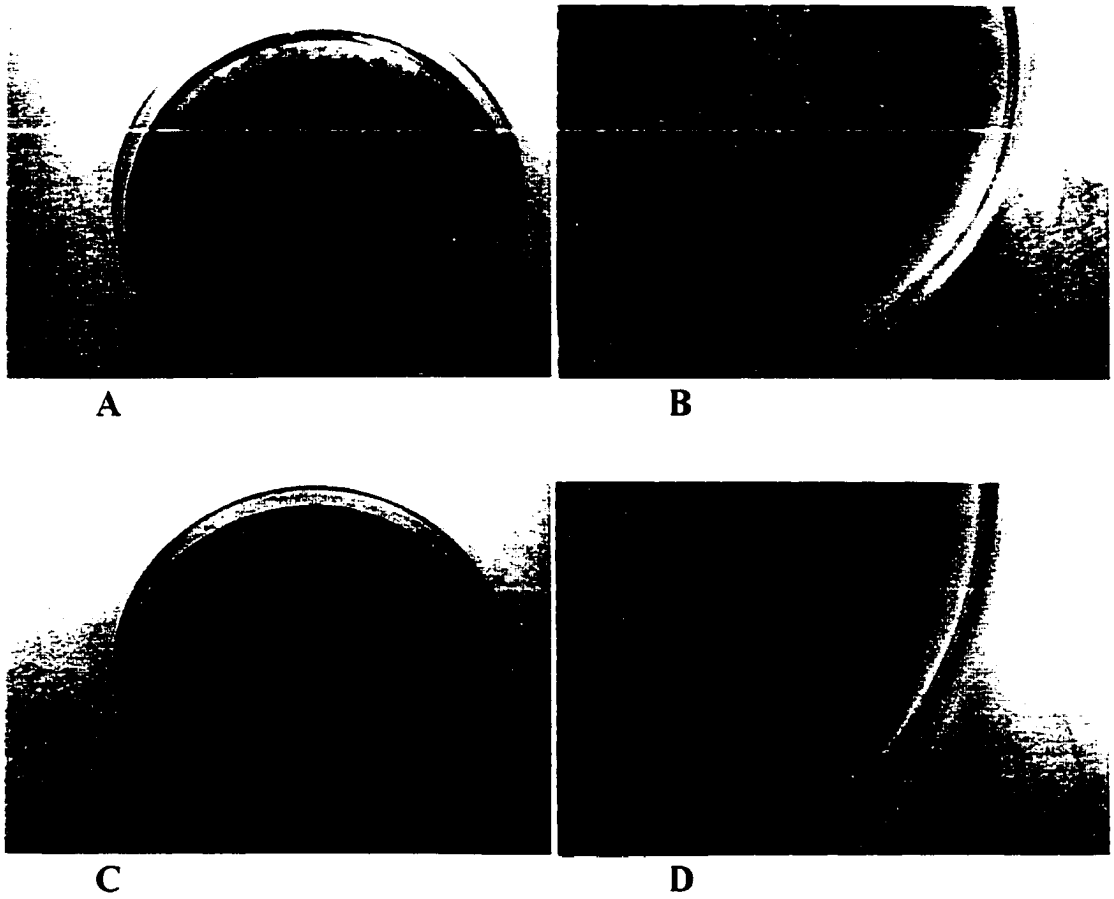


Figure 2.1: Cultures of *Colletotrichum trifolii* on PDA plates; A- & B-, race 1; C- & D-, race 2.

Fourteen Sar plants, sixteen SarAR plants, and eleven Arc plants were evaluated for resistance or susceptibility to race 1 and 2 of *C. trifolii*. All Sar plants were susceptible to both races of the fungus. As expected, not all plants grown from seeds of "cultivars" carrying resistance to the two races were resistant to the fungus. Out of sixteen SarAR plants, three were resistant to both races and six were resistant only to race 1. Seven out of eleven Arc plants were resistant to race 1 and all of them were susceptible to race 2. From this preliminary selection, two plants of each cultivar were chosen and re-evaluated. Results from this experiment are presented in Figures 2.2 and 2.3.

No symptoms of the infection could be detected after examination by eye or light microscopy in alfalfa leaves four days after infection analysis. However, symptoms were detected seven days after fungal inoculation. Wilting and dark brown to black specks could be seen by eye on plants susceptible to *C. trifolii*. The specks corresponded to setae emerging from acervuli. No fungal structures were detected in non-infected leaves (only Arc cultivar shown: Figure 2.2, A). A differential response to infection by race 1 and race 2 was observed for the Arc plants selected for further investigation. No acervuli were detected in leaves infected with race 1 of *C. trifolii* (incompatible interaction; Figure 2.2, B), whereas many acervuli that stained

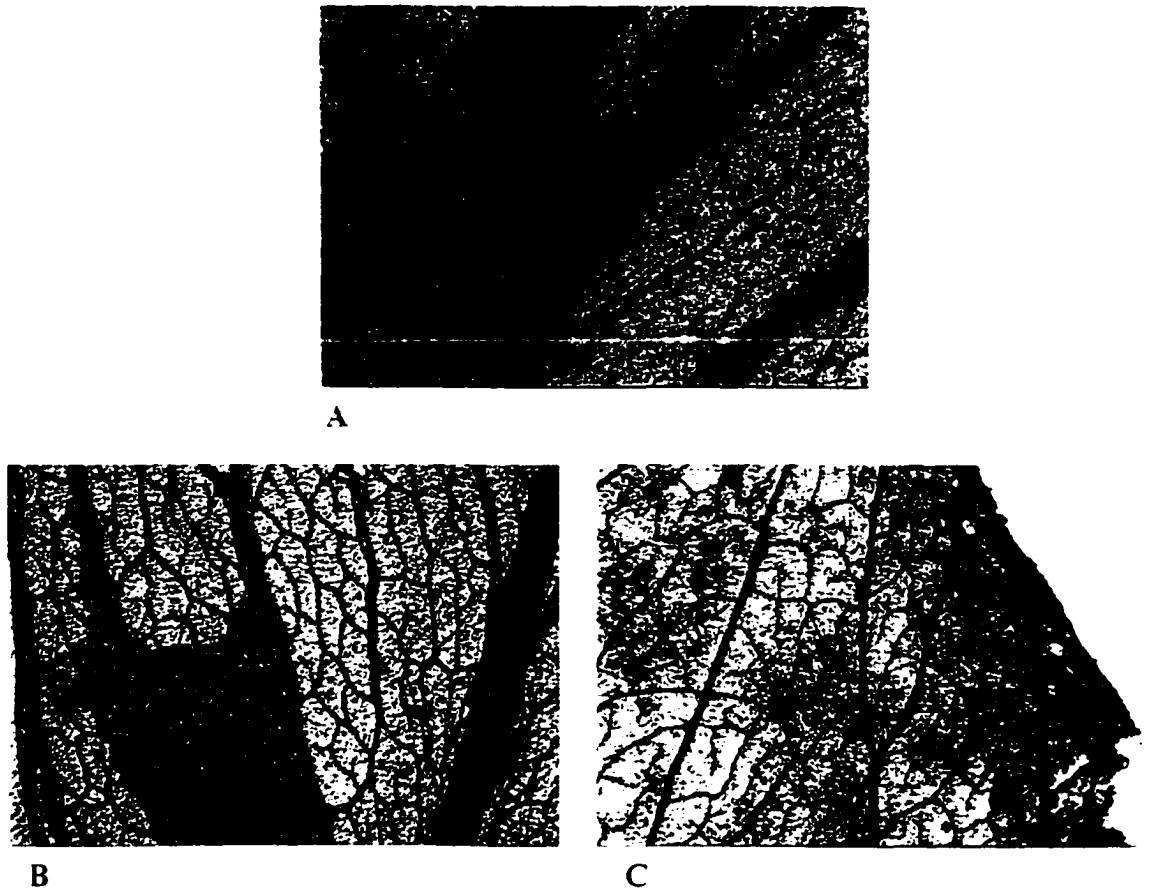


Figure 2.2: Reactions of Arc cultivar leaves to race 1 and race 2 of *Colletotrichum trifolii*. 7 days after infection. Arc is resistant to race 1 and susceptible to race 2. (A) Water-inoculated. (B) Arc/race 1: incompatible interaction. (C) Arc/race 2: compatible interaction. Some of the fungal structures (acervuli) detected in (C) are indicated by red arrows.

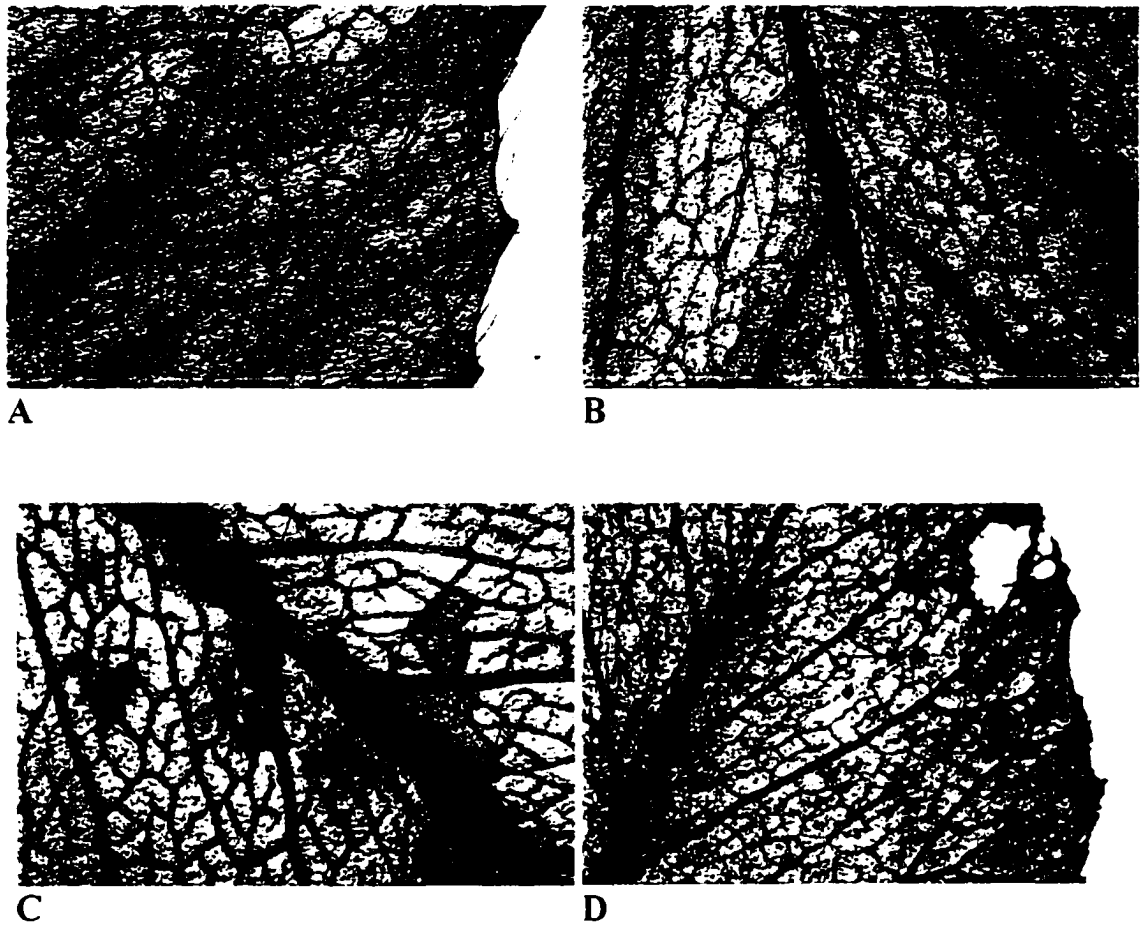


Figure 2.3: Reactions of SarAR and Sar cultivar leaves to race 1 and race 2 of *Colletotrichum trifolii*, 7 days after infection. SarAR is resistant to race 1 and race 2, Sar is susceptible to both races. (A) SarAR/race 1: incompatible interaction. (B) SarAR/race 2: incompatible interaction. (C) Sar/race 1: compatible interaction. (D) Sar/race 2: compatible interaction. Some of the fungal structures detected in (C) and (D) are indicated by red arrows.

blue were present in leaves infected with race 2 of the fungus (compatible interaction, Figure 2.2, C). SarAR when inoculated with either race didn't show any symptoms of disease (Figure 2.3, A and B). Sar leaves were covered with acervuli after infection by race 1 or 2 of the fungus (Figure 2.3, C and D). Additionally, some conidia were seen.

Another experiment was carried out to observe potential necrosis that may be associated with a HR. Leaves were wounded with a tracing wheel and then dipped into fungal suspension or water for control. The wounding seemed to affect the resistance/susceptibility to the fungus. Instead of having no acervuli present in their leaves when the tracing wheel was not used, Arc and SarAR cultivars developed few acervuli when infected by race 1 or either race of *C. trifolii,* respectively. However, more of these fungal structures were seen for Arc and Sar cultivars when infected by race 2 or either race of the fungus, respectively.

Interestingly, a brownish color was observed around the site of wounding, when examined under the microscope or by eye, as shown in Figures 2.4 and 2.5. Very little necrosis was observed for water inoculated leaves (Figure 2.4, A; data not shown for SarAR and Sar), or the Sar cultivar infected by either race (Figure 2.5, C and D). SarAR showed a little more necrosis than Sar (Figure 2.5, A and B) but Arc had distinct brown circles

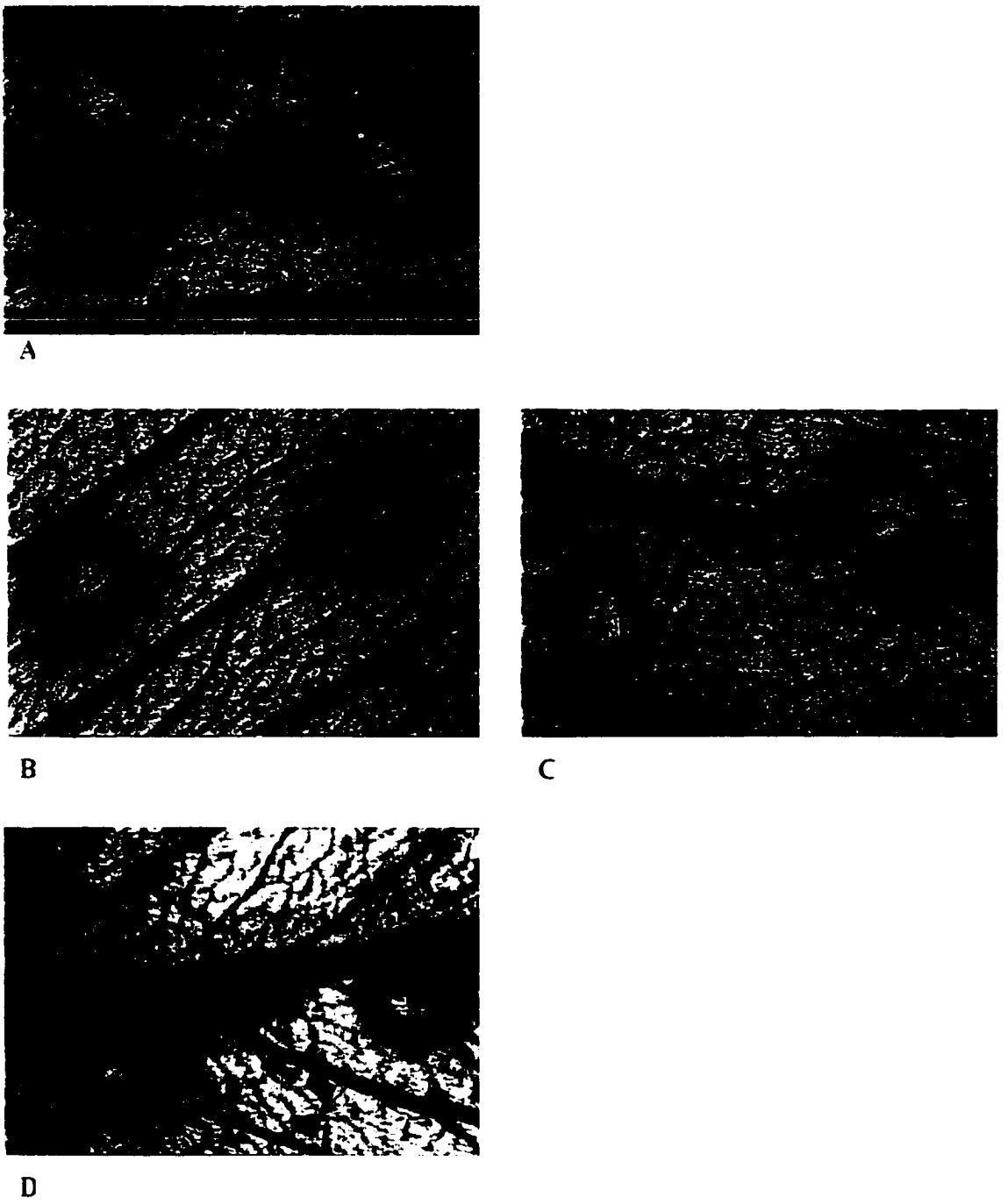


Figure 2.4: Reactions of Arc cultivar leaves to race 1 and race 2 of *Colletotrichum trifolii*, 7 days after infection with prior wounding. Arc is resistant to race 1 and susceptible to race 2. (A) Water-inoculated. (B) (C) Arc/race 1: incompatible interaction from two different infections. (D) Arc/race 2: compatible interaction. Some of the fungal structures detected in (B) (C) and (D) are indicated by red arrows.

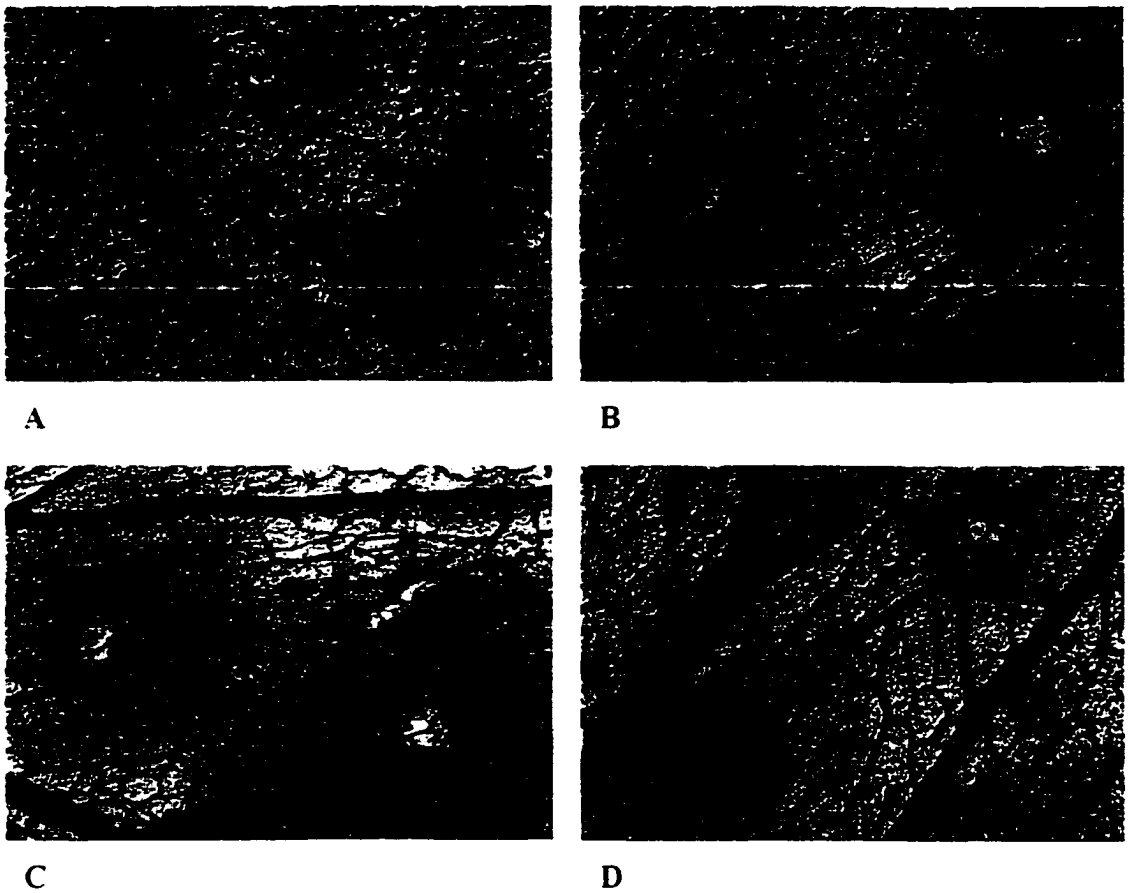


Figure 2.5: Reactions of SarAR and Sar cultivar leaves to race 1 and race 2 of *Colletotrichum trifolii*, 7 days after infection, with prior wounding. SarAR is resistant to race 1 and race 2, Sar is susceptible to both races. (A) SarAR/race 1: incompatible interaction. (B) SarAR/race 2: incompatible interaction. (C) Sar/race 1: compatible interaction. (D) Sar/race 2: compatible interaction. Some of the fungal structures are indicated by red arrows.

around the wound sites (Figure 2.4, B, C and D). Another interesting aspect was the spread of the fungus around wound sites detected with lactophenol cotton blue staining. As expected, in the compatible interactions the fungus had spread from the wound sites (Figure 2.4, D; Figure 2.5, C and D), but this did not occur in incompatible interactions (Figure 2.4, B and C), where the fungus seemed trapped inside the wounding sites (Figure 2.4, B and C). Unexpectedly, the fungus had spread beyond the wound site in SarAR leaves infected by either race of *C. trifolii* (Figure 2.5, A and B).

2. *Induction of chitinase and glucanase activities in alfalfa leaves infected by C. trifolii.*

Differences in the patterns of acidic glucanase isoforms were observed in the extracts of uninfected leaves of the three different cultivars (Figure 2.6, A) and were further confirmed (Figure 5.6). Isoforms Ga1 and Ga2 were present in Arc, whereas Ga2 and Ga3 were detected in Sar. Ga1 and Ga3 were observed in SarAR. All cultivars seemed to have the same acidic chitinase isoform patterns (Figure 2.6, B). Ca2 and Ca3 are two separate isoforms with similar pIs. An additional band (Ca1) appeared in some samples but presented less activity than Ca2 and Ca3.

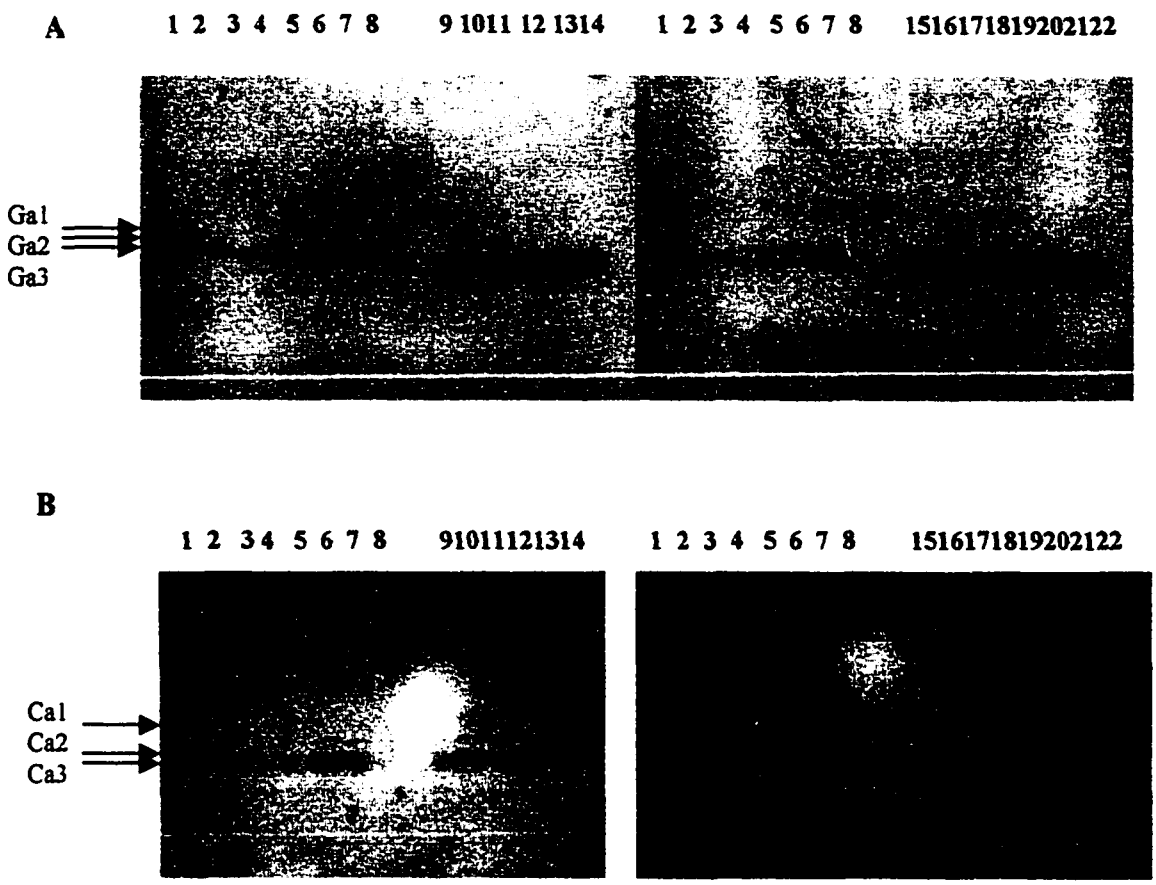


Figure 2.6: Glucanase and chitinase isoforms separated by anodic native PAGE from extracts of non-infected leaves. Crude enzyme extracts were loaded in each lane of a 12% polyacrylamide gel. Glucanase activity was revealed after incubation of polyacrylamide gels for 45 min at 37°C in laminarin solution (A). Chitinase activity in agarose gels (containing 0.04% glycol chitin) overlaid with acrylamide gels was revealed after incubation for 1 h at 37°C (B). Different isoforms were defined based on mobility and are noted with arrows. Lanes 1 to 8: Arc extracts. Lanes 9 to 14: Sar extracts. Lanes 15 to 22: SarAR extracts.

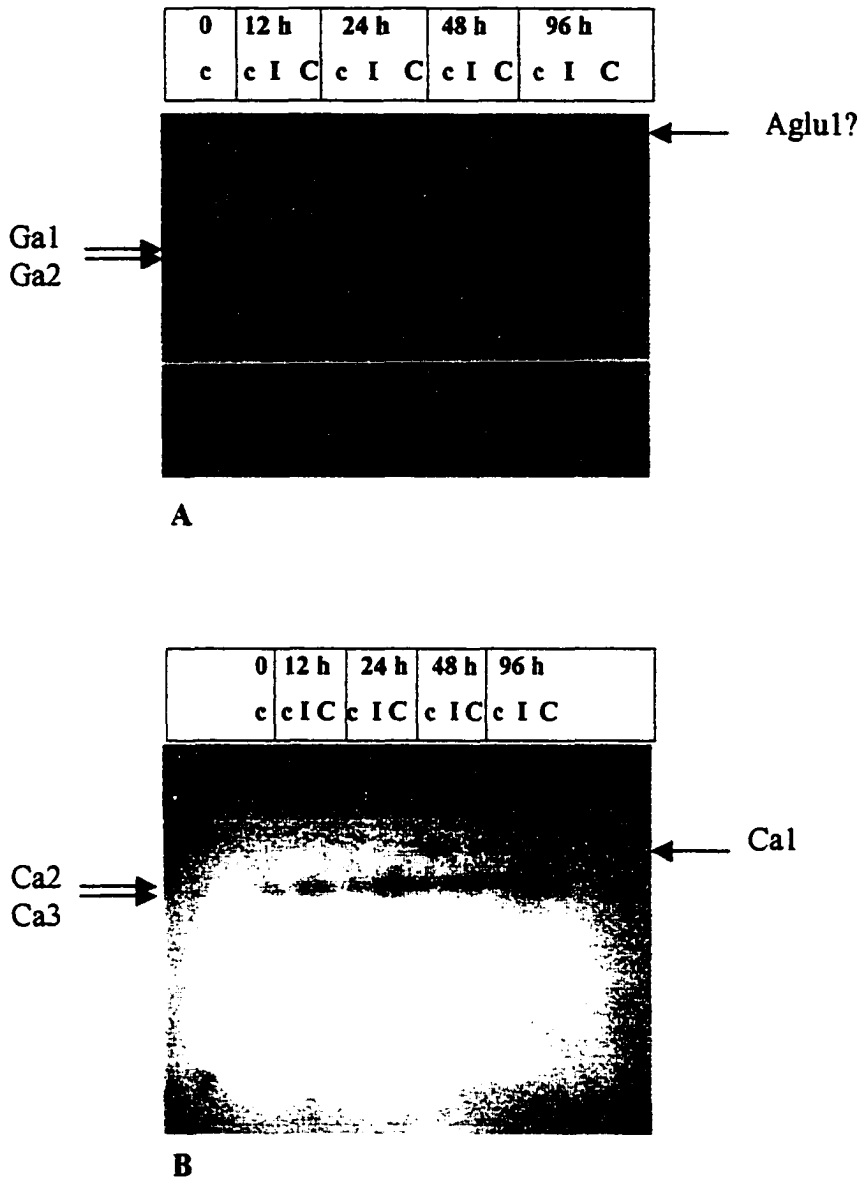


Figure 2.7: Glucanase (A) and chitinase (B) isoforms separated by anodic native PAGE from Arc leaf extracts inoculated with race 1 and race 2 of *C. trifolii* represented by I (incompatible interaction) and C (compatible interaction) respectively. Mock-inoculated leaf extracts are represented by c. Crude enzyme extracts (100 µg of total proteins) were loaded in each lane of a 12% polyacrylamide gel. Glucanase activity was revealed after incubation of polyacrylamide gels for 45 min at 37°C in laminarin solution (A). Chitinase activity in agarose gels (containing 0.04% glycol chitin) overlaid with acrylamide gels was revealed after incubation for 1 h at 37°C (B). Different isoforms were defined based on mobility and are noted with arrows.

Using a colorimetric assay of chitinase and glucanase, I found that there were no significant differences in glucanase and chitinase activity between resistant and susceptible alfalfa leaves of any of the cultivars infected by *C. trifolii.* This conclusion was supported by results of native PAGE analysis (Figure 2.7, A and B).

The two acidic glucanases, Ga2 and Ga3, and the three acidic chitinases Ca1, Ca2 and Ca3 detected in non-infected leaves (Figure 2.6) are present in Arc leaves during the infection by *C. trifolii* (Figure 2.7) but as mentioned above, there was no significant increase in the activity of these isoforms. An additional isoform with lower mobility than Ga1 appeared in race 1 or 2 infected leaves 96 h after inoculation as well as in non-infected leaves. This isoform is most likely the Aglu1 glucanase isoform detected in alfalfa roots (Chapter 4, Figures 4.5 and 4.11).

3. Phytoalexin response in alfalfa leaves infected by C.trifolii.

HPLC was used for the determination of free medicarpin levels in the leaves of the three different cultivars during compatible and incompatible *C. trifolii* interactions. Results of the medicarpin analysis are presented Figures 2.8, 2.9 and 2.10. Accumulation of medicarpin was observed only in the Arc cultivar and not in the SarAR or Sar cultivars. Levels of medicarpin in Sar

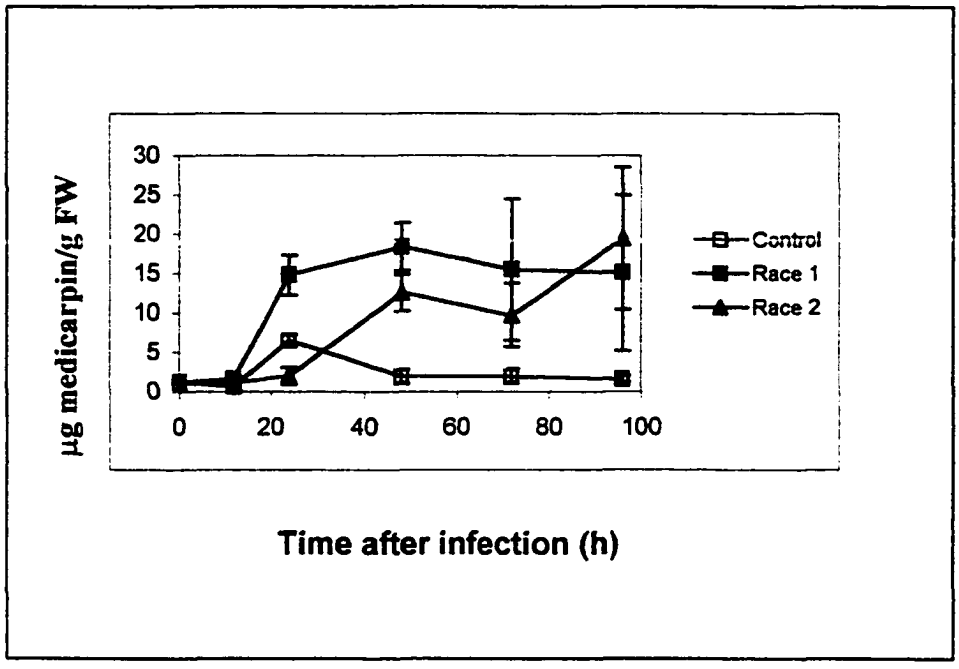


Figure 2.8: Time course of accumulation of the phytoalexin medicarpin in Arc cultivar leaves inoculated with avirulent and virulent races of *C. trifolii*, race 1 and race 2, respectively. Open symbols represent non-infected leaves (control) and closed symbols represent infected leaves. The data represent the mean of two determinations, one of each made on extracts from leaves infected by two independent batches of fungal inoculum. Vertical lines through the data points represent standard errors of the means.

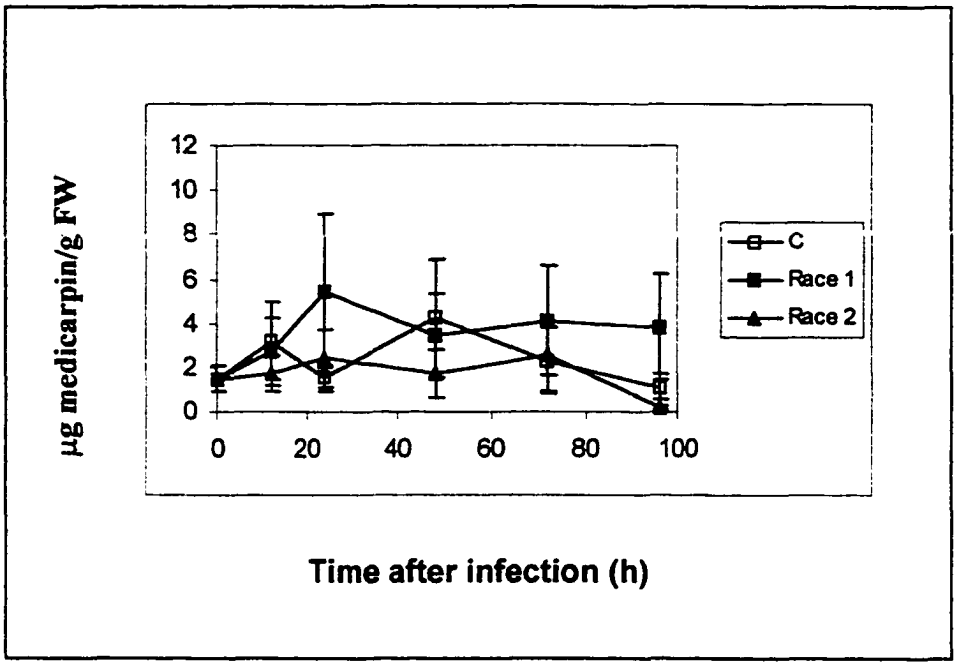


Figure 2.9: Time course of accumulation of the phytoalexin medicarpin in Sar cultivar leaves inoculated with virulent races of *C. trifolii*, race 1 and race 2. Open symbols represent non-infected leaves (control) and closed symbols represent infected leaves. The data represent the mean of two determinations, one of each made on extracts from leaves infected by two independent batches of fungal inoculum. Vertical lines through the data points represent standard errors of the means.

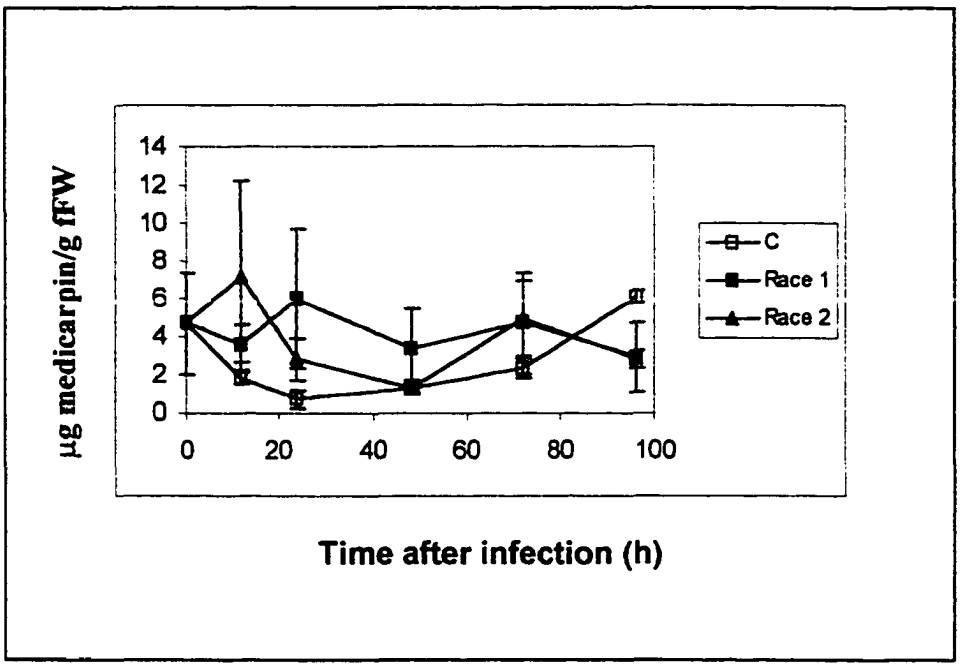


Figure 2.10: Time course of accumulation of the phytoalexin medicarpin in SarAR cultivar leaves inoculated with avirulent races of *C. trifolii,* race 1 and race 2. Open symbols represent non-infected leaves (control) and closed symbols represent infected leaves. The data represent the mean of two determinations, one of each made on extracts from leaves infected by two independent batches of fungal inoculum. Vertical lines through the data points represent standard errors of the means.

and SarAR leaves infected by either race of the fungus remained low throughout the experiment [≤ 7 μg/g FW] (Figure 2.9 and 2.10). Medicarpin content increased more rapidly in the incompatible interaction between *C. trifolii* and Arc than in the compatible interaction (Figure 2.8). The level of medicarpin started to increase 12 h after infection with race 1, and remained higher than the level in Arc leaves infected with race 2 for up to 72 h. Medicarpin content was 70% greater in the incompatible interaction than in the compatible interaction, 24 h after inoculation. The difference between the two interactions was not as obvious for later time points. The same maximum level of medicarpin was reached in both interactions (18-20 μg/g FW), albeit the phytoalexin level reached a maximum earlier in race 1 infected Arc leaves (48 h) than in race 2 infected Arc leaves (96 h).

In one of my experiments, HPLC analysis of alfalfa leaves from Arc cultivar infected by both races of *C. trifolii* revealed that no accumulation of free medicarpin was observed. HPLC profiles of extracts from Arc leaves infected by race 1 or 2 and from mock-inoculated leaves are shown only for 72 h after inoculation (Figure 2.11 to 2.13, A). Analysis of all the peaks present in the extracts with computer analysis software comparing each UV spectrum with spectra of authentic standards revealed that several compounds had very similar absorption spectra to that of free medicarpin

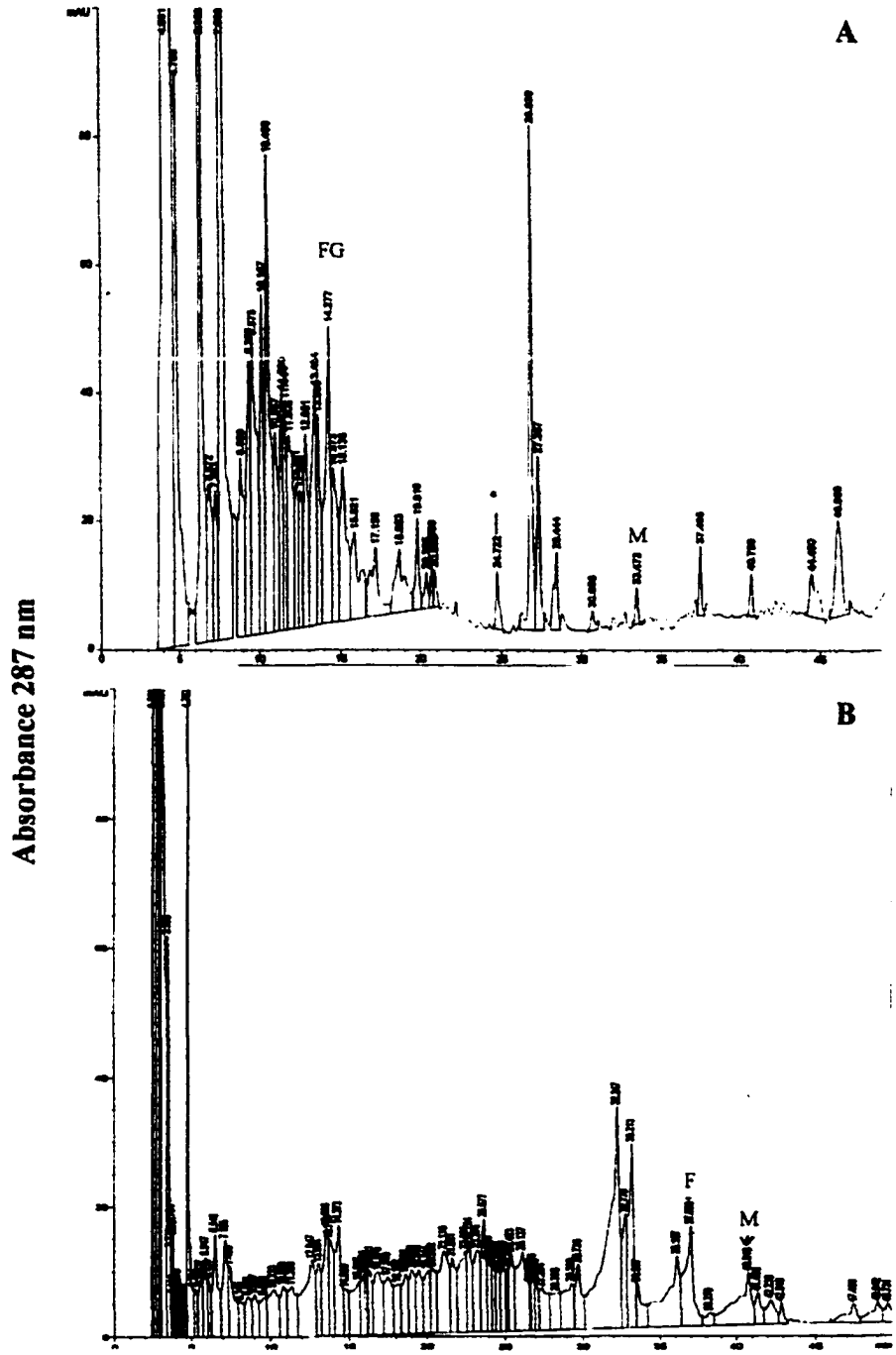


Figure 2.11: HPLC analysis of non-infected leaf extracts from Arc 72 h after water immersion, with eluant monitored at 287 nm (A, B). Same extract treated with β-glucosidase (B). M = medicarpin; F = formononetin; FG = formononetin-7-*O*-glucoside; * = glycosylated medicarpin.

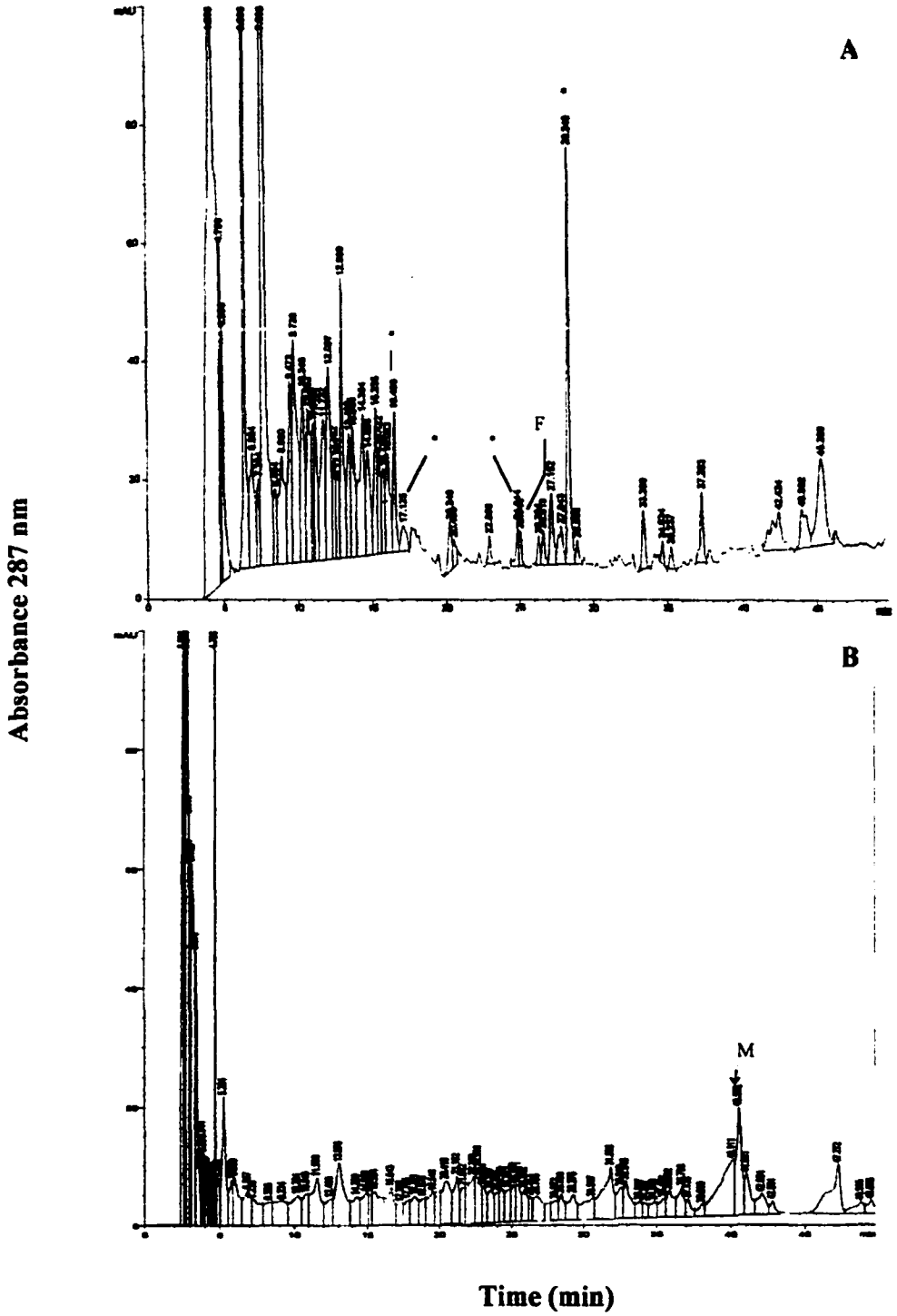


Figure 2.12: HPLC analysis of extracts of Arc leaves infected with *C. trifolii* Race 1 (72 h after inoculation), with eluant monitored at 287 nm (A). Same extract treated with β-glucosidase (B). M = medicarpin; F = formononetin; FG = formononetin-7-*O*-glucoside; * = glycosylated medicarpin.

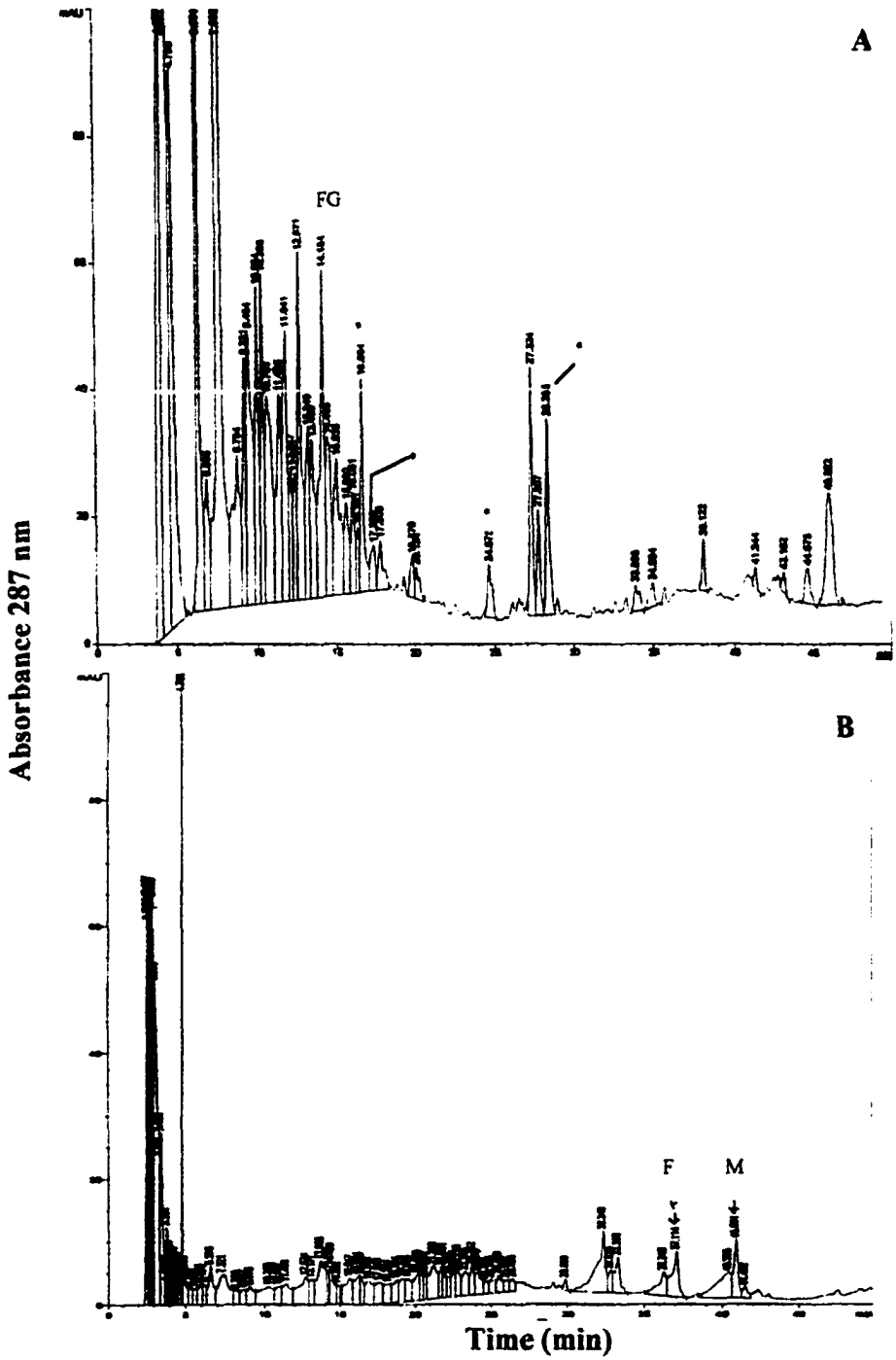


Figure 2.13: HPLC analysis of extracts of Arc leaves infected with *C. trifolii* Race 2 (72 h after inoculation), with eluant monitored at 287 nm (A). Same extract treated with β-glucosidase (B). M = medicarpin; F = formononetin; FG = formononetin-7-*O*-glucoside; * = glycosylated medicarpin.

as illustrated in Figure 2.14. Free medicarpin usually has a retention time around 35-36 min, but the putative conjugates were eluted at 16.5, 17.2, 24.6 and 28.4 min. Previous studies in alfalfa have identified several conjugates of medicarpin (MG: medicarpin-3-*O*-glucoside or MGM: medicarpin-3-*O*-glucoside-6"-*O*-malonate) or its intermediates (FG:formononetin-7-*O*-glucoside or FGM: formononetin-7-*O*-glucoside-6"- *O*-malonate) as compounds present in elicited cells (Kessmann *et al.*, 1990b) and in roots of healthy alfalfa plants (Tiller *et al.*, 1994).

To determine if the compounds eluting at 16.5, 17.2, 24.6 and 28.4 min were glycosylated derivatives of medicarpin, part of the extracts were digested with β-glucosidase. Treatment of crude extracts of infected leaves with β-glucosidase resulted in the appearance of medicarpin (M) eluting around 40.5 minutes, and a concomitant disappearance of compounds with UV spectra similar to that of medicarpin eluting at 16.5, 17.2, 24.6 and 28.4 min (Figures 2.11-2.13, B). It was therefore concluded that compounds with similar spectra to that of medicarpin were most likely glycosylated medicarpin conjugates. Tiller *et al.* (1994) identified MGM in alfalfa root extracts eluting at 22-23 min, which is close to the retention time of some of the medicarpin conjugates detected here. Further analysis of these conjugates is not yet conducted and it is necessary to determine if these

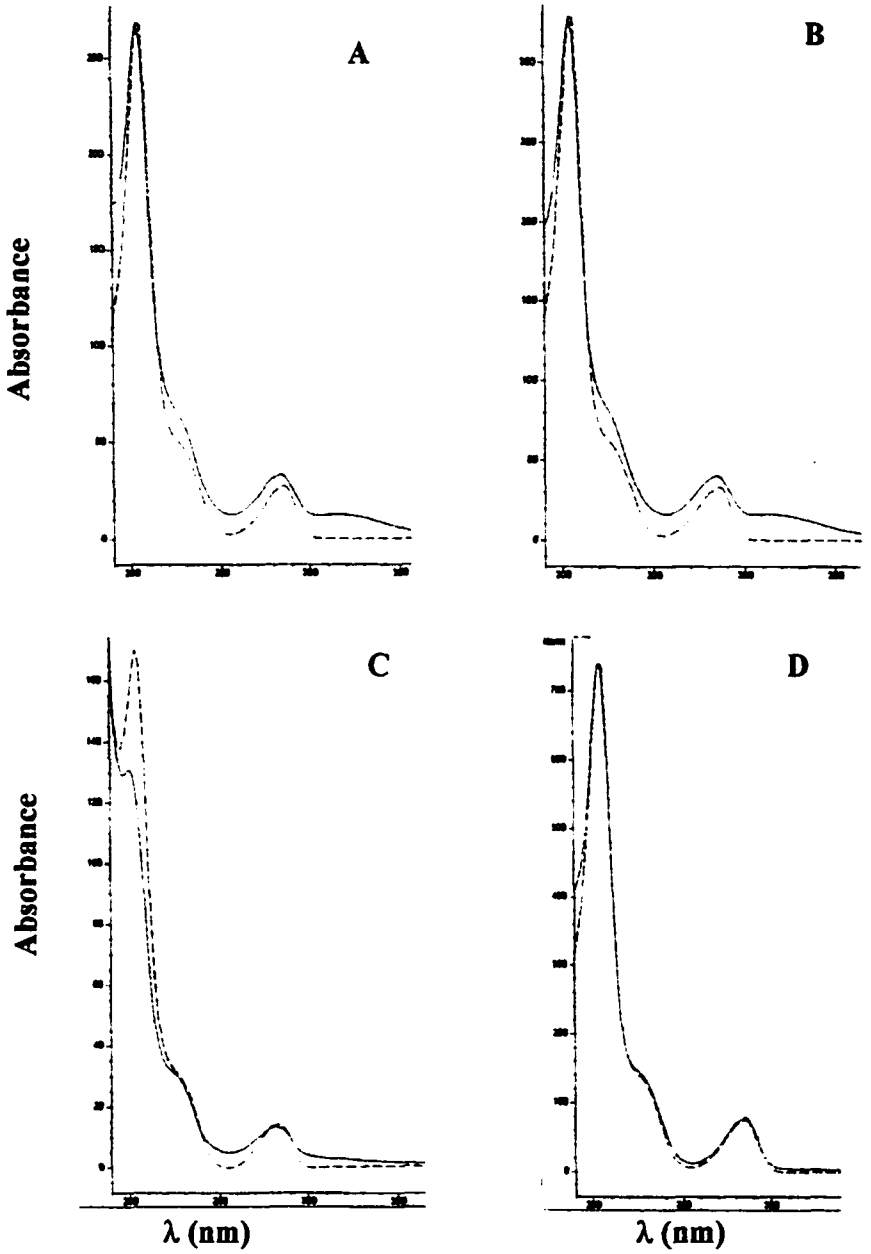


Figure 2.14: UV absorption spectra of authentic medicarpin (------), and compounds eluting at 16.5 min (A), 17.3 min (B), 24.6 min (C) and 28.4 min (D) from *C. trifolii* infected leaf extracts (——). Percentages of identity between (A), (B), (C), (D) and medicarpin: 97%, 97%, 92% and 99%, respectively.

compounds are identical to MGM identified previously by Kessmann *et al.* (1990b) or Tiller *et al.* (1994).

In race 2 infected leaves and in non-infected leaves, a compound with a UV spectrum like that of formononetin-7-O-glucoside (FG) (Figures 2.11 and 2.13, A, Figure 2.15) eluting at 14.5 min was detected. The extracts incubated with β-glucosidase gave rise to a formononetin peak (F) eluting at 37 min (Figures 2.11 and 2.13, B). Formononetin (F) was only detected in the incompatible interaction (elution time of 25 min) (Figure 2.12, A) and was also present 96 h after inoculation. In the incompatible interaction FG was only detected 48 h after inoculation (data not shown).

Non-infected leaves of Arc cultivar contained relatively low levels of medicarpin in either their free or conjugated forms (Table 2.1) except for 96 h after inoculation. A significant increase of glycosylated medicarpin was observed in an incompatible interaction (race 1 infected leaves) 48 h after inoculation. The level of medicarpin conjugates stayed high up to 72 h and then disappeared at 96 h after inoculation (Table 2.1). The accumulation of putative medicarpin conjugates in the compatible interaction (race 2 infected leaves) occurred later (72 h after inoculation) than in the incompatible interaction. The conjugates remained present at high levels until 96 h after

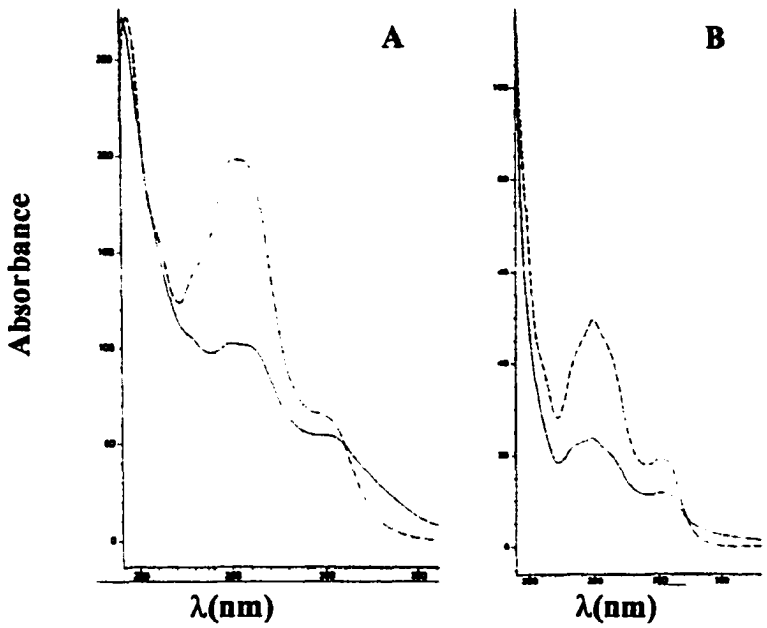


Figure 2.15: UV absorbtion spectra of authentic standards (------), [formononetin-7-*O*-glucoside (A) and formononetin (B)] and compounds eluting at 14.5 min (A) and 25 min (B) from *C. trifolii* infected leaf extracts (——). Percentages of identity between (A) and formononetin-7-*O*-glucoside: 93%; (B) and formononetin: 97%.

Table 2.1: Concentrations of free (M) and conjugated medicarpin (MC) in a compatible/incompatible interaction between Arc and *C. trifolii*. Concentrations are expressed in µg/g FW of tissue. c, control; I, incompatible interaction; C, compatible interaction.

Samples		MC	MC	MC	MC	M
		Retention times (minutes)				
		28.9	24.6	17.3	16.7	33.9
0	c	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2
	c	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2
6H	I	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2
	C	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2
	c	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2
12 H	I	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2
	C	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2
	c	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2
24 H	I	4.4	≤0.2	≤0.2	≤0.2	≤0.2
	C	6	≤0.2	≤0.2	≤0.2	≤0.2
	c	3.6	≤0.2	≤0.2	≤0.2	≤0.2
48 H	I	13.3	2.9	≤0.2	8.8	≤0.2
	C	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2
	c	≤0.2	3.1	≤0.2	≤0.2	1.4
72 H	I	20	2.6	1.4	7	≤0.2
	C	10.9	4	13.9	12.5	2.4
	c	13.4	≤0.2	≤0.2	9.3	≤0.2
96 H	I	≤0.2	12.3	≤0.2	≤0.2	4.5
	C	13.2	≤0.2	19.4	≤0.2	3

inoculation (Table 2.1).

Reanalysis of earlier HPLC data revealed that medicarpin conjugates were also detected in other experiments. No major changes in the quantity of medicarpin conjugates were observed for Sar and SarAR cultivars throughout the infection as noted earlier for free medicarpin. For the Arc cultivar, one putative conjugate of medicarpin eluting at around 21 min seemed to increase at the same time following challenge by either race of the fungus, which is not in agreement with the results presented in Table 2.1. However, total medicarpin (free and conjugates) accumulated earlier (24 to 48 h after inoculation) in an incompatible interaction compared to a compatible interaction (48 to 72 h after inoculation). Conjugates of medicarpin were also detected in other alfalfa plants (2.14 and A21A plants, transformed from Regen SY cultivar, Chapter 4) after fungal infection. Four conjugates with the following retention times 43.4, 36.2, 30.7, and 22.1 were detected in *Phoma medicaginis* infected leaves 96 h after inoculation. The same conjugates except for the 43.4 min conjugate were detected in roots of the same plants, along with F and FG.

Formononetin-7-O-glucoside (FG), the conjugate of formononetin was detected in Sar and SarAR after infection by either race of the fungus although lesser amounts were detected compared to the amount detected in

Arc. FG was detected earlier in an incompatible interaction (24 h after inoculation) compared to a compatible interaction (48 h after inoculation) for the Arc cultivar.

4. Northern-blot analysis of several defense genes in the alfalfa/C.trifolii interaction

Total RNAs were isolated from Arc leaves infected with *C. trifolii* race 1 (incompatible) and *C. trifolii* race 2 (compatible) at 0, 6, 12, 24, 48, 72 and 96 h after inoculation. The same tissues were producing medicarpin conjugates (see above). Total RNAs were electrophoresed and hybridized with different alfalfa cDNAs: one glucanase cDNA (*aglu1)* and cDNAs encoding enzymes involved in medicarpin biosynthesis (PAL: phenylalanine ammonia-lyase, CHR: chalcone reductase, CHS: chalcone synthase and IFR: isoflavone reductase). Mock-inoculated leaves were included in the experiment as a control. The results of this experiment are presented in Figure 2.16. Similar results were obtained in a separate experiment where total RNAs were isolated from Arc leaves infected by a different fungal inoculum, and hybridized with the same cDNAs. Only data from the first experiment are presented (Figure 2.16).

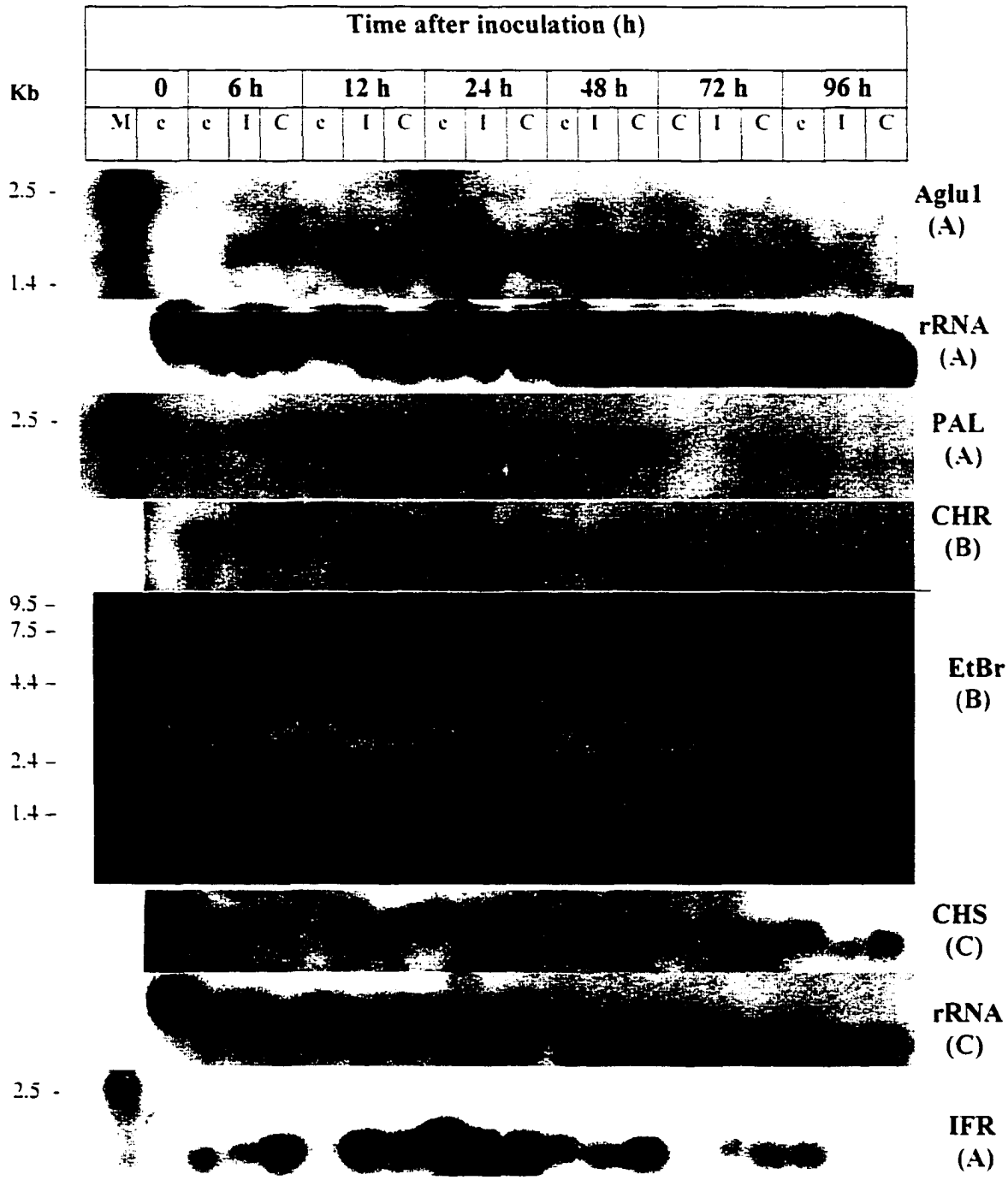


Figure 2.16: See following page for legend.

Figure 2.16: Northern blot analysis of total RNA (10 μg) isolated from mock-inoculated alfalfa leaves [c], alfalfa leaves infected by *C. trifolii* race 1 (incompatible interaction, I), and alfalfa leaves infected by *C. trifolii* race 2 (compatible interaction, C). Tissues were frozen in liquid nitrogen at 0, 6, 12, 24, 48, 72, and 96 h after inoculation. RNA size markers are indicated on the left side of the figure (M). RNAs were hybridized with different ^{32}P labeled cDNA probes: *Aglu1, PAL, CHR, CHS* and *IFR.* Staining of RNAs with ethidium bromide (B) or hybridization with 18S soybean rRNA (A) (C) to check the quantity of RNAs loaded in each lane are indicated below each blot (Aglu1, PAL, CHR, CHS and IFR).

Low levels of hybridization were observed for all the probes in non-infected leaves except for the 24 h time point. In addition, the glucanase cDNA probe presented a high level of hybridization in non-infected leaves for the 48 h time point, in both experiments.

The mRNAs encoding Aglu1 glucanase accumulated in both compatible and incompatible interactions. Transcripts of *aglu1* started to accumulate 12 h after inoculation in leaves infected by either race of *C. trifolii.* Levels of glucanase transcripts were greater in race 1 infected alfalfa leaves compared to race 2 infected leaves from 12 to 24 h after inoculation (Figure 2.16). This trend seemed to be reversed from 48 to 72 hours after inoculation, although in the second experiment higher levels of *aglu 1* mRNAs were present in the incompatible interaction compared to the compatible interaction 48 h after inoculation. Low levels of glucanase gene transcripts were detected at early (6 h) or late stages (96 h) of the infection (Figure 2.16).

The mRNAs encoding the enzymes involved in medicarpin biosynthesis (PAL, CHR, CHS, and IFR) accumulated in both compatible and incompatible interactions. In the compatible interaction, the mRNA levels increased rapidly 6 h after inoculation, then decreased at 12 h to increase again 24 h after inoculation. Transcripts were present in slightly

lower quantity 48 h after inoculation for the *CHR*, *CHS* and *IFR* genes, and in lower quantity for the *PAL* gene. Lower levels of transcripts were detected for the later stages of the infection. In the incompatible interaction, mRNA levels started to increase 12 h after inoculation and stayed at the same level up to 24 h or slightly increased with the exception of *PAL* mRNAs that slightly decreased 24 h after inoculation. As noted for the compatible interaction, lower levels of transcripts were detected for the later stages of the infection (Figure 2.16). In the second experiment the levels of mRNAs during both compatible and incompatible interactions were similar to those just described. One difference was observed in the incompatible interaction where mRNA levels of *PAL*, *CHR*, *CHS* and *IFR* increased up to 48 h after inoculation instead of 24 h.

The same blots presented in Figure 2.16 were hybridized with *C4H* (cinnamic acid 4-hydroxylase), *IFS* (isoflavone synthase), and *IOMT* (isoflavone-*O*-methyltransferase) cDNAs but no signals were detected with any of these probes. Very weak signals were obtained with the *CHI* (chalcone isomerase) probe (data not shown). No differences in *CHI* transcripts between the incompatible and the compatible interaction were observed except 12 h after inoculation where more mRNAs were present in the incompatible interaction.

Northern-blot analysis showed that genes encoding PAL, CHR, CHS, and IFR were induced earlier (6 h after inoculation) in a compatible interaction compared to an incompatible interaction. However, the same tissues used for northern-blot analysis contained medicarpin conjugates that accumulated earlier in the incompatible interaction (48 h) compared to the compatible interaction (Table 2.1). In addition, free medicarpin accumulated earlier in an incompatible interaction (24 h) between alfalfa and *C. trifolii* compared to a compatible interaction (Figure 2.8). These observations led us to investigate the time course of accumulation for the same defense-related gene transcripts for earlier stages of this infection.

Arc leaves were infected by *C. trifolii* (race 1 and 2) and total RNAs were isolated 0, 2 and 4 h after inoculation. In parallel, the outcome of the infection was checked 7 days after inoculation under the microscope (Figure 2.17). As expected and shown previously in Figure 2.2, Arc leaves were resistant to race 1 and susceptible to race 2 of *C. trifolii.* Many acervuli could be seen for the compatible interaction (Figure 2.17, C).

Northern-blot analysis of RNA from Arc leaves 2 and 4 h after inoculation by the two races of *C. trifolii* with probes corresponding to genes encoding PAL and CHR revealed that low levels of transcripts were detected 2 h after inoculation (Figure 2.18). Levels of *CHR* gene transcripts were

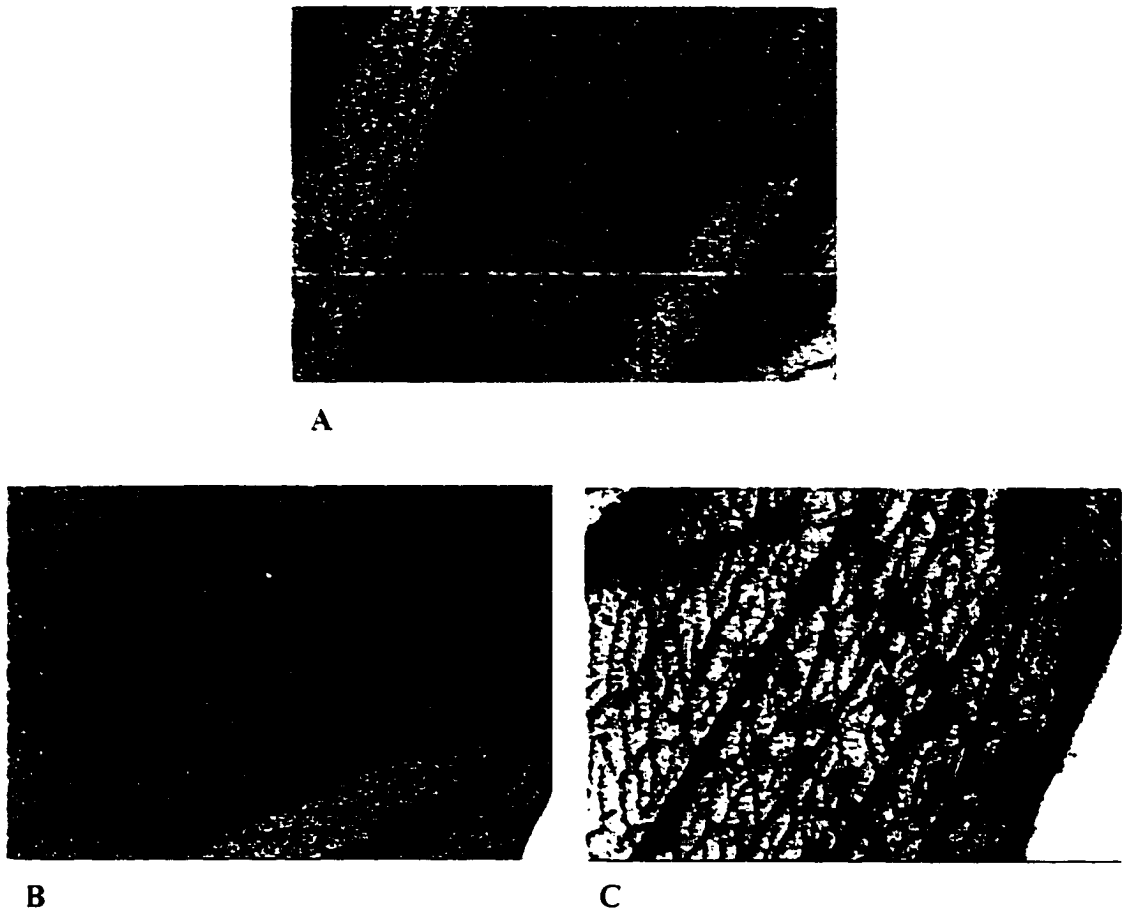


Figure 2.17: Reactions of Arc leaves to race 1 and race 2 of *Colletotrichum trifolii*. 7 days after infection. Arc is resistant to race 1 and susceptible to race 2. (A) Mock inoculated. (B) Arc/race 1: incompatible interaction. (C) Arc/race 2: compatible interaction. Some of the fungal structures detected in (C) are indicated by red arrows. Cf. following page for total RNA extraction.

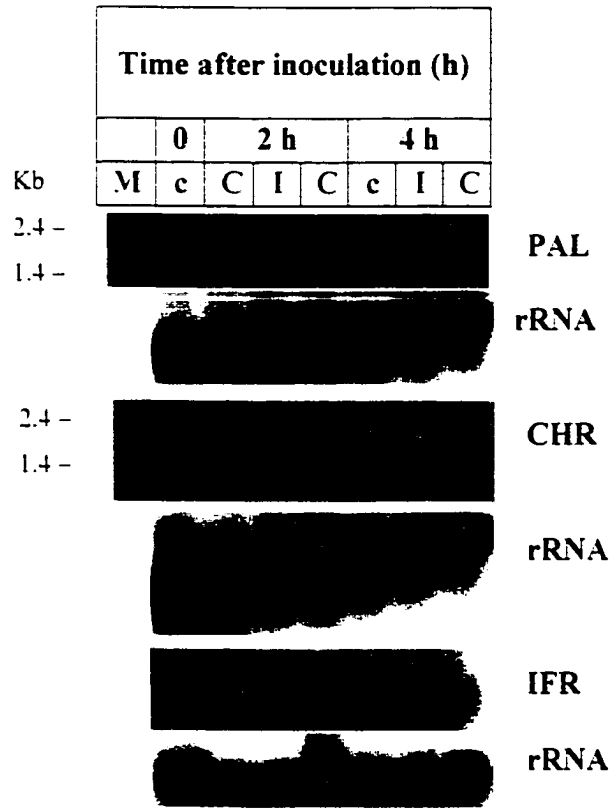


Figure 2.18: Northern blot analysis of total RNA (10 µg) isolated from non infected Arc alfalfa leaves [c], Arc leaves infected by *C. trifolii* race 1 (incompatible interaction, I), Arc leaves infected by *C. trifolii* race 2 (compatible interaction, C). Tissues were frozen in liquid nitrogen at 0, 2 and 4 h after inoculation. RNA size markers are indicated in the left side of the figure (M). RNAs were hybridized with different ^{32}P labelled cDNA probes:, *PAL*, *CHR*, and *IFR*. The same blots hybridized with 18S soybean rRNA showing the quantity of RNAs loaded in each lane are shown below each blot.

approximately the same 2 h after inoculation after taking into account the difference in loading RNAs from race 2 Arc infected leaves. However, after longer exposures of the hybridized RNA blots (7 days instead of 2), more *PAL* and *CHR* mRNAs were present in the incompatible interaction 4 h after inoculation compared to the compatible interaction. *IFR* transcripts were also detected as early as 2 h after inoculation for both interactions, and were present at a low level 4 h after inoculation in the compatible interaction. However, high quantities of *IFR* mRNAs were present in the incompatible interaction 4 h after inoculation (Figure 2.18). Low levels of transcripts of the three genes were detected in non-infected tissues if we exclude the *CHR* gene at the 0 time point where RNAs were overloaded as illustrated by the rRNA hybridization of the same blot. No *Aglu1* or *CHS* gene transcripts were detected in Arc leaves infected by the two races of the fungus at 2 and 4 h after inoculation (data not shown).

DISCUSSION

Reactions of alfalfa leaves to C. trifolii infection

Race 1 & 2 of *C.trifolii* could be distinguished on PDA plates as illustrated in Figure 2.1. Race 2 has previously been reported to not be different to race 1 except in its ability to infect the Arc cultivar (Dickman, 1995). Different

variants of each race have been isolated (O'Neill *et al.*, 1989), therefore, other race 2 isolates may not show the same results.

The differential responses of each cultivar (Arc, Sar and SarAR) were clearly demonstrated with respect to resistance or susceptibility to each race (Figures 2.2-2.5). As reported previously on cotyledons (O'Neill and Saunders, 1994) incompatible and compatible interactions in alfalfa leaves exhibited visible differences within 7 days after inoculation. According to previous reports, appressoria form and mature in about 20 h in both resistant and susceptible cotyledons, but hyphae only develop in the susceptible ones after 48 h. Masses of hyphae (acervuli) that break through the epidermis were visible after four days on the susceptible cultivar, Sar (Churchill *et al.*, 1988; O'Neill and Saunders, 1994). O'Neill and Saunders (1994) were able to detect a HR on cotyledons of the cultivar Arc. However, in our system no acervuli were observed before 7 days after inoculation. In addition, no HR was evident on leaves of resistant cultivars such as reported by Maher *et al.* (1993). The potential ability of each cultivar to develop a HR was evaluated by developing an assay involving wounding of the leaves (Figure 2.3 and 2.5). The degree of browning was more intense in resistant leaves than in susceptible ones. In addition, a few acervuli to very few acervuli were found around the necrotic zone. The fact that no acervuli were visible in alfalfa

leaves until 4 days after inoculation, and no HR was noted in resistant cultivars may be explained by the different method of infection used in our experiments, along with a possible difference in the inocula or the material used (cotyledons vs. leaves). Indeed, it has been reported that the time course for fungal development can vary greatly from one study to another (O'Neill and Saunders, 1994).

Alfalfa is autotetraploid, and its seeds are heterogeneous, therefore only a portion of the seeds carries a given resistance gene. In our experiments, Sar was 100% susceptible to both races of the fungus, whereas approximately 64% and 56% of Arc and SarAR plants were resistant to race 1, respectively. SarAR was only 19% resistant to race 2. Results presented here on anthracnose disease evaluation with alfalfa leaves are in agreement to what has been previously published on alfalfa seedlings (Elgin and Ostazeski, 1982; O'Neill *et al.*, 1989).

Induction of glucanases and chitinases

The accumulation of β-1,3-glucanases and chitinases during compatible and incompatible interactions has been reported in several plant pathogen interactions. Specific isoforms of the enzymes accumulated in both resistant and susceptible soybean hypocotyls and leaves, bean leaves and

tomato leaves when challenged with *Phytophthora megasperma, Colletotrichum lindemuthianum* and *Alternaria solani,* respectively. The accumulation of glucanase and chitinase presented in the studies noted above occurred between 36 and 48 h after inoculation with the pathogens and occurred earlier in resistant leaves than in susceptible leaves (Yi and Hwang, 1996; Dann *et al.,* 1996; Lawrence *et al.,* 1996). Yi and Hwang (1996) showed that certain isoforms were synthesized and accumulated in leaves and hypocotyls of soybean following infection by *P. megasperma,* whereas others were present constitutively in healthy plant tissues, and only certain isoforms were induced and accumulated.

Our results with the colorimetric assay indicated that infection of alfalfa leaves from Arc, Sar or SarAR by *C. trifolii* did not seem to induce the accumulation of β-1,3-glucanases or chitinases. Similar results with Arc have been reported by Maher *et al.,* (1993). This conclusion was supported by results of native PAGE gels where no significant changes in the glucanase and chitinase patterns were observed. The two acidic glucanase isoforms, Ga1 and Ga2 accumulated in both susceptible and resistant leaves 4 days after inoculation. In addition an isoform with lower mobility than Ga1 (probably Aglu1) was detected. However, these isoforms were also detected in the mock-inoculated leaves, therefore, the accumulation must

have occurred in response to wounding caused by leaf excision, not inoculation. An example of induction of chitinases and glucanases after wounding was shown in chickpea by Cabello *et al.* (1994).

In our study, three glucanase isoforms designated Ga1, Ga2 and Ga3 seemed to be constitutively expressed in alfalfa leaves and *C. trifolii* did not induce their production. However, the observation that Ga1 was present in the cultivars Arc and SarAR, but not in Sar, and that Arc and SarAR are resistant to race 1 or both races, respectively, and Sar susceptible to both races, suggests that it might play a role in early stages of the infection. Ga1 is an acidic protein and is likely to be secreted and could either inhibit fungal growth or produce elicitors. In the latter model, since the Sar cultivar does not produce Ga1 constitutively, Sar may not release elicitors at an early stage of the infection and thereby delay defense responses causing increased susceptibility. More experiments need to be done to determine if Ga1 plays any role in the induction of host defenses in alfalfa after *C. trifoli* infection.

Induction of phytoalexin production

Compounds with UV spectra similar to that of medicarpin and FG (formononetin-7-*O*-glucoside) were induced in the alfalfa cultivar Arc during infection by *C. trifolii* (race 1 and 2). The presence of conjugates of

medicarpin (medicarpin 3-*O*-glucoside-6"-*O*-malonate: MGM) and its precursors (FG) have been reported in non-infected and *Rhizobium meliloti*-infected alfalfa roots and in elicited alfalfa cells (Tiller *et al.*, 1994; Kessmann *et al.*, 1990b). Low levels of conjugated or free forms of medicarpin and formononetin have been detected in non-infected leaves (Tiller *et al.*, 1994) in agreement with our results. Further analysis is required to determine if the conjugates of medicarpin are malonylated.

The relevance of induction of medicarpin conjugates and FG during *C. trifolii* infection may be that they act as antifungal components, although generally isoflavonoids loose their anti-fungal activity following glycosylation (Dr. Dixon, personal communication). Conjugates of medicarpin and formononetin may also act as storage forms of preformed medicarpin. The release of medicarpin and formononetin from preformed compounds could be mediated by the action of glucosidases of host or pathogen origin. They would be released during *C. trifolii* infection if availability of carbon sources for phenylpropanoid biosynthesis was limited as Tiller *et al.* (1994) suggested in roots of alfalfa. Kessmann *et al.* (1990b) demonstrated that treatment of elicited cells with an inhibitor of PAL activity resulted in the release of medicarpin from a preformed conjugate. Conjugates could also be used for later stages of the infection or in the

eventuality of another fungal attack. The release of medicarpin from preformed conjugates induced in response to a previous infection was suggested by O'Neill (1996) who showed that tissues accumulating medicarpin after an infection by *C. trifolii* race 1 were protected from a subsequent infection by race 2.

Medicarpin at a concentration of 0.1 mM inhibits the growth of *C. trifolii* by only 10% (Blount *et al.*, 1992) and O'Neill (1996, unpublished data) observed that *C. trifolii* hyphae were insensitive to medicarpin which suggest that it may not be the factor limiting fungal growth. The accumulation of medicarpin and medicarpin conjugates occurred 24 to 48 h after inoculation and after the formation of appressoria (20 hours), indicating that medicarpin and its conjugates could act as inhibitors of secondary spore germination occurring at a later stage of the infection, as suggested by O'Neill and Saunders, (1994). Further investigations *in vitro* are needed to determine if medicarpin and its conjugates have any effect on spore germination or hyphal growth of *C. trifolii.*

A differential accumulation of medicarpin and its conjugates was observed in Arc leaves during infection by virulent or avirulent isolates of *C. trifolii.* Medicarpin levels started to increase 24 h after inoculation and reached maximal levels 48 h after inoculation in Arc leaves infected by race

1 of *C. trifolii*. Medicarpin conjugates and FG were also induced earlier in the incompatible interaction compared to the compatible interaction; nevertheless the maximum levels reached were about the same for both interactions. The results presented here for leaves of the cultivar Arc when challenged by race 2 of *C. trifolii* are not in agreement with those reported previously for alfalfa cotyledons or stems where no phytoalexin response was observed in compatible interactions (O'Neill and Saunders, 1994; Baker *et al.*, 1989). Nevertheless, the differential response observed between incompatible and compatible interactions confirmed the potential role of phytoalexins in restricting disease development in alfalfa leaves inoculated with *C. trifolii*. The timing of the production of medicarpin appears to be critical to the outcome of the interaction.

Northern-blot analysis

In our studies, northern-blot analysis revealed an early accumulation of several gene transcripts in both compatible and incompatible interaction of the alfalfa cultivar Arc with *C. trifolii*. Transcripts of *PAL*, *CHR* and *IFR* genes were detected as early as 2 h after inoculation in both interactions. However, the levels of mRNAs were not much higher than in the mock-inoculated alfalfa leaves. Higher levels of transcripts of these three genes

were present in Arc leaves 4 h after inoculation with the avirulent race of *C. trifolii* (race 1) compared to leaves inoculated with the virulent race of the fungus (race 2). The four genes involved in the phytoalexin biosynthesis pathway (*PAL, CHR, CHS* and *IFR*) were strongly expressed 6 h after inoculation in the compatible interaction, but the *PAL, CHR* and *IFR* genes seemed to be induced earlier (4 h) in the incompatible interaction. Differential transcript accumulation of *CHS* and *IFR* was also observed in alfalfa between compatible and incompatible bacterial interaction (*Xanthomonas campestris* [virulent race] and *Pseudomonas syringae* pv. *pisi* [avirulent race]) (Esnault *et al.*, 1993).

High levels of mRNAs of *PAL, CHR, CHS* and *IFR* preceded the previously described increases in isoflavonoids in the infected alfalfa leaves (Figure 2.8), as reported for elicited alfalfa cultures (Ni *et al.* 1996). The co-accumulation patterns throughout the course of the *C. trifolii* infection for these transcripts suggest a complex regulation of the activation of these defense genes such as Ni *et al.* (1996) suggested.

Aglu1 transcripts reached their maximal level 12 h after inoculation in the incompatible interaction, whereas the *aglu1* transcripts in the compatible interaction reached their maximal level at a later time point. The level of *Aglu1* mRNAs in resistant leaves remained higher than in susceptible leaves

up to 24 h after inoculation while the opposite was seen from 48 to 72 h after inoculation. A strong signal was detected in mock-inoculated leaves 24 h and 48 h after immersion in water, suggesting that a wounding response caused by the cutting of the stem with scissors was occurring. This is in agreement with results from our gel analysis of glucanase activity where Aglu1 was induced for the 96 h time point. This wounding response has also been reported in alfalfa by Baldridge *et al.* (1998) who showed that transcripts of glucanase and chitinase were highly induced in roots after excision during the study of alfalfa resistance to nematodes. Effects of wounding have also been reported for genes involved in medicarpin biosynthesis in alfalfa roots by Baldridge *et al.* (1998) for *PAL, CHS* and *IFR,* in alfalfa cotyledons by McKhann and Hirsch (1994) for *CHI* and *CHS,* and in alfalfa stems by Abrahams *et al.* (1995) for *CHS.* In this last report the *CHS* transcripts accumulated 24 h after wounding as in our experiments.

Transcripts of *Aglu1* were detected earlier in an incompatible interaction compared to a compatible interaction; however, the protein was not detected in activity gels. Aglu1 was present 96 h after infection for both interactions but was most likely induced by a wounding response. This suggests that Aglu1 may not play an important role in the infection. However, the protein may be produced in quantities not detectable in the

activity gel assay at earlier times, but still could release elicitors from the fungal cell walls and thus be involved in plant defense.

CONCLUSIONS

In our study, we provide evidence for a differential induction of medicarpin and its conjugates between compatible and incompatible interaction in the alfalfa/*C. trifolii* gene-for-gene system. The accumulation of phytoalexin medicarpin, related compounds, and the transcripts of the genes involved in their biosynthesis were induced earlier in the incompatible interaction compared to the compatible interaction. The effect of medicarpin and its conjugates on the growth of *C. trifolii* needs to be investigated as well as the importance of the induction of medicarpin conjugates and the role of preformed medicarpin.

Several defense genes were induced more rapidly during an incompatible interaction compared to a susceptible interaction of alfalfa with *C. trifolii* suggesting the possible involvement of elicitors in this interaction. *Aglu1* mRNA synthesis was induced more rapidly in the incompatible interaction compared to the compatible interaction, but occurred later than for the genes involved in medicarpin synthesis. In terms of our hypothesis that elicitors are involved in the interaction, and that glucanases are releasing

elicitors from *C. trifolii* cell walls, Aglu1 most likely is not releasing specific elicitors triggering early defense responses but rather general elicitors because the phytoalexin response is induced before *aglu1* gene expression. However, other glucanases such as Ga1 constitutively present in Arc may release specific elicitors from the fungal cell walls. I therefore proceeded to investigate the possible involvement of elicitors in this interaction and the potential role of glucanases in their production.

CHAPTER 3

INVESTIGATION OF THE INVOLVEMENT OF ELICITORS IN THE ALFALFA/*COLLETOTRICHUM TRIFOLII* INTERACTION

Incompatible interactions between plants and pathogens are associated with a sequence of complex reactions that lead to resistance. They are presumed to occur as follows: recognition of pathogen avirulence determinants (elicitors) by plant receptors, activation of signal transduction pathways and finally triggering of plant defenses leading to resistance (Dixon *et al.*, 1994). Typical elements of the plant defense system include the HR, the production of PR proteins and AOS, the strengthening of plant cell walls, and the accumulation of phytoalexins, which are low-molecular weight antimicrobial compounds (Dixon and Lamb, 1990). In addition to localized resistance, systemic acquired resistance (SAR) can also develop, and is manifested if the plant is subjected to subsequent microbial attack (Ryals *et al.*, 1996).

Production of phytoalexins has been proposed to be a key step in determining resistance in several gene-for-gene systems (see Chapter 1, 6.2). Phytoalexins can be induced by race-specific elicitors that are direct or indirect products of avirulence genes, or by general, non-specific elicitors

that are released from either fungal or plant cell walls (Ebel and Scheel, 1997). Examples of race specific elicitors encoded by avirulence (*avr*) genes include *avr4* and *avr9* from the tomato pathogen *Cladosporium fulvum* (Van Kan *et al.*, 1991) and *nip1* from the barley pathogen *Rhynchosporium secalis* (Knogge *et al.*, 1994). Several non-specific elicitors have been identified such as chitin (Ebel and Mithöfer, 1998), oligogalacturonides (Boudart *et al.*, 1995), glycoproteins (Baillieul *et al.*, 1995), elicitins (Ricci *et al.*, 1989) and harpins (Wei *et al.*, 1992). In the soybean/*Phytophthora megasperma* interaction, phytoalexins are induced by active carbohydrate elicitors released from the mycelial walls of the fungus (Yoshikawa *et al.*, 1981; Keen *et al.*, 1983). The enzyme releasing the elicitor from the fungal cell walls was shown to be a β-1,3-endoglucanase (Keen and Yoshikawa, 1983).

The mechanisms for resistance to anthracnose disease caused by the fungal pathogen *Colletotrichum trifolii* in alfalfa have not yet been elucidated. The interaction follows a gene-for-gene system in which single dominant resistant genes (*an1* and *an2*) provide resistance to race 1 and 2 of *C. trifolii*, respectively. In addition, the production of the phytoalexin medicarpin has been proposed to play a role in plant defense (See also discussion in Chapter 2, Baker *et al.*, 1989; O'Neill and Saunders, 1994). Alfalfa plants exhibit immunity to virulent isolates of *C. trifolii* if they have

previously been challenged by avirulent isolates of *C. trifolii* (O'Neill, 1996). These results, along with those presented in Chapter 2 where a rapid accumulation of several defense gene transcripts occured during the infection of alfalfa by *C. trifolii,* suggest that elicitors may be involved in this interaction. By the nature of the interaction (gene-for-gene), specific elicitors, perhaps along with general elicitors, are expected to be involved in determining the resistance or susceptibility of the host cultivar to one race of the fungus.

Our objective was to establish primary evidence for the involvement of elicitors in the interaction and to evaluate the degree of specificity of these elicitors. As our laboratory has been interested in the PR protein β-1,3-glucanase which is induced during pathogen infection and may be involved in the release of elicitors (Keen and Yoshikawa, 1983), our experiments were mainly focused on the potential release of oligosaccharides (glucans) from the cell walls of *C. trifolii.* Results of these studies are presented in this chapter.

MATERIALS AND METHODS

Fungal isolates and plant cultivars. C. trifolii was grown at 24°C on PDA plates and in an asparagine-glucose medium (Keen, 1975). Calcium nitrate

was used instead of calcium carbonate (Appendix 2).

Alfalfa (*Medicago sativa*) was maintained under standard greenhouse conditions and cut every 3 to 4 weeks. Different cultivars were used: Arc, Sar, SarAR, and Regen SY transformants 2.14 and A21A. Arc is resistant to race 1 and susceptible to race 2 of *C. trifolii* whereas Sar and SarAR are susceptible and resistant to both races of the fungus, respectively (Chapter 2). 2.14 plants were transformed with the *aglu1* glucanase gene under the control of the CaMV 35S promoter and A21A plants were transformed with an empty vector (Chapter 4).

Isolation of mycelial cell walls. Races 1 and 2 of *C. trifolii* were grown at room temperature for 3 weeks in asparagine-glucose medium. Cell walls were isolated according to Yoshikawa *et al.* (1981). Mycelia were collected by filtration and pulverized in a mortar with liquid nitrogen. The resulting powder was resuspended in 20 ml of 20 mM Tris-HCl (pH 7.2) and sonicated for 5 minutes (repeated 2 times) to release components from inside the cell walls. The suspension was centrifuged at 100 g for 5 minutes. The pellet was resuspended and washed 3 times with the same buffer.

Cell walls were sonicated as described above and were washed with deionized water at least 7 times. Isolated cell walls were put on a slide with

lactophenol cotton blue and observed under the microscope to check if the cell walls were properly sonicated. The purpose of the sonication was to break the mycelia in order to minimize the possibility that compounds other than the ones released from the cell walls by different treatments could interact in the testing of the activity of the putative elicitor samples. Fungal mycelia usually stain blue with addition of lactophenol cotton blue. Most of the isolated cell walls did not stain blue and, therefore were successfully sonicated. Finally, cell walls were lyophilized and stored at 4°C. Walls were resuspended in 20 mM potassium acetate (pH 5.2) just before use.

Release of putative elicitors from fungal cell walls.

1. Release by commercially available enzymes.

Cell walls (15 mg) from race 1 or race 2 of *C. trifolii* were resuspended in 3 ml of 20 mM potassium acetate (pH 5.2) and incubated at 37°C for different time periods with the following commercial enzymes: pronase (Sigma Cat. No. 81750), laminarinase (commercial glucanase, Sigma Cat. No. L5272) and chitinase (Sigma Cat. No. C6137). Control treatments consisted of replacing cell walls or enzymes by an equal volume of buffer. Lyophilized enzymes (6 mg) were resuspended in 3 ml of the buffer above. Aliquots were centrifuged for 5 minutes at 15,000 g and passed through Acrodisk

filters (0.2 μm) (Millipore, Fisher Cat. No. 102206D) to remove cell walls. When specified, samples were filtered further using a Centricon 3 (Amicon Cat. No. 4202) for 45 minutes according to the procedure specified by the manufacturer. This procedure was used to remove the added enzymes and other molecules with a MW ≥ 3,000 Da. Filtrates were assayed for released carbohydrates by a modification of the anthrone method (Ashwell, 1957). Briefly, 1 g of anthrone (Sigma Cat. No.A1631) was dissolved in 500 ml of sulfuric acid. The reagent (5 ml) was first added to chilled tubes, followed by the samples (100-400 μl). A standard curve was produced by adding different volumes of a 1 mg/ml glucose solution (0, 25, 50, 75, 100, 200, 300, and 400 μl) to the anthrone reagent. All volumes (samples and standards) were adjusted to 400 μl with sterile deionized water. All tubes were carefully vortexed and heated for 16 minutes in a boiling waterbath. When tubes had cooled down, readings were made at 625 nm using a Beckman 7500 diode array spectrophotometer. Results were expressed in μg glucose equivalents per ml.

2. Release by autoclaving.

Isolated cell walls were resuspended in 20 mM potassium acetate (pH 5.2) and autoclaved for 30 minutes. Cell walls were centrifuged and filtered as

described above. Carbohydrate contents were determined by the anthrone method.

3. Release by plant extracts.

Alfalfa leaves (10 g) were frozen and ground in liquid nitrogen. Different alfalfa cultivars were tested in this experiment: Arc, Sar, and SarAR. Buffer (20 ml of 0.1 M sodium acetate, pH 5.0) was added and tissues were homogenized in a mortar. Homogenates were centrifuged for 10 minutes at 10,000 g. Supernatants were brought to 90% saturation by addition of solid ammonium sulfate, stirred for 1 h at 4°C, and centrifuged as stated above. Pellets were dissolved in 5 ml of 0.1 M sodium acetate (pH 5.0) and dialyzed overnight against 10 mM sodium acetate (pH 5.0) with 6,000-8,000 MW cut off dialysis tubing (Spectrum, Fisher Cat. No. 08670B). The preparation was then centrifuged and further concentrated by filtration with a Centricon 10 filter (Amicon Cat. No. 4205). The resulting retentate (0.5 ml) was incubated with resuspended cell walls (2 mg in 0.5 ml of 20 mM potassium acetate buffer, pH 5.2) for 4 h at 37°C, centrifuged, and filtered with Acrodisk filters (Millipore) and Centricon 3 filters as described earlier. Filtrates were assayed for released carbohydrates by the anthrone method. Results are expressed in μg glucose equivalents per mg of total protein in the extract used for the experiment.

Elicitor bioassays.

1. Cuttings assay.

The following alfalfa cultivars were used in the experiment: Arc, Sar, SarAR, and Regen SY transformants 2.14 and A21A. Alfalfa stems supporting the three youngest trifoliate leaves were cut with scissors and immediately placed into Eppendorf tubes containing 150-300 µl of the potential elicitor preparation or water (control). The absorption of the samples was complete within 3 to 4 h incubation at room temperature. The cuttings were then supplied with autoclaved deionized water until harvest. Cuttings were harvested 24 h after the initial exposure to putative elicitor samples and immediately frozen in liquid nitrogen. Medicarpin content of the leaf tissues (0.5 to 1 g) was determined according to the procedure described in Chapter 2 (isoflavonoid extraction). Glucanase and chitinase activities were also measured as described in Chapter 2.

2. Wounding assay.

Trifoliate leaves (9 per plate) from Arc, Sar and SarAR alfalfa cultivars were excised and placed on moistened filter paper in petri dishes. Leaves were wounded with a pinwheel (✱——) on their undersurface. A drop containing 50 µl of each putative elicitor was placed onto each wound site. Droplets were recovered with a pipet after 24 h at room temperature, pooled, and

wounded tissues were immediately frozen in liquid nitrogen. Recovered droplets were analyzed for medicarpin and its intermediates by HPLC. Tissues were extracted for isoflavonoid analysis and the samples were then separated by HPLC. Medicarpin content was determined.

3. Cell cultures.

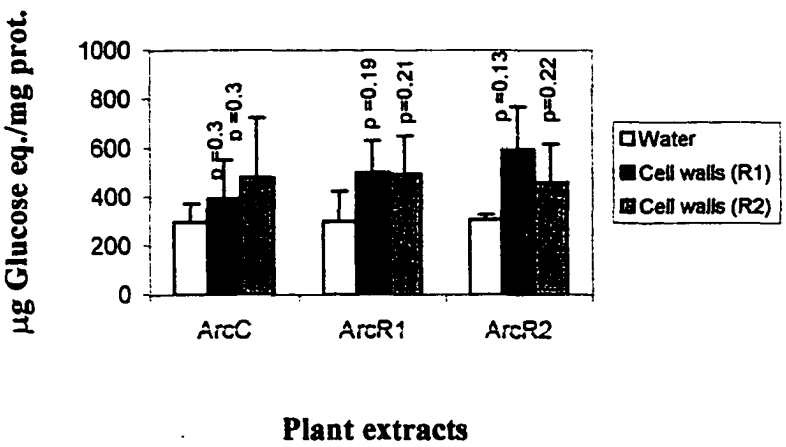
Cell suspension cultures of alfalfa (*Medicago sativa* L. cv. Apollo) were initiated and maintained as described by Kessmann *et al.* (1990a). Cells were transferred to fresh Schenk and Hildebrandt (SH) medium (Appendix 2) every 10 days. On the fourth day after the transfer, elicitor preparations were added to the cells. An elicitor derived from Baker's yeast (Schumacher *et al.*, 1987) previously shown to induce medicarpin and enzymes involved in its biosynthesis (Edwards and Dixon, 1991; Tiller *et al.*, 1994) was used as a positive control in our experiments. The yeast elicitor was added to the cell cultures at a final concentration of 50 μg glucose equivalents per ml of culture. An equal amount of water was added to control cultures. Cells were harvested 48 hours later by vacuum filtration on nylon mesh. The cells were immediately frozen in liquid nitrogen and stored at –80°C. Medicarpin content was determined in the leaf tissues.

RESULTS

1. *Release of putative elicitors from fungal cell walls.*

Incubation with extracts from uninfected plant cultivars (Arc, Sar, and SarAR) did not lead to the release of any carbohydrates from race 1 or race 2 *Colletotrichum trifolii* cell walls (data not shown). Because the factor releasing putative elicitors could be induced during the infection of the plant by *C. trifolii*, the procedure used to prepare the putative elicitors was modified. Cuttings of alfalfa plants (Arc, Sar and SarAR) were infected with a fungal spore suspension as described in Chapter 2. Tissues were harvested 48 hours after inoculation and extracts were prepared as described in the Materials and Methods for this chapter. Incubation of cell walls from either race with extracts from Sar and SarAR extracts did not result in any significant production of carbohydrates (data not shown). Incubation of fungal cell walls (race 1 and 2) with infected Arc alfalfa extracts did not lead to the liberation of more carbohydrates compared to incubation with non-infected Arc alfalfa extracts (Figure 3.1, panel A). Taking into consideration the high amount of carbohydrates present in the extracts, very little carbohydrate was released from the cell walls. All the elicitor preparations were further tested for elicitor activity.

A-



B-

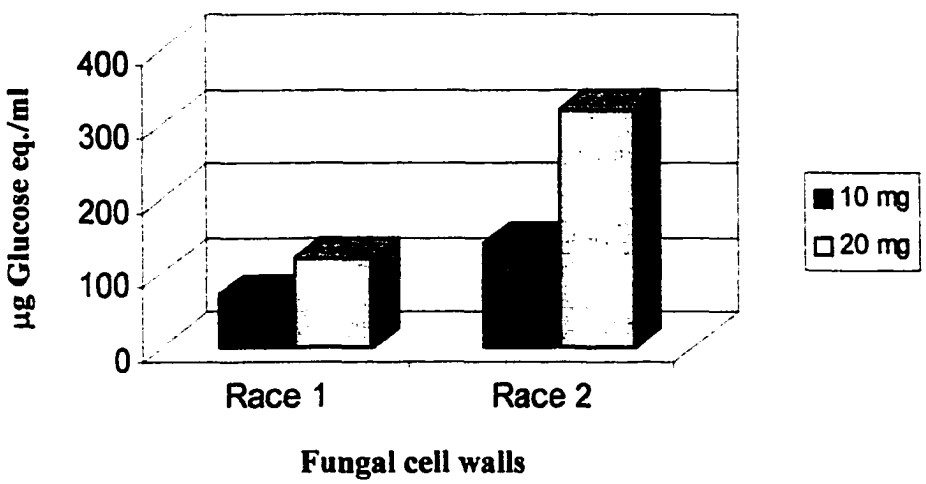


Figure 3.1: Release of carbohydrates from *Colletotrichum trifolii* cell walls. (A) Cell walls incubated with Arc extracts previously infected by the fungus (Arc R1: race 1 infected leaves; Arc R2: race 2 infected leaves harvested 48 h after inoculation) or non-infected Arc extracts (Arc C). Race 1 or 2 cell walls (4 mg/ml) were incubated with extracts for 4 h at 37°C. The water control involved incubation of the extract in the absence of cell walls. (B) Cell walls were autoclaved for 30 min. Samples were centrifuged and filtered with Acrodisk filters and assayed for carbohydrate content. Results are expressed in µg glucose equivalents per mg of total protein and represent the mean and standard errors from 3 independent trials in (A). Statistical analysis indicated no significant differences between Race 1 or 2 and control treatments using t test. Levels of significance are indicated on the columns. Results are expressed in µg glucose equivalents/ml and represent the data from one trial with one replicate in (B).

Other treatments were carried out to release putative elicitors from the fungal cell walls. Autoclaving cell walls resulted in the liberation of 5.2 ± 0.9 μg and 5.6 ± 0.6 μg equivalent glucose per mg of race 1 and race 2 *C. trifolii* cell walls, respectively (mean of 6 replicates from 3 different experiments ± standard errors). Figure 3.1 (panel B) show the result of one such experiment with one replicate where the carbohydrate content in putative elicitor fractions prepared by autoclaving was increased with the quantity of fungal cell walls used in the experiment.

The release of carbohydrates after incubation of mycelial cell walls of *C. trifolii* with pronase was not a linear increase with time. However, the longer the cell walls were incubated with the enzyme (4 h) the greater was the level of released carbohydrates (Figure 3.2, A). Chitinase did not significantly release carbohydrates from either race 1 or race 2 cell walls (Figure 3.2, B). Fungal cell walls incubated with glucanase generated levels of carbohydrates increasing linearly from 20 min to 24 h (Figure 3.3, A). In addition, for any incubation time, glucanase released higher levels of carbohydrates from the fungal cell walls than did any other enzyme.

2. *Elicitor activity assay*

Treatment of alfalfa cell cultures with elicitor preparations from the cell

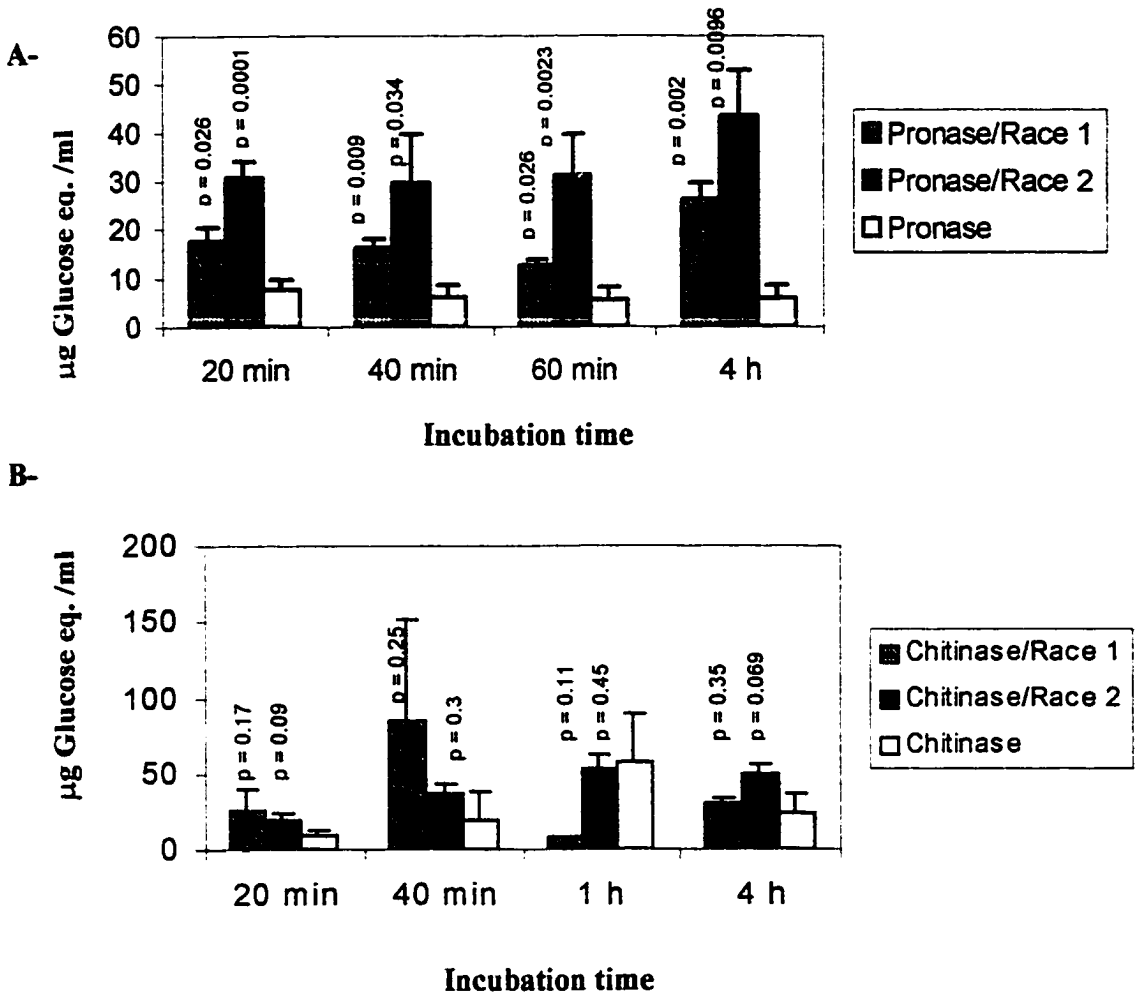


Figure 3.2: Time course of the release of carbohydrates from *Colletotrichum trifolii* cell walls incubated with pronase (A) and chitinase (B). Race 1 or 2 cell walls (15 mg in 20 mM potassium acetate, pH 5.2) were incubated at 37°C with enzymes (6 mg in 20 mM potassium acetate, pH 5.2) for the indicated times. The controls included incubation of the enzymes without cell walls, indicated on the figure by the name of the enzyme alone, or cell walls without enzymes (data not shown). Samples were centrifuged and filtered through Acrodisk filters and assayed for carbohydrate content. Results are expressed in µg glucose equivalents per ml and represent the mean and standard errors from 2 independent trials with 2 replicates each. The levels of significance of differences between chitinase/Race 1 or 2 and chitinase and pronase/Race 1 or 2 and pronase are indicated on the columns (t test).

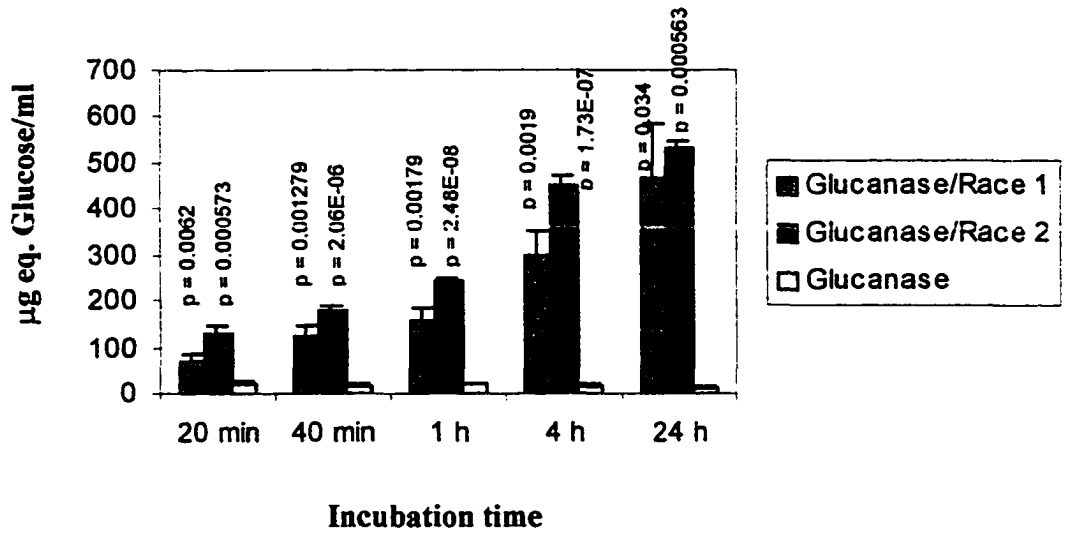


Figure 3.3: Time course of the release of carbohydrates from *Colletotrichum trifolii* cell walls incubated with glucanase. Race 1 or 2 cell walls (15 mg in 20 mM potassium acetate, pH 5.2) were incubated at 37°C with the enzyme (6 mg in 20 mM potassium acetate, pH 5.2) for the indicated times. The controls consisted of incubation of the enzymes without cell walls, indicated on the figure by the name of the enzyme alone, or cell walls without enzymes (data not shown). Samples were centrifuged and filtered through Acrodisk filters and assayed for carbohydrate content. Results are expressed in µg glucose equivalents per ml and represent the mean and standard errors from 3 independent trials with 2 replicates each. The levels of significance of differences between glucanase/Race 1 or 2 and glucanase are indicated on the columns (t test).

walls of *C. lindemuthianum* or from Baker's yeast resulted in the accumulation of the phytoalexin medicarpin (Kessmann *et al.*, 1990b; Dalkin *et al*, 1990; Tiller *et al.*, 1994). The study of different plant defenses during the alfalfa/*C. trifolii* interaction carried out in our laboratory (Chapter 2) indicated that medicarpin production is an important plant defense in alfalfa. Therefore, the phytoalexin response appeared to be the best parameter to use to assess the activity of potential elicitors.

Several different bioassays were set up to determine the ability of the different putative elicitors to induce the production of medicarpin in alfalfa tissues. One of them consisted of dipping the stem of freshly cut alfalfa trifoliate leaves (3-4 youngest) in the sample to be tested. Measurements of medicarpin content were made after 24 h, as the medicarpin response was shown previously to be induced in resistant Arc alfalfa plants 24 h after inoculation by the fungus (Figure 2.8). HPLC analysis of extracts of Arc leaves revealed that no significant amount of medicarpin was produced for any of the putative elicitor preparations tested: cells walls incubated with alfalfa extracts (from non-infected and infected plants), glucanase, chitinase, pronase or autoclaved cell walls. Similar results were observed for the Sar, SarAR cultivar and the 2.14 transgenic alfalfa line.

The second elicitor bioassay consisted of wounding the lower surface of alfalfa leaves using a tracing wheel and depositing samples to be tested in droplets onto the wounds. Three different elicitor samples were tested on the Arc cultivar in this experiment: *C. trifolii* cell walls treated with (1) glucanase, (2) non- infected Arc extracts, or (3) heat by autoclaving. Droplets were recovered and analyzed by HPLC along with extracts from the wounded tissues. No peak of medicarpin or any of its conjugates was detected in the HPLC profiles.

The last elicitor bioassay was carried out with alfalfa cell cultures, because plant tissues did not seem to respond to any of the putative elicitors tested. Using cell cultures, there was no response with any of the putative released elicitors. The results are presented in Table 3.1. Formononetin-7-*O*-glucoside (FG) level in yeast elicitor treated cells (positive control) was about threefold higher than in cells treated with water. However, no induction of phytoalexin synthesis was observed for any of the putative elicitors tested (Table 3.1). Medicarpin levels were about the same in elicited and non-elicited cell culture extracts. In other experiments, the ability of different elicitor preparations to induce free or conjugated medicarpin, or some of its intermediates could not be determined. Indeed, the production of phytoalexins was not induced in alfalfa cell cultures even by the yeast

elicitor that was shown previously to induce isoflavonoid compounds in alfalfa cell cultures (Kessmann *et al.*, 1990b; Dalkin *et al.*, 1990).

Table 3.1: Effects of elicitors on isoflavonoid levels in alfalfa cell suspension cultures. M, medicarpin, FG, formononetin-7-*O*-glucoside. M and FG were determined by HPLC analysis with the eluant monitored at 254 and 287 nm. Their retention times were 16.5 minutes and 14.1 minutes, respectively.

Elicitor		FG[1]	M[1]
• Yeast		1100	64
• Water (negative control)		376	74
• *C. trifolii* race 1 cell walls:	Autoclaved	222	38
	Glucanase-treated	210	30
	No treatment	224	76
• *C. trifolii* race 2 cell walls:	Autoclaved	205	36
	Glucanase-treated	370	60
	No treatment	434	64
• Glucanase (control)		310	44

[1] Formononetin-7-*O*-Glucoside and medicarpin levels are expressed as mAU/g tissue. Data represent the values of one determination.

DISCUSSION

Purification and characterization of race-specific elicitors in a gene-for-gene plant/pathogen interaction is an important step in understanding the biochemical events occurring in the recognition process. The accumulation of phytoalexins has been shown to be an important factor in the expression of resistance in several gene-for-gene systems. The race-cultivar specific interaction between alfalfa and *C. trifolii* is characterized by an earlier induction of the phytoalexin defense response in the alfalfa cultivar Arc when inoculated with an avirulent race of the pathogen (resistance) than when inoculated with a virulent race of the pathogen (susceptibility) (Chapter 2). This result supports the hypothesis that specific and/or general elicitors may be involved in this interaction.

In the eventuality that elicitors were released *in vivo* in the interaction and the factor releasing putative elicitors was a glucanase, attention was given to isolate and characterize carbohydrates released from the fungal cell walls. No carbohydrates were released from the fungal cell walls when they were incubated with alfalfa extracts from infected or non-infected tissues. The release of carbohydrates from fungal cell walls was achieved by autoclaving fungal cell walls or by incubating them with pronase which was reported to release the β-1,3-glucan elicitor from *Phytophthora magasperma*

cell walls (Ayers *et al.*, 1976; Keen *et al.*, 1983). The most effective treatment in releasing carbohydrates was by incubating the fungal cell walls with the commercial glucanase laminarinase.

To verify our hypothesis that there were elicitors (specific and/or non-specific) involved in the alfalfa/*C. trifolii* interaction, it was necessary to develop a bioassay in order to determine the activity of the putative elicitor. The phytoalexin response was chosen as a marker for elicitor activity as it was shown to be differentially induced in an incompatible interaction compared to a compatible interaction (Chapter 2). The incubation of samples on wounded alfalfa leaves did not induce any phytoalexin response although similar assays using wounded cotyledons, leaves or hypocotyls have been successfully used in several systems (Ayers *et al.*, 1976, Yoshikawa *et al.*, 1981, Graham and Graham, 1995; Schweizer *et al.*, 2000). Another assay which consists in the absorption of putative elicitors by plant cuttings has been successfully used to test the activity of elicitins from *Phytophthora* ssp. in several plant species by monitoring the development of a HR (Kamoun *et al.*, 1993), and oligogalacturonides in bean cuttings by determining the activities of glucanases and chitinases (Boudart *et al.*, 1998). For all putative elicitor preparations tested, the absorption of the samples by alfalfa cuttings did not lead to any accumulation of medicarpin nor to an increase in

glucanase and chitinase activities in the alfalfa leaves. The fact that there was no induction of these three plant defenses supported the results presented in Chapter 2 for glucanase and chitinase activities where no differential response was observed. However results presented here for medicarpin production are not in total agreement with what I reported previously where there was an accumulation of medicarpin in both interactions, albeit detected earlier in the resistant plants. One possible explanation of our results would be that the elicitor did not migrate to the leaves and thus was unable to induce any response. A recent study could support this hypothesis. Côté *et al.*, (2000) developed a dipping elicitor assay by using four-day-old *Medicago truncatula* seedlings with roots2 to 4 cm in length. Accumulation of phenylpropanoid compounds was observed in root tissues after absorption of glucan oligosaccharides or the hepta-β-glucoside first characterized by Sharp *et al.* (1984) (Côté *et al.*, 2000). In our investigation, another elicitor bioassay consisting of injecting putative elicitors with a syringe in alfalfa leaves did not induce any medicarpin response (data not shown).

Alfalfa cell cultures were used as an alternative to the two bioassays mentioned earlier. The advantage of this system is that it provided us with a positive control, the yeast elicitor that reportedly induces the accumulation

of medicarpin in alfalfa cell cultures. However, this elicitor was injected into alfalfa leaves and did not induce any medicarpin response (Dr. X-Z He, personal communication) and could not be used as a positive control for the other bioassays. The disadvantage of using cell cultures was that they were not initiated from the cultivar Arc, therefore the specificity of the putative elicitors could not be fully elucidated. The elicitation of the phytoalexin response in cell culture was low, and the yeast elicitor did not induce any medicarpin accumulation. The only difference between elicited and non-elicited cell cultures was a three-fold increase in the level of a conjugate of formononetin, observed in only one experiment. Based on these observations, the activity of the putative elicitors could not be determined.

In this chapter, we focused on characterizing a potential elicitor from *C. trifolii* cell walls, however none was detected. An alternative model in which elicitors would be released from plant cell walls could explain the observed host response. In this case the fungus would have the elicitor releasing factor instead of the host. Preliminary results showed that the race 2 of *C. trifolii* grown in a synthetic medium described by Di Pietro and Roncero (1996) has a different glucanase and chitinase isoform compared to race 1 of the fungus. Further investigation is needed to confirm this observation and determine if this glucanase and chitinase have any effect in

producing elicitors, and if these elicitors are associated with disease resistance. In the hypothesis that elicitors are released from plant cell walls by the fungus, another method other than directly infecting plant tissues should be carried out to release elicitors because no elicitor was present in plant extracts 48 h after inoculation when compared to mock-inoculated extracts (Figure 3.1, A).

In conclusion, it was not possible to determine if elicitors were involved in the alfalfa/*C. trifolii* interaction. The other possibility that putative elicitors are generated from plant cell walls needs to be further explored. In addition, the induction of the phytoalexin response by putative elicitor preparations needs to be reinvestigated by using alfalfa seedling roots instead of alfalfa cuttings. The specificity of elicitors could be determined and elicitors further characterized. This would be an important step in understanding the early molecular events that take place in alfalfa when attacked by *C. trifolii.* This assay would also have been a nice system to evaluate the role of glucanases in plant defense in generating elicitors by testing if extracts from alfalfa plants overexpressing a particular glucanase can release active elicitors from fungal cell walls.

CHAPTER 4

CHARACTERIZATION OF TRANSGENIC ALFALFA ISOLINES OVEREXPRESSING GLUCANASE AND/OR CHITINASE GENES

The protection of plants against microbial pathogens including fungi, remains a challenge to agriculture. The strategies commonly used for protecting crop plants include the development of resistant cultivars by plant breeders and the use of pesticides. Chemical control is limited due to the adaptability of pathogens. In addition, pesticides are tremendously costly and represent a danger to the environment. Therefore, other strategies such as genetic engineering are being developed, these approaches have shown some successes.

The involvement of chitinases and glucanases in plant defense has been proposed in many plant/pathogen interactions (Chapter 1). These enzymes have been chosen for engineering increased resistance against fungal pathogens in several plants (Broglie *et al.*, 1991; Benhamou *et al.*, 1993; Neuhaus *et al.* 1991a, 1992; Sela-Buurlage *et al.*, 1993; Yoshikawa *et al.*, 1993;; Vierheilig *et al.*, 1993; Jach *et al.*, 1995; Zhu *et al.*, 1994; Masoud *et al.*, 1996; Grison *et al.*, 1996; Keller *et al.*, 1999; Kobayashi *et al.*, 2000). Zhu *et al.* (1994) obtained an increased resistance against *Cercospora*

nicotianae by constitutively expressing both a rice chitinase and an alfalfa glucanase in transgenic tobacco. Resistance was higher in plants expressing both genes than in plants expressing either gene alone.

Masoud *et al.*, (1996) introduced the same transgenes into alfalfa, but the results differed from those in tobacco. The results indicated that transgenes could be expressed in transgenic alfalfa individually but not in tandem. In addition, out of 4 different fungi tested, improved protection was obtained only with the oomycete *Phytophthora megasperma* in transgenic alfalfa plants constitutively expressing the inducible alfalfa β-1,3-glucanase gene *aglu1* (Masoud *et al.*, 1996). The objective of this chapter was to characterize the different transgenic alfalfa lines for chitinase and glucanase activities and to explore the possibility that overexpressing one plant defense gene (chitinase) can lead to the release of elicitors thus inducing other plant defenses such as the production of glucanases.

MATERIALS AND METHODS

Materials. Medicago sativa plants, genetically transformed by Masoud *et al.*, (1996) were used for this study. A rice basic chitinase gene (*rch10*) and an alfalfa acidic glucanase gene (*aglu1*) were introduced into alfalfa (cv. Regen SY) as summarized in Figure 4-1. Plants were maintained in the greenhouse

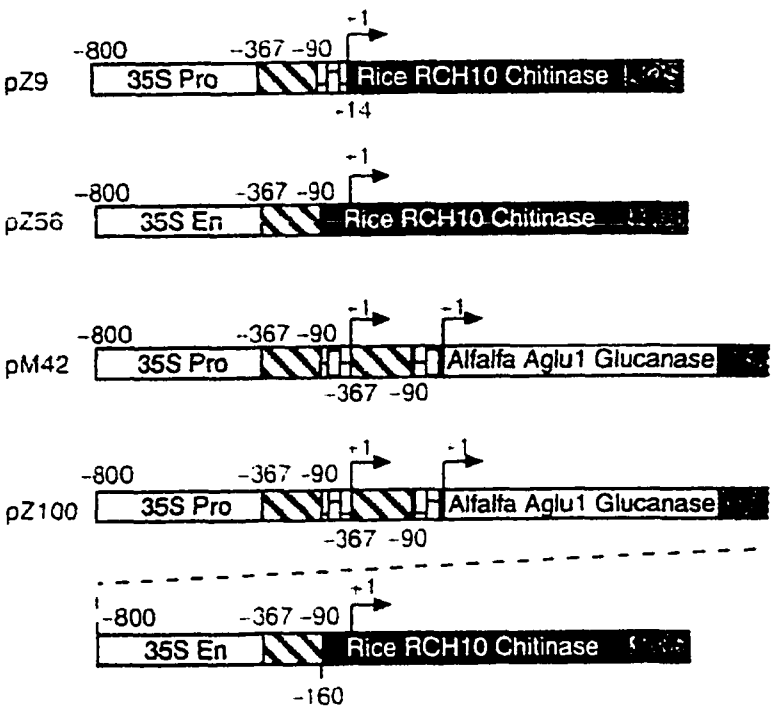


Figure 4.1: Schematic representation of the chimaeric rice chitinase (*RCH10*) and acidic alfalfa glucanase (*Aglu1*) transgenes used for alfalfa transformation. All constructs shown are in the binary vector pBI101. NOS=nopaline synthase 3' terminator. In pZ9, a complete 35S promoter/enhancer from +1 to –800 is fused to the rice chitinase at position +14. In pZ56, the 35S promoter from +1 to –90 is absent, and transcription from the rice chitinase transcription start (+1) is driven from the chitinase's own promoter sequence from +1 to –160, and the 35S enhancer (-90 to –800). In pM42, expression of the alfalfa glucanase is driven by a 35S promoter with the +1 to –367 region duplicated, and a single enhancer region (-367 to –800). In pZ100, the chimaeric genes in pM42 and pZ56 are placed in tandem (Masoud *et al.*, 1996).

by stem cuttings. Lines 2.14 and 2.34 were transformed with pM42 construct, 1.18 and 1.1 with pZ9 construct, A3A and A3I with pZ100 construct. A21A plants were transformed with an empty vector. 4D and K5 plants were original plants used for the transformations (Masoud *et al.*, 1996).

Colorimetric glucanase assay. Plant protein extracts to be analyzed were prepared as described in Materials and Methods, Chapter 2. The colorimetric glucanase assay has also been described in Materials and Methods, Chapter 2.

Radiometric chitinase assay. Radioactive chitin was prepared by acetylation of chitosan with tritiated acetic anhydride (Molano *et al.*, 1977). Chitosan (100 mg) was ground in a mortar with 2 ml of 10 % (v/v) aqueous acetic acid. The mortar was covered with parafilm and allowed to stand overnight at room temperature until complete dissolution of the polysaccharide. In the morning, 9 ml of methanol were added, mixed, and the solution was filtered through glass wool on a Buchner funnel. The filtrate was placed in a beaker on a magnetic stirrer and 150 µl of tritiated acetic anhydride (1 mCi, 2 µmol) were added. After about 1 min, the mixture formed a gel and was incubated

for 30 min at room temperature. The gel was then cut with a spatula into small pieces and homogenized in methanol. The dispersed chitin was then filtered with a medium porosity sintered-glass funnel and washed several times with water. The chitin was finally resuspended in 0.02% (w/v) sodium azide to a final concentration of 15 mg/ml. The regenerated chitin, although finely dispersed, tended to clog pipets, therefore disposable tips were cut to enlarge the opening and facilitate pipeting.

The chitinase assay consisted of 0.8 mg (53 µl) of radiolabeled chitin (0.011 mCi/mg), 50 µl of 100 mM sodium phosphate buffer pH 6.4, and 147 µl of crude enzyme samples, or water for blank samples. The reaction was stopped after 1-1.5 h at 37°C with the addition of 250 µl of 10% trichloroacetic acid. After centrifugation (2,000 g for 5 min), the radioactivity in a 200 µl volume of the supernatant was determined by scintillation counting.

Chitinase and glucanase activity gels. These procedures have been described in Materials and Methods, Chapter 2. Chitinase and glucanase isoforms are named C for chitinase or G for glucanase, a for acidic, b for basic, and numbered sequentially from the lowest protein mobility (1) to the highest protein mobility.

Isoelectric Focusing Electrophoresis. IEF was carried out using a Novex pre-cast IEF apparatus. The gel cassette was rinsed with distilled water, the tape at the bottom of the cassette was peeled off, and the comb removed. The 10 X cathode buffer (arginine free base: 3.5 g; lysine free base: 2.9 g up to 100 ml) was diluted to 1 X and degassed for 10 min. The upper chamber was filled with 1 X cathode buffer. The 10 X anode buffer (phosphoric acid 85%: 4.7 g up to 100 ml) was diluted to 1 X and poured into the lower chamber. Protein samples (150 μg) and IEF molecular weight protein markers (Biorad) were loaded onto the gel with the same volume of 2 X loading buffer (10 X cathode buffer: 2 ml; glycerol: 3 ml up to 10 ml). The gel was run for 1 h at 100 V, 1 h at 200 V, and 30 min at 500 V. The chitinase and glucanase isoforms were revealed as described previously.

DNA gel blot analysis.

1. *Total DNA extraction.*

About 2 g of freshly harvested leaves were rapidly frozen in liquid nitrogen and ground in a mortar and pestle. DNA extraction buffer 2 X CATB (Appendix 3), was added to the ground leaf material. The mixture was incubated at 65°C for 30 min. A half volume of chloroform-isoamyl alcohol (24:1; v/v) was added and the samples were homogenized. Samples were

then centrifuged at 10,000 g for 10 min. The supernatant was collected and first incubated with RNase (10 μg/ml) at 37°C for 30 min, then with proteinase K (20 mg/ml) at 37°C for 1 h. One fifth of a volume of 10% CATB, and one half of a volume of chloroform-isoamyl alcohol were then added. The mixture was centrifuged at 10,000 g for 10 min. DNA was precipitated by adding 1 volume of precipitating buffer (Appendix 3). DNA samples were homogenized, incubated for 10 min at room temperature, and centrifuged at 10,000 g for 15 min. Pellets were resuspended in TE and incubated at 65°C until completely dissolved. DNA was precipitated with two volumes of 100% ethanol at -80°C for 10 min. After centrifugation at 10,000 g for 10 min, pellets were resuspended in TE and DNA was purified with phenol/chloroform. DNA was reprecipitated in 1 volume of isopropanol and one tenth of a volume of 3 M sodium acetate at -80°C. Pellets were collected by centrifugation (10,000 g for 10 min), washed twice with 70% ethanol, dried by speed-vacuum and resuspended in sterile water.

Another method of plant DNA extraction developed by Amersham LIFE SCIENCE, using a nucleon resin, was also used according to the manufacturers' instructions.

2. *Restriction enzyme digestion and agarose gel electrophoresis.*

DNA (10-20 μg) was digested according to the conditions recommended by the manufacturer (Promega) using either 10 units of *Eco*RV or *Hin*dIII, 1 X of appropriate enzyme buffer and DNA, in a volume of 20 μl. After digestion (5 h at 37°C) DNA fragments were separated on 1% agarose gels in 1 X TAE (Appendix 3). Gels contained ethidium bromide (2 μg/50 ml) to allow the visualization of DNA under UV light. 6 X agarose loading buffer (Appendix 3) was added to the samples to a final concentration of 1 X prior to loading. The gel was run at 80 V for 45 min to 1 h.

3. *DNA transfer to nylon membranes.*

Transfer was carried out according to protocols from NEN Research Products. Briefly, DNA fragments were transferred to nylon membranes (Hybond N+, Amersham) by capillary transfer using 0.4 M NaOH as the transfer solution for 4-18 h. The membrane was then washed with 2 X SSC buffer (Appendix 3) and dried with 3 MM paper. DNAs were fixed to the membranes by baking at 80°C for 2 h under vacuum.

4. *DNA hybridization.*

Several hybridization techniques were used, the most successful being carried out as follows. The membrane was wetted in 2 X SSC buffer for 2 min and incubated in prehybridization solution (Appendix 3) inside a sealed

plastic bag at 65°C for at least 4 h. Prehybridization buffer was discarded and replaced by hybridization buffer (Appendix 3) containing (α^{32}P d-CTP) radiolabeled probe (Ready to Go DNA labelling Beads [dCTP], Amersham Pharmacia Biotech). The hybridization was carried out overnight at 65°C. The following day the membrane was washed twice at room temperature, once at 50°C starting with 2 X SSC and finishing at 0.2 X SSC. The membrane was then placed into an X-ray cassette with autoradiographic film (Hyperfilm-*MP*, Amersham) at -80°C for at least 24 h. The film was developed and fixed with Kodak GBX solutions.

PCR. DNA was isolated according to the procedure described in the previous paragraph. General conditions were set according to Promega PCR protocols. The reactions were performed in a total volume of 50 µl. The reaction contained the 1 X manufacturer's Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol of each primer, 500 ng of plant DNA and 2.5 U of Taq polymerase. PCR reactions were performed in a thermocycler (Perkin-Elmer). Parameters such as the concentrations of $MgCl_2$, DNA and primers (35S forward primer: 5' CCTTCGCAAGACCCTTCC 3', Rch10 reverse primer: 5' CATCAGGTCGAAGAGCGAGC 3') were adjusted according to results. The following program was used: denaturation (1 min at 94°C),

annealing* (30 sec at 55°C) and extension (1 min at 72°C then 2 min at 72°C for the last cycle) for 30 cycles.

*Program was made according to the annealing temperature which was calculated as 5 degrees lower than the lowest melting temperature (tm) of the two primers. Calculation of tm: 2 × (A/T) + 4 × (G/C).

The band to be amplified from plants carrying the *rch10* transgene was predicted to be around 350 bp.

RESULTS

1. *Measurement of glucanase and chitinase activities in transgenic alfalfa plants.*

Glucanase and chitinase activities were assayed in alfalfa plants expressing the alfalfa *aglu1* transgene (2.14 and 2.34), the rice *rch10* gene (1.18 and 1.1) and in plants expressing both transgenes (A3A and A3I). The results are presented in figures 4.2 and 4.3.

Glucanase activity was significantly higher in 2.14 and A3A alfalfa plants that are transformed with the acidic glucanase gene *aglu1* alone, or in tandem with the *rch10* gene, respectively, compared to control plants (Figure 4.2). However, this was not observed for 2.34 and A3I plants also carrying the *aglu1* transgene. Unexpectedly, *rch10* transformed plants had

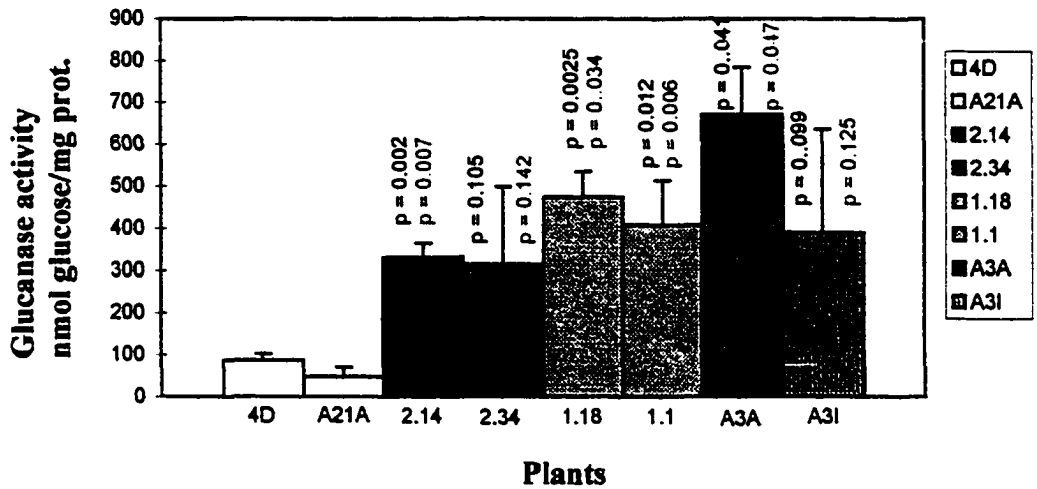


Figure 4.2: Glucanase activity in leaf extracts of alfalfa plants transformed with the empty-vector pBI121 (4D & A21A), the *aglu1* transgene construct pM42 (2.14 & 2.34), the *rch10* transgene construct pZ9 (1.18 & 1.1) and the tandem construct pZ100 (A3A & A3I). Glucanase activity was assayed by measuring the release of reducing sugars from the substrate laminarin. Vertical bars represent means +/- standard errors of three different samples for each plant assayed. The levels of significance of differences between transgenic plants and 4D or A21A are indicated in black and red on the columns, respectively (t test).

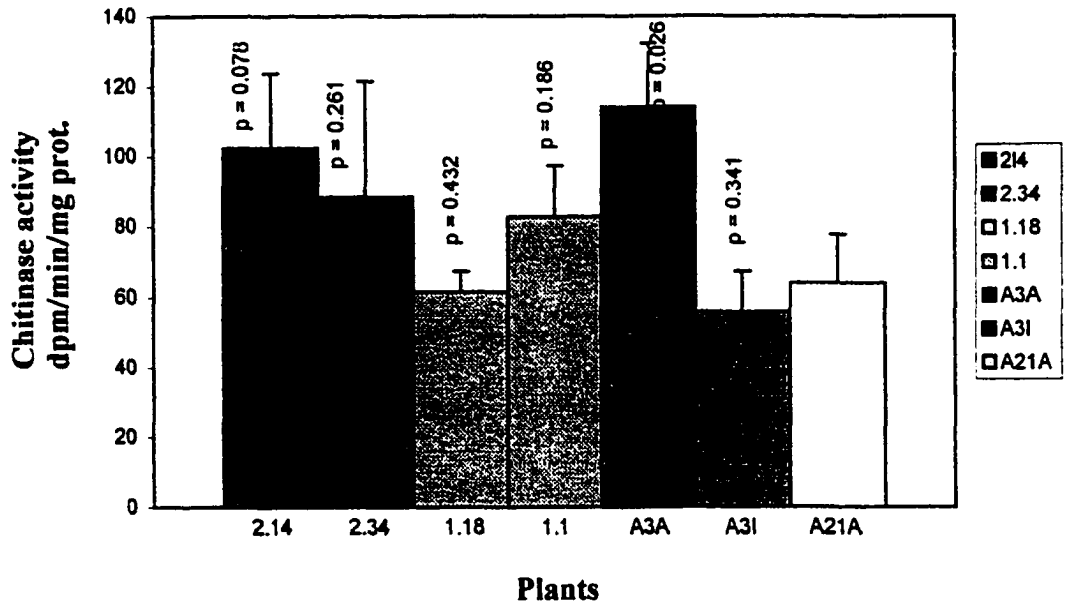


Figure 4.3: Chitinase activity in leaf extracts of alfalfa plants transformed with *aglu1* transgene construct pM42 (2.14 & 2.34), with *rch10* transgene construct pZ9 (1.18 & 1.1), the tandem construct pZ100 (A3A & A3I), and with the empty vector pBI121 (A21A). Chitinase activity was assayed by counting the ^{3}H corresponding to NAG released from radiolabeled chitin. Vertical bars represent means +/- standard errors of five determinations. The levels of significance of differences between transgenic plants and A21A are indicated in black on the columns (t test).

higher glucanase activity compared to control plants, with their levels increased about six-fold.

In contrary, chitinase activity measured in extracts from transgenic plants was not significantly higher compared to the empty vector transformed plants A21A except for the A3A transformant (Figure 4.3). These results were further confirmed using the chitinase colorimetric assay described in Chapter 2.

2. *Detection of glucanase isoforms.*

Several acidic isoforms and one basic isoform were detected in extracts from transgenic plants on 15% native PAGE gels (Figures 4.4, 4.5 and 4.6). As the same amounts of proteins were loaded on the gels, the overall intensity of isoform bands was greater for transgenic plants compared to non-transformed plants K5 (cv. Regen SY), plants from cv. Apollo, and A21A plants transformed with the empty vector although the difference was not as distinct.

The acidic isoform Ga4 was present in plants transformed with either transgene alone or with both transgenes, as well as in non-transformed plants, or plants transformed with the empty vector. Ga5 was only detected in Regen SY plants (transformed, or non-transformed) and not in Apollo

Figure 4.4: Acidic β1,3 glucanase isoforms separated by native PAGE (anodic system) from alfalfa leaves. Crude enzyme extracts (150 μg) were loaded in each lane of a 15% polyacrylamide gel. Glucanase activity was revealed after incubation of polyacrylamide gels for 45 min at 37°C in laminarin solution. Different isoforms were defined based on mobility and are noted with arrows. Plants 2.14, 2.34, 1.18, 1.1, A3A, A3I, A21A, Apollo, K5; lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, respectively.

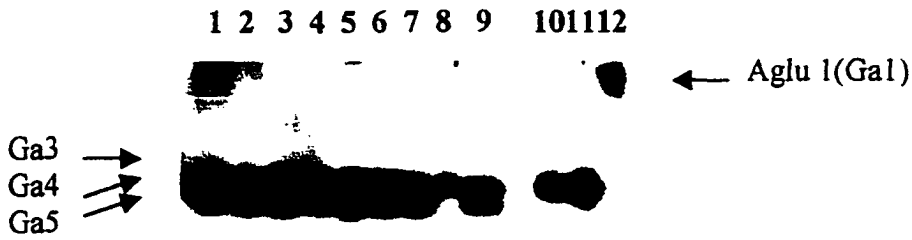


Figure 4.5: Acidic β1,3 glucanase isoforms separated by native PAGE (anodic system) from alfalfa leaves. Crude enzyme extracts from leaves and one extract from roots (150 μg, unless indicated otherwise) were loaded in each lane of a 15% polyacrylamide gel. Glucanase activity was revealed after incubation of polyacrylamide gels for 1 h at 37°C in laminarin solution. Different isoforms were defined based on mobility and are noted with arrows. Plants 2.14, 2.34, 1.18, 1.1, A3A, A3I, A21A, Apollo, K5, Apollo (75 μg), Apollo, Apollo (root, 20 μg); lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 respectively.

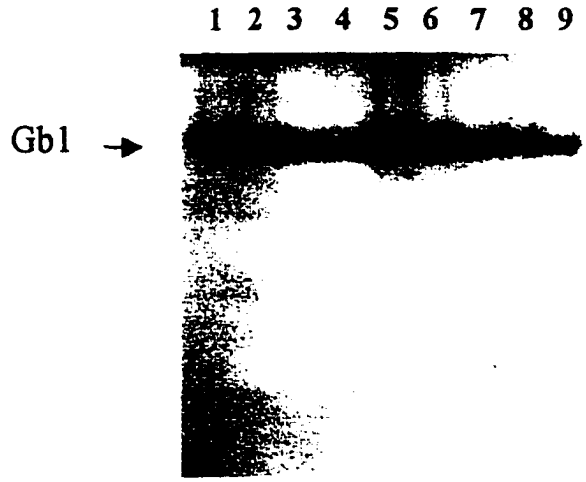


Figure 4.6: Basic β1,3 glucanase isoforms separated by native PAGE (cathodic system) from alfalfa leaves. Crude enzyme extracts (150 μg) were loaded in each lane of a 15% polyacrylamide gel. Glucanase activity was revealed after incubation of polyacrylamide gels for 45 min at 37°C in laminarin solution. Different isoforms were defined based on mobility and are noted with arrows. Plants 2.14, 2.34, 1.18, 1.1, A3A, A3I, A21A, Apollo, K5; lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, respectively.

extracts. The Ga3 isoform was present in all transgenic plant extracts but not in non-transformed plants A21A and K5 (Figure 4.4). If gels were incubated for two hours instead of 45 min, I was able to detect the Ga3 isoform in A21A and K5 plants. However, the bands appeared more diffuse (Figure 4.5).

Aglu1 is constitutively expressed in roots as reported previously by Masoud *et al.* (1996). We were able to detect Aglu1 on IEF gels in root samples (data not shown). The pI of Aglu1 was experimentally determined to be around 5.1. The pI of Aglu1 (*aglu 1* accession number: U27179.1) determined using the program "ExPasy" available on the web @ Expasy.ch. was 6.43, as previously reported by Maher *et al.*, (1990). On native gels, Aglu 1 (Ga1) was detected in root extracts (Figure 4.5, lane 12) and had the lowest mobility of the glucanase isoforms in accordance with its relatively low acidic pI. Unexpectedly, it was not detected in any of the plants transformed with the *aglu1* transgene.

Aglu1 has been shown previously to be induced after fungal infection (Maher *et al.*, 1993). To determine if the endogenous *aglu1* gene was still induced after fungal infection, 2.14 plants were infected with *Phoma medicaginis* (Figure 4.7). An Aglu1 band similar to the one present in the

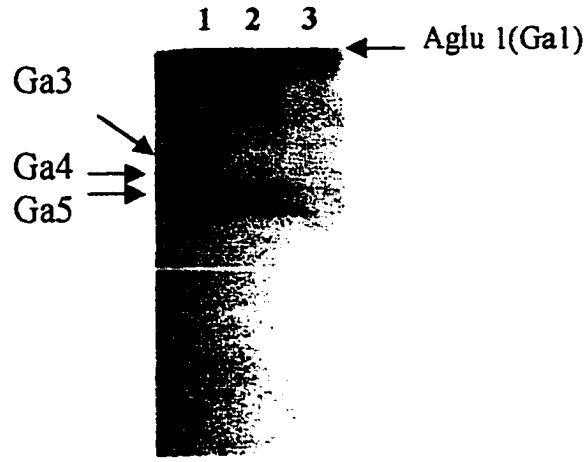


Figure 4.7: Acidic β1,3 glucanase isoforms separated by native PAGE (anodic system) from alfalfa leaves. Crude enzyme extracts from *Phoma medicaginis*-infected leaves (96 h after inoculation) and one extract from non-infected alfalfa roots were loaded in each lane of a 15 % polyacrylamide gel (100 μg and 20 μg of total protein, respectively). Glucanase activity was revealed after incubation of polyacrylamide gels for 1 h at 37°C in laminarin solution. Different isoforms were defined based on mobility and are noted with arrows. Lanes 1,2: Plants 2.14, A21A, respectively. Lane 3: root extract.

root extract was detected 4 days after inoculation in both 2.14 and A21A plants.

One basic glucanase (Gb1) was detected in both transgenic and non-transformed plants (Figure 4.6). Gb1 was constitutively expressed in alfalfa leaves from both Regen SY and Apollo.

3. *Detection of chitinase isoforms*

The acidic isoform Ca2 was present in all alfalfa extracts examined (Figure 4.8). Rch10 (Ca1) was expressed in alfalfa plants transformed with pZ9 (transgene containing rice chitinase alone) but not in plants transformed with pZ100 (transgene containing rice chitinase and alfalfa glucanase). Reported to be a basic chitinase, surprisingly Rch10 was detected in the anodic system for acidic proteins. The predicted pI calculated by the Expasy program mentioned earlier was 5.51, consistent with the presence of Rch10 in the acidic gel system. An additional band (Ca3) was present for 1.1, 1.18, A3A and A3I isolines. No significant difference in band intensity was detected between plant extracts if we exclude the presence of Rch10 in the two isolines.

Two basic chitinase isoforms (Cb1 and Cb2) were expressed in all alfalfa plants of cv. Regen SY (transformed or non-transformed) and non-

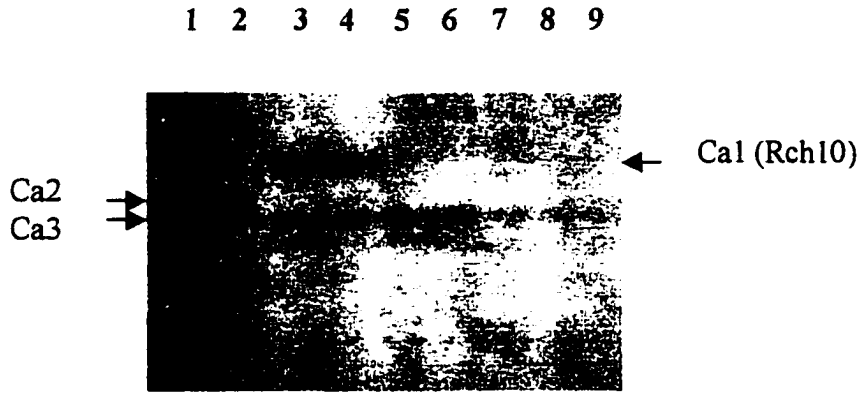


Figure 4.8: Acidic chitinase isoforms separated by native PAGE (anodic system) from alfalfa leaves. Crude enzyme extracts (150 μg) were loaded in each lane of a 15% polyacrylamide gel. Chitinase activity in agarose gels (containing 0.04% glycol chitin) overlaid with acrylamide gels was revealed after incubation for 1 h at 37°C. Different isoforms were defined based on mobility and are noted with arrows. Plants 2.14, 2.34, 1.18, 1.1, A3A, A3I, A21A, Apollo, K5; lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, respectively.

transformed plants from the Apollo cultivar (Figure 4.9). Just like acidic chitinases, no significant difference in the level of expression between the plants was observed.

4. *Expression of Rch10 in alfalfa and correlation with high glucanase activity*

To test the hypothesis that constitutive expression of a chitinase gene in alfalfa was responsible for the higher level of glucanase activity observed in transgenic plants (Figure 4.2), a population of plants resulting from a cross between 1.1 plants (cv. Regen SY) and Apollo plants (cv. Apollo) was tested for glucanase activity (Figure 4.10). The objective was to demonstrate that the chitinase transgene was segregating with high glucanase activity. Apollo was used as a pollen donor to avoid the depression in the progeny from self-pollinated plants.

Progeny plants were classified in 4 different groups using the t test. A significant increase in glucanase activity was observed for the following plants: 1, 2, 5, 7, 9, 19, 23, 24, 27, 28, 33, 36 and 38 compared to the Apollo parent. This group had the same glucanase activity level as the transgenic alfalfa line 1.1 displaying high glucanase activity (Figure 4.10-A). Plant numbers 32 and 37 had similar glucanase activity level to 1.1 and were

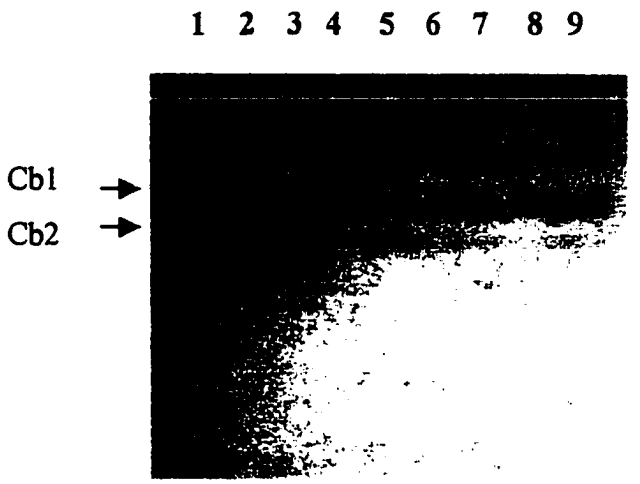


Figure 4.9: Basic chitinase isoforms separated by native PAGE (cathodic system) from alfalfa leaves. Crude enzyme extracts (150 μg) were loaded in each lane of a 15% polyacrylamide gel. Chitinase activity in agarose gels (containing 0.04% glycol chitin) overlaid with acrylamide gels was revealed after incubation for 1 h at 37°C. Different isoforms were defined based on mobility and are noted with arrows. Plants 2.14, 2.34, 1.18, 1.1, A3A, A3I, A21A, Apollo, K5; lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, respectively.

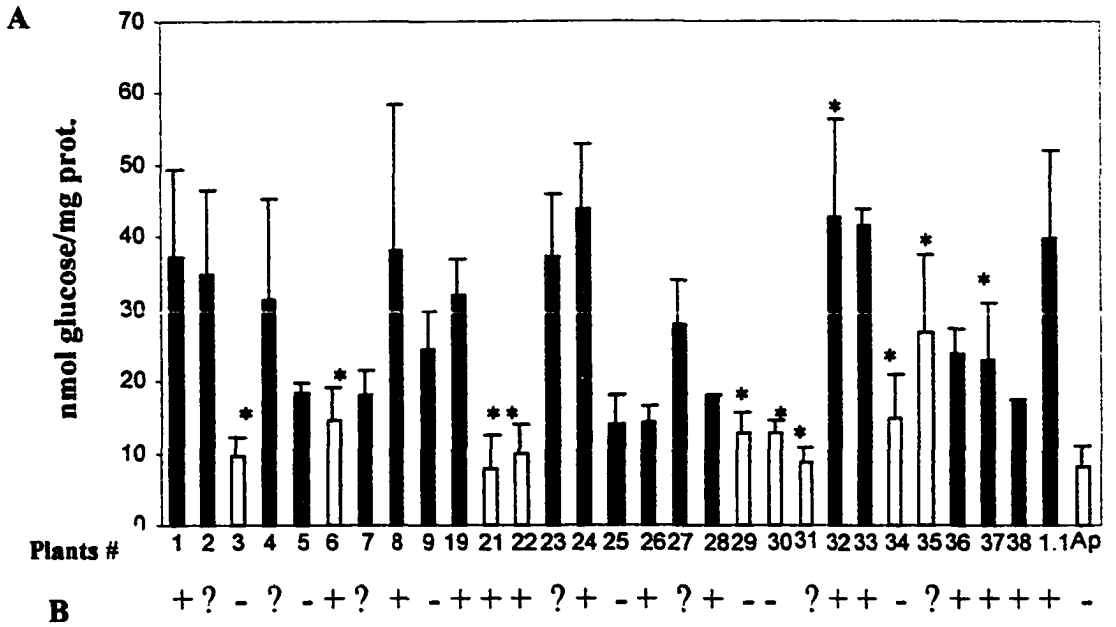


Figure 4.10: (A) Glucanase activity in leaf extracts of alfalfa plants resulting from a cross between 1.1 transgenic alfalfa plants harboring the *rch10* transgene and Apollo plants. 1.1 and Apollo were assayed for glucanase activity and used as controls in the analysis. They are expressing respectively high and low glucanase activity, represented by black and white colors. Glucanase activity was assayed by measuring the release of reducing sugars from the substrate laminarin. Vertical bars represent means +/- standard errors of three experiments. Black and white colors correspond to high and low glucanase activity levels, respectively, determined by t test analysis ($p = 0.05$). Plants with levels of glucanase activity significantly different ($p = 0.07$) from 1.1 or Apollo levels are indicated by an asterix on each column. Columns with black stripes are plants with glucanase levels that are not significantly different from levels in 1.1 or Apollo. (B) Results of analysis for presence (+) or absence (-) of Rch10 in alfalfa leaves of the plants cited above.

statistically different from Apollo for p = 0.07. Another group of plants (3, 6, 21, 22, 29, 30, 31, 34, and 35) had glucanase activity levels comparable to the Apollo line and were statistically different from 1.1 for p = 0.067-0.079. Finally, the remainder of the plants (4, 8, 25, and 26) exhibited glucanase activity that was not significantly different from glucanase activity of 1.1 or Apollo.

In order to determine which of these plants carried the *rch10* gene, southern-blot analysis and PCR were first carried out on the DNA of the progeny plants. Unfortunately no conclusive data were obtained. A second approach was to run plant extracts on 15% native PAGE gels and look for the chitinase Rch10 isoform. The results are presented in Figures 4.10-B and 4.11.The following plants were shown to express Rch10: 1, 6, 8, 17, 19, 21, 22, 24, 26, 28, 32, 33, 36, 37 and 38. Rch10 was not detected in the following plants: 3, 5, 9, 25, 29, 30, and 34. The presence of Rch10 in plants # 2, 4, 7, 23, 27, 31 and 35 could not be elucidated as these plants died between the glucanase activity determination (Figure 4.10, A) and native gel analysis (Figure 4.11-4.13). In the group of plants with high glucanase activity, nine out of eleven tested were also expressing Rch10 and two did not express Rch10. On the other hand, among the plants displaying low glucanase activity, four out of seven tested did not express Rch10 but three

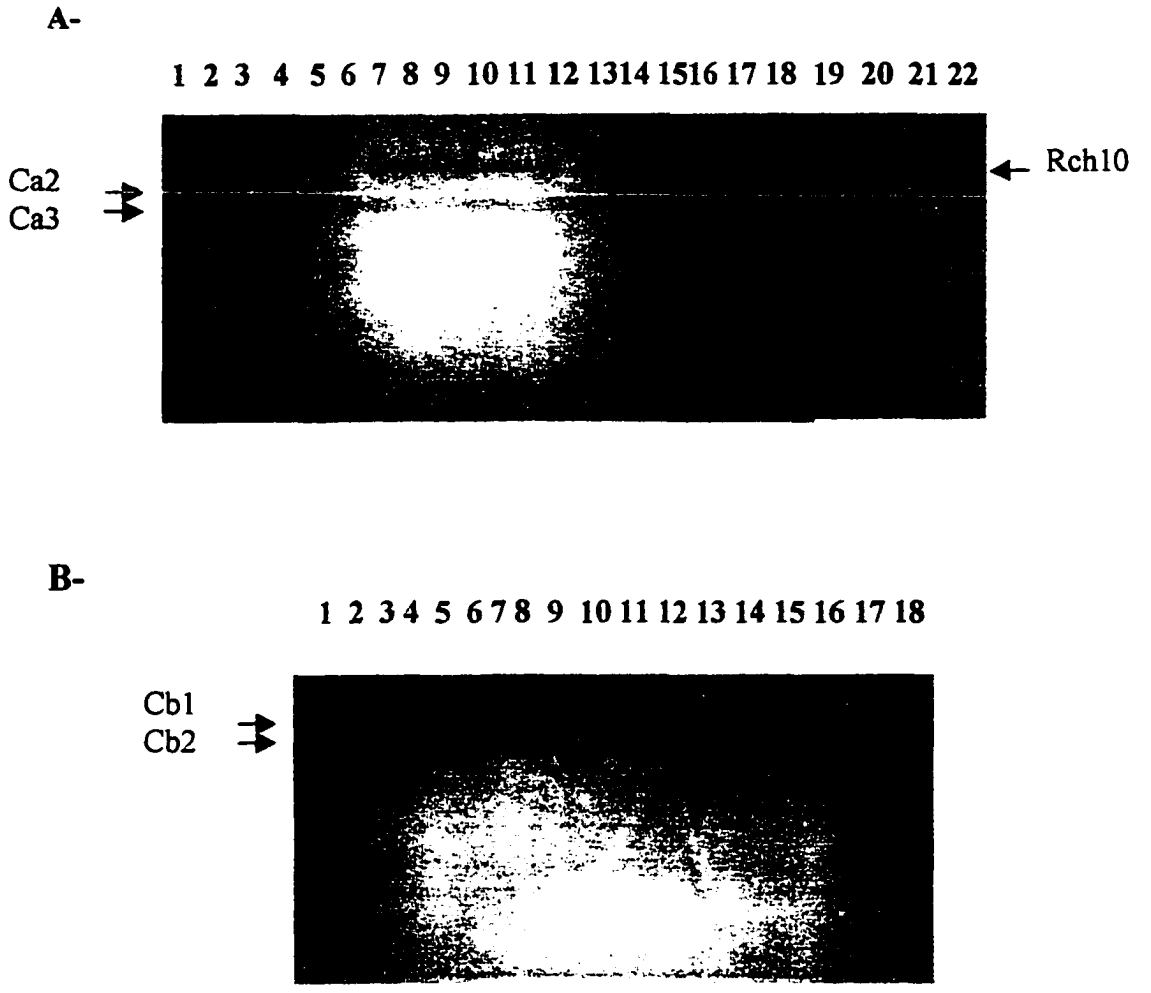


Figure 4.11: Chitinase activity gels after native PAGE (anodic system, A; cathodic system, B) from progeny of a cross between the transgenic alfalfa plant 1.1 transformed with *rch10* and a plant from cultivar Apollo. Crude enzyme extracts (150 µg) were loaded in each lane of a 15% polyacrylamide gel. Chitinase activity in agarose gels (containing 0.04% glycol chitin) overlaid with acrylamide gels was revealed after incubation for 1 h at 37°C. Different isoforms were defined based on mobility and are noted with arrows. Lanes 1 (plant #1), 2 (#3), 3 (#5), 4 (#6), 5 (#8), 6 (#9), 7 (#17), 8 (#19), 9 (#21), 10 (#22), 11 (#24), 12 (#25), 13 (#26), 14 (#28), 15 (#29), 16 (#30), 17 (#32), 18 (#33), 19 (#34), 20 (#36), 21(#37) and 22 (#38).

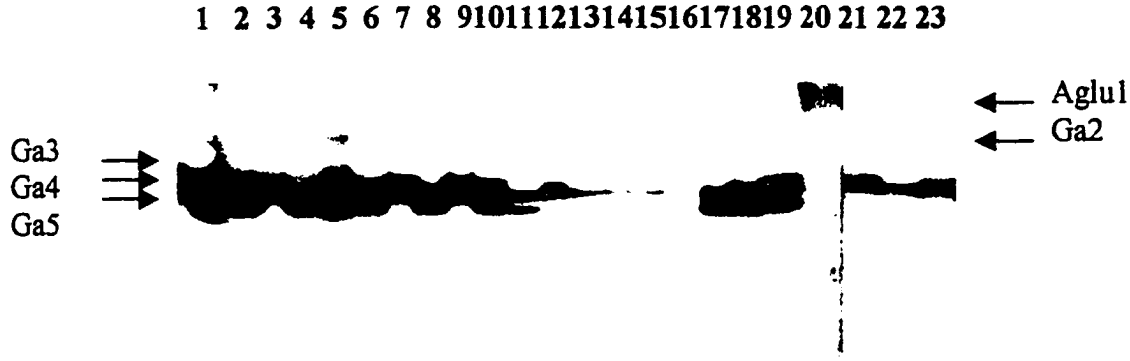


Figure 4.12: Glucanase activity gels after native PAGE (anodic system) from progeny (lane 1- 23) of a cross between the transgenic alfalfa plant 1.1 transformed with *rch10* and a plant from cultivar Apollo. Crude enzyme extracts (150 μg, except for root extract 20 μg) were loaded in each lane of a 15% polyacrylamide gel. Glucanase activity was revealed after incubation of polyacrylamide gels for 45 min at 37°C in laminarin solution. Different isoforms were defined based on mobility and are noted with arrows. Lanes 1 (plant #1), 2 (#3), 3 (#5), 4 (#6), 5 (#8), 6 (#9), 7 (#17), 8 (#19), 9 (#21), 10 (#22), 11 (#24), 12 (#25), 13 (#26), 14 (#28), 15 (#29), 16 (#30), 17 (#32), 18 (#33), 19 (#34), 20 (Root extract from Apollo), 21 (#36), 22 (#38) and 23 (#37).

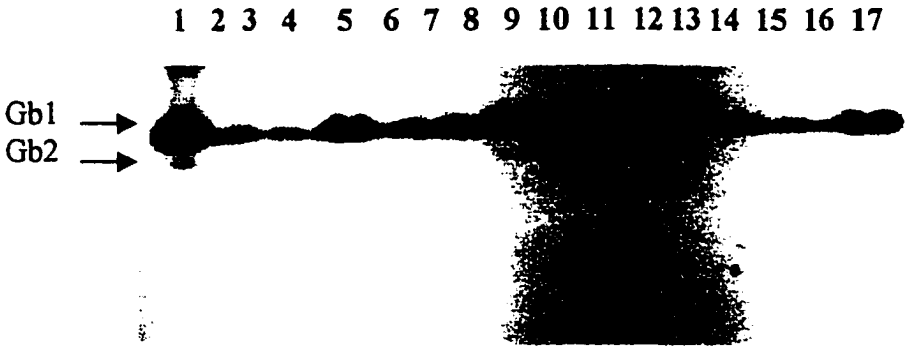


Figure 4.13: Glucanase activity gels after native PAGE (cathodic system) from progeny (lane 1- 17) of a cross between the transgenic alfalfa plant 1.1 transformed with *rch10* and a plant from cultivar Apollo. Crude enzyme extracts (150 μg) were loaded in each lane of a 15% polyacrylamide gel. Glucanase activity was revealed after incubation of polyacrylamide gels for 45 min at 37°C in laminarin solution. Different isoforms were defined based on mobility and are noted with arrows. Lanes 1 (plant #1), 2 (#3), 3 (#5), 4 (#6), 5 (#8), 6 (#9), 7 (#17), 8 (#19), 9 (#21), 10 (#22), 11 (#24), 12 (#25), 13 (#26), 14 (#28), 15 (#30), 16 (#32), and 17 (#33).

were expressing it. Therefore I could not clearly demonstrate that overexpression of *rch10* in alfalfa leaves leads to higher level of glucanase activity.

One to three acidic and two basic chitinase isoforms were detected in the extracts of each progeny from the cross between 1.1 and Apollo on 15% native activity gels (Figure 4.11). At least two acidic and one basic glucanase isoforms were detected in the alfalfa extracts on 15% native PAGE run with anodic and cathodic buffers, respectively (Figures 4.12 and 4.13). The results, which are summarized in Tables 4.1 and 4.2, revealed a complex pattern for the population of progeny from the cross, although there is a more homogeneous isoform pattern for basic enzymes compared to acidic enzymes. Ga2 and Gb2 are two isoforms that were not detected in parent extracts (1.1 or Apollo) and that are present in three different plants expressing the *rch10* transgene: 1, 8 and 21.

DISCUSSION

Masoud *et al.* (1996) placed an alfalfa glucanase gene (*aglu1*) and a rice chitinase gene (*rch10*) alone or in tandem into constitutive expression vectors and introduced them into alfalfa. Only plants overexpressing *aglu1* displayed resistance against the fungal pathogen *Phytophthora megasperma*

Table 4.1: Glucanase isoforms in alfalfa (*Medicago sativa)* leaves. Ga = acidic glucanase, Gb = basic glucanase. Isoforms were numbered from the lowest protein mobility to the highest. * = Presence of isoform. ? = Not determined.

	Ga1 (Aglu1)	Ga2	Ga3	Ga4	Ga5	Gb1	Gb2
Transformants (Regen SY)							
2.14			*	*	*	*	
2.34			*	*	*	*	
1.1			*	*	*	*	
1.18			*	*	*	*	
A3A			*	*	*	*	
A3I			*	*	*	*	
A21A				*	*	*	
K5 (non-transformed)				*	*		
Apollo							
Ap			*	*		*	
Ap (Root)	*						
Progeny (1.1*Apollo)							
1		*	*	*	*	*	*
3			*	*	*	*	
5			*	*		*	
6				*	*	*	
8		*	*	*	*	*	*
9				*	*	*	
17			*	*		*	
19				*	*	*	
21			*	*		*	*
22			*	*	*	*	
24				*	*	*	
25			*	*		*	
26				*	*	*	
28			*	*		*	
29			*	*		*	
30			*		*	*	
32				*	*	*	
33				*	*	*	
34			*	*	*	*	
36			*	*		?	?
37			*	*		?	?
38				*		?	?

Table 4.2: Chitinase isoforms in alfalfa (*Medicago sativa)* leaves. Ca = acidic chitinase, Cb = basic chitinase. Isoforms were numbered from the lowest protein mobility to the highest. * = Presence of isoform. ? = Not determined.

	Ca1 (Rch10)	Ca2	Ca3	Cb1	Cb2
Transformants (Regen SY)					
2.14		*		*	*
2.34		*		*	*
1.1	*	*	*	*	*
1.18	*	*	*	*	*
A3A		*	*	*	*
A3I		*	*	*	*
A21A		*		*	*
K5 (non-transformed)		*		*	*
Apollo					
Ap		*		*	*
Progeny (1.1*Apollo)					
1	*	*	*	*	*
3		*		*	*
5		*		*	*
6	*	*		*	*
8	*	*	*	*	*
9		*		*	*
17	*	*	*	*	*
19	*	*	*	*	*
21	*	*	*	*	*
22	*	*	*	*	*
24	*	*		*	*
25		*		*	*
26	*	*		*	*
28	*	*		*	*
29		*		*	*
30		*		*	*
32	*	*	*	*	*
33	*	*	*	*	*
34		*		*	*
36		*		?	?
37	*	*		?	?
38	*	*		?	?

(Masoud *et al.*, 1996). Glucanase and chitinase activities have been analyzed in these alfalfa transgenic plants. Plants transformed with either gene alone or in combination had higher glucanase activities compared to control plants. Native gel analysis of protein extracts from the different transgenic lines showed that Aglu1 was no longer produced in the plants. This phenomenon, where transgenes are inactivated in a number of transformed plants is called gene silencing. Gene silencing has been observed in several plants transformed with constructs designed to overexpress one or several genes, including glucanases and chitinases (Napoli *et al.*, 1990; De Carvalho *et al.*, 1992; Neuhaus *et al.*, 1991a; Hart *et al.*, 1992; De Carvahlo-Niebel *et al.*, 1995). There are several types of gene suppression: the mutual suppression of transgenes, the silencing of the trangenes or the endogenous genes, and the cosuppression of both transgenes and endogenous genes. The silent state may be the result of transcriptional inactivation of the genes or post-transcriptional processes (De Carvahlo Niebel *et al.*, 1995). Recently, Van Eldik *et al.* (1998) and Holtorf *et al.* (1999) demonstrated that silencing of β-1,3-glucanase genes was associated with degradation of transcripts. In our case, further analysis is needed to determine if there is a reduced level of transgene transcripts and a change in their size. However, it seems that only the transgenes are suppressed, as endogenous glucanases (Ga4 and Ga5)

present in the parental line K5 are also present in the transgenic lines (Figure 4.2 and 4.3). In addition, the endogenous *aglu1* gene does not seem to be affected by the silencing as it is induced after infection by the fungal pathogen *Phoma medicaginis* (Figure 4.7). Neither *aglu1* nor *rch10* were expressed in the double transformant lines A3A and A3I as also mentioned by Masoud *et al.*, (1996), suggesting there might be a mutual suppression of the transgenes and/or a suppression of each transgene individually.

Gene silencing can be stochastic where the inactivation is often variable in different parts or in different developmental stages of the same plant, and where only some plants in a population show the effect as reported in *Nicotiana sylvestris* plants containing a tobacco chitinase (Hart *et al.*, 1992). Our results might be another example of stochastic silencing as the *aglu1* gene was originally expressed at both the RNA and protein level in alfalfa plants transformed with the transgene alone (Masoud *et al.*, 1996). In addition, environmental conditions could strongly influence gene silencing as reported by Holtorf *et al.*, (1999) and Hart *et al.*, (1992).

The fact that plants transformed with *aglu1* alone or in tandem displayed higher glucanase activities while the gene was silenced suggests that mechanisms inducing endogenous glucanases must be occuring. Ga4 and Ga5 are present in higher levels in transgenic lines compared to the

parental line. However, a small induction was also observed in the empty vector transformed line compared to the parental line. The decrease in Aglu1 activity caused by silencing in alfalfa plants may be compensated by an increase in Ga4 and Ga5 endogenous glucanases or by production of another glucanase isoform (Ga3). A compensation mechanism has been described by Beffa *et al.* (1993) where antisense tobacco transformants could compensate for the class I glucanase deficiencies by producing another glucanase isoform.

Analysis of glucanase activities in transgenic alfalfa lines expressing the rice chitinase by the colorimetric or native gel assays revealed unexpectedly higher levels than in non-transformed plants and similar levels as in plants transformed with the *aglu 1* transgene. Therefore, the analysis of glucanase activity in transgenic alfalfa plants led us to question if overexpressing the rice chitinase triggered glucanase gene expression. Zoubenko *et al.*, (1997) showed that introducing a RIP (ribosome inactivating protein) into tobacco triggered other plant defenses such as induction of PR proteins. Transgenic plants expressing the RIP were resistant to the fungus *Rhizoctonia solani* (Zoubenko *et al.*, 1997). However in the present work, a correlation between the expression of *rch10* with a high level of glucanase activity could not be clearly established. Rch10 was

detected as an acidic protein, and this was confirmed by using the Expasy program that determined its pI to be 5.5. Rch10 may have been called a basic protein in reference to its homology to class I chitinases that are mostly basic proteins. In the population of plants (from the cross 1.1 * Apollo), most of the plants with high glucanase activity were expressing Rch10 but on the other hand, among the plants expressing Rch10 three had low levels of activity. In addition, among the plants with low glucanase activity about half of them were not expressing Rch10, but two plants not expressing Rch10 presented high glucanase activity. Levels of glucanase activity in four plants determined in three different experiments presented high variability; therefore they could not be classified after statistical analysis by t test with plants displaying high or low glucanase activities, adding to the difficulty of demonstrating the hypothesis. In addition, almost half of the plants presented probability higher than 0.05. This could be explained by the low number of samples used in the determination, and the variability occuring during the assay for a given plant.

The study of glucanase and chitinase isoform pattern revealed more homogeneity for basic enzymes compared to acidic enzymes. Two glucanases, Ga2 and Gb2, were induced in three plants carrying Rch10 suggesting that Rch10 could be the inducing factor. However, this may not

be the case as Ga2 and Gb2 were not present in all the plants expressing Rch10.

Levels of chitinase activity in alfalfa plants transformed with *rch10* alone or in tandem with *aglu1* did not differ from those in plants transformed with *aglu1*, or control plants except for A3A, although the increase was less than two-fold. These data confirmed what Masoud *et al.* (1996) observed for plants with the transgenes introduced in tandem, however plants transformed with rice chitinase alone seemed not to display high chitinase activity anymore. However, no colorimetric assay for chitinase activity was presented by Masoud *et al.* (1996). The colorimetric assay carried out in our study did not reveal significant differences in the level of chitinase activity between *Rch10* transformed alfalfa plants and non-transformed plants. The presence of Rch10 in plant extracts may not make a significant difference that is detectable with the colorimetric assay.

Genetic engineering has proven to be a good alternative to economically costly and environmentally undesirable chemical control. Enhancing disease resistance has been achieved by overexpressing genes encoding plant defense proteins (PR proteins for the most part). Most of the plants constitutively expressing a transgene do not have abnormal phenotypes, but the presence of the transgene constitutively may represent a

stress to the plant that may respond by not expressing the introduced genes over time, as illustrated in our study. Introducing defense genes that are only induced during the infection might represent an alternative and has been favored where the introduced genes are under control of promoters inducible by fungal attack instead of the constitutive 35S CaMV promoter (Logemann *et al.*, 1992; Keller *et al.*, 1999).

In addition, introducing genes that directly or indirectly generate elicitors may lead to broader resistance than introducing isolated plant defense genes because the elicitors produced trigger a battery of defense genes (Keller *et al.*, 1999). Glucanases have been suggested to be involved in the release of elicitors (Mauch and Staëhelin, 1989; Keen and Yoshikawa, 1983, Yoshikawa *et al.*, 1993) and still represent good candidates to engineer enhanced resistance in alfalfa although the transgenes can become suppressed over time.

Indeed, the plant seems to induce other glucanase isoforms or induce endogenous glucanases that could also release active elicitors. The hypothesis that glucanases produce elicitors have been demonstrated previously *in vitro* by Mauch and Staëhelin (1989), Keen and Yoshikawa (1983), and Yoshikawa *et al.* (1990) and suggested *in vivo* by Yoshikawa *et al.* (1993). The release by glucanases of potential elicitors that would induce

other plant defenses in alfalfa during a fungal infection was investigated by genetically engineering alfalfa plants to overproduce glucanases (Chapter 5).

CHAPTER 5

CONSTITUTIVE EXPRESSION OF A β-1,3-GLUCANASE IN ALFALFA: SUSCEPTIBILITY TO *C.TRIFOLII* CORRELATES WITH ABSENCE OF MEDICARPIN PRODUCTION

Plants have developed an array of defense strategies to respond to fungal attacks. These have been discussed in Chapter 1. Glucanases are hydrolytic enzymes that can be induced in plants after pathogen attack or treatment by elicitors or ethylene (Boller, 1985; Vogeli-Lange *et al.*, 1988; Ward *et al.*, 1991). β-1,3-glucanases hydrolyze β-linked glucans that are present in both fungal and plant cell walls. Beside their role in plant defense, glucanases have been implicated in several processes in plant physiology and development such as germination, growth, pollen germination, fertilization, microsporogenesis, and flowering (reviews by Simmons, 1994; Beffa and Meins Jr, 1996).

Glucanases play a direct role in plant defense by digesting fungal cell walls, as has been reported *in vitro* for several plant glucanases (Mauch *et al.*, 1988a,b; Sela-Buurlage *et al.*, 1993; Ji and Kúc, 1996), and an indirect role by releasing elicitors from fungal or plant cell walls (Mauch and Staëhelin, 1989; Keen and Yoshikawa, 1983). Glucanase genes have been

expressed in plants alone or in combination with other plant defense proteins such as chitinases. Results of these studies have been encouraging and suggest that glucanase expression may provide protection against fungal pathogens in tobacco (Yoshikawa *et al.*, 1993; Sela-Buurlage *et al.*, 1993; Jach *et al.*, 1995; Zhu *et al.*, 1994) and alfalfa (Masoud *et al.*, 1996).

The objective of this chapter was to reevaluate the potential of constitutive expression of glucanases to increase the resistance to fungi in one major crop species, alfalfa (*Medicago sativa*). More importantly, we were interested in gaining insight into the potential role of glucanases in plant defense via released elicitors. Yoshikawa *et al.* (1993) introduced a β-1,3-endoglucanase from soybean into tobacco that was previously shown to release an active glucan elicitor in soybean. Transgenic plants displayed resistance against several fungi. It was suggested that the overexpressed glucanase was playing an important role in conferring resistance by releasing elicitors, although this was not directly demonstrated. The possibility that overexpressing a glucanase in alfalfa leads to production of elicitors potentially resulting in the induction of other plant defenses is addressed herein. The medicarpin response was chosen for this study as it has been implicated in defense of alfalfa against fungal pathogens and is an elicitor-inducible response (Chapter 2, Baker *et al.*, 1989; Blount *et al.*,

1992; O'Neill and Saunders, 1994; He and Dixon, 2000).

MATERIALS AND METHODS

Construction of a chimeric binary vector, pGA482aglu1, containing the alfalfa glucanase aglu 1 cDNA. The glucanase gene *aglu1* (GenBank accession number U27179) was first cloned into pRTL2 (3 Kb) (Restrepo *et al.*, 1990) and the expression cassette was re-cloned into the binary vector pGA482 (13.2 Kb) (An, 1986). The plasmid pRTL2 contains a 35S promoter with duplicated enhancer, the TEV leader (tobacco etch potyvirus leader: 5' non-translated region) and initial coding sequence of TEV polyprotein from pTL-7SN, a short multiple cloning site and the 35S poly(A) signal. The 5' TEV non-translated region was removed and replaced by the non-translated region of the *aglu 1* cDNA during the construction of the transgene.

The *Aglu 1* cDNA originally in pGEM3Zf(+) (Promega Cat. No. P2271) was subcloned into pGEM7Zf(+) (Promega Cat. No. P2251) to produce compatible enzyme sites (*XhoI/Sma*I or *Sma*I/*Xba*I) on both sides of the cDNA to facilitate introduction into pRTL2. pGEM7Zf(+) (1 μg) and 5 μl of a mini-preparation of pGEM3Zf(+)/*aglu 1* were digested with *Eco*RI (10 units) for 2 h at 37°C. Plasmids were digested with *Eco*RI, and treated with alkaline phosphatase (Promega) for 30 minutes to prevent

recircularization. Digested plasmids were run on a 1% agarose gel using 1 X TAE (Appendix 3). One band of 3 Kb (plasmid pGEM7Zf(+)) and 1.4 Kb (*aglu1*) were excised and purified using DNA purification resin from a Wizard PCR preps kit (Promega Cat. No. A7181). DNAs were resuspended in 20 µl of sterile deionized water. *Aglu 1* cDNA was ligated into vector pGEM7Zf(+) in a ratio of 1:3, with 3 µl of 100 mM DTT, 1.5 µl of 10 mM ATP, 3 µl of 10 X ligase buffer, 0.5 µl of ligase (1 U/µl, Promega) and water up to 30 µl. Ligation was carried out for 16 h at 16°C. The ligation product was transformed into *E. coli* DH5α competent cells by heat shock treatment. Ten different colonies were picked from ampicillin LB medium (50 µg/ml) plates and analyzed by restriction enzyme digestion (*Eco*RI). Orientation was checked with *Stu*I/ *Xho*I.

Recombinant pGEM7/*aglu 1* and pRTL2 plasmids were digested with *Xho*I/*Sma*I or *Sma*I/*Xba*I. Digested plasmids were run on a 1% agarose gel and bands were cut out and purified as described earlier. *Aglu1* cDNA fragments were ligated into pRTL2 for 16 h at 16°C as described above. The DNA products were transformed into *E. coli* DH5α. Recombinants were identified by ampicillin selection (50 µg/ml) and by restriction enzyme digestion. The orientation of the inserts was checked by *Eco*RV/*Stu*I digestion.

Selected pRTL2/*aglu1* plasmids were digested with *Hin*dIII producing a ~2.8 Kb fragment consisting of the 35S promoter with duplicated enhancer, the full *aglu1*cDNA and the 35S terminator. The binary vector pGA482 was first digested with *Hin*dIII and then treated with alkaline phosphatase for 30 min. The *Hin*dIII fragment was inserted into pGA482 and pGA482bar using the same ligation conditions as above. These vectors carry the left and right tDNA borders from the *Agobaterium* Ti plasmid ensuring the transfer of cDNA into the plant genome. The vector pGA482bar, as its name indicates, has an additional gene compared to pGA482, the *bar* gene responsible for resistance to the herbicide phosphinothricin (PPT). This vector is commonly used in transformation of *Medicago truncatula* and *Medicago sativa.* Ligation products were transformed into *E. coli* DH5α. The recombinants were selected in kanamycin medium (25 μg/ml), analyzed by restriction enzyme digestion and sequenced. The DNA sequencing group of the Noble Foundation performed DNA sequencing on two selected plasmids (pGA482-I2, pGA482bar-I10). Plasmids were sequenced using the 35S promoter primer in the forward direction and the 35S terminator primer in the reverse direction. DNA sequence analysis was performed with the BLAST programs available at the NCBI (National Center for Biotechnology Information) web

page (www.ncbi.nlm.nih.gov).

Plasmids pGA482/*aglu1* were transferred into *Agrobacterium tumefaciens* LBA4404 via electroporation. Transformants were selected on media with 150 μg/ml rifampicin and 25 μg/ml kanamycin.

Plant transformation. One day before plant infection, 25 ml of YEP medium (Appendix 4) with 25 μg/ml kanamycin in a 250 ml sterile flask were inoculated with 3 loops of fresh *Agrobacterium*. Bacteria were grown at 30°C under shaking (250 rpm) for approximately 18 h. The next day, the *Agrobacterium* suspension was transferred into a 40 ml sterile Oakridge tube and centrifuged at 7,000 rpm for 10 min at 4°C. The pellet was kept on ice until plant infection. Just before infection, the *Agrobacterium* was resuspended in 10 ml of A1 medium (Appendix 4).

Around 50 trifoliate leaves of alfalfa cultivar Regen SY were collected at a time. The second or third youngest trifoliate leaves were chosen as very young leaves do not survive surface sterilization and very old leaves do not regenerate very well. Leaves were washed several times with tap water to remove dust and transferred into a sterile magenta box. Work was performed a laminar flow hood. The box was filled with 70% ethanol (~ 200 ml) and agitated for 10 seconds. Ethanol was discarded and chlorox

solution (Appendix 4) was added. The box was gently shaken for about 1.5 min. Leaves were then rinsed 3 times with sterile deionized water (~ 200 ml).

Trifoliates were then transferred into petri dishes (15 x100 mm) with 2-3 ml of sterile deionized water. Leaves were cut with a sterile scalpel: edges and veins were removed. The water was removed with a sterile Pasteur pipet, and the resuspended *Agrobacterium* was added and incubated for 10 min. Explants were transferred to sterile filter papers tp dry, and then were blotted on A1 medium plates (10-12 pieces/plate; Appendix 4). Plates were maintained for 4 days in the dark at 22°C. The fifth day after infection, explants were transferred to selection medium A2 (Appendix 4) and cultured under low light at 25°C. Explants were transferred to fresh medium every 2 weeks. When pro-embryo callus mass began to form, explants were transferred to a lower 2,4 D medium (A3 medium, Appendix 4) under high light with a 16 h photoperiod. Once globular embryos were developed they were transferred to A4 hormone free medium (Appendix 4). Embryos were kept on this medium until the first trifoliate developed and until roots grew 2 to 5 mm long. Plants were then transferred to A5 rooting medium (Appendix 4) in GA7 boxes. Once plants had well-developed roots and 4 to 5 trifoliate leaves, they were transferred to soil. Plants issued from the same callus were

maintained *in vitro* in A6 rooting medium (Appendix 4).

Analysis of alfalfa transformants.

1. *PCR.*

The presence of the *aglu1* transgene in kanamycin-resistant plants was tested by PCR. Genomic DNA was isolated with the kit "Dneasy" (Qiagen Cat. No. 69104) according to the procedure specified by the manufacturer. DNA was resuspended in 100 µl of TE, pH 8.0. PCR reactions were performed in a total volume of 50 µl containing 1 X Taq polymerase buffer, 200 µM dNTPs, 50 pmol of each primer, ~200 ng of plant DNA and 0.5 U of Taq polymerase (Promega). The 35S promoter (5'-CCTTCGCAAGACCCTTCC-3') and 35S terminator (5'-TAATAAAATGTTTATGTTTATGTATGATTA-3') primers were used as the 5' and 3' primers, respectively. PCR reactions were performed in a thermocycler (Perkin-Elmer) as follows: denaturation (1 min at 94°C), annealing (30 sec at 53°C), and extension (1 min at 72°C then 2 min at 72°C for the last cycle) for 25 cycles. *Aglu1* cDNAs from original plasmids pGA482-I2 and pGA482barI-10 were amplified according to the conditions described above and were used as a control.

2. *Detection of Aglu 1 in native glucanase activity gel*

The procedure has been described in the Materials and Methods, Chapter 2.

3.*HPLC analysis*

Isoflavonoids were extracted in acetone, which was evaporated under nitrogen and the residue resuspended in methanol. HPLC was carried out as described earlier (Materials and Methods, Chapter 2).

Bioassays. Leaves from transgenic plants were infected with *Colletotrichum trifolii* (race 1 and 2). The fungus was cultured on potato dextrose agar plates for 2-4 weeks at room temperature. Spore suspensions were prepared as described in Chapter 2 (Materials and Methods) and their concentrations adjusted to 10^6 spores/ml. Freshly cut trifoliate leaves were dipped in the spore suspensions and incubated in petri dishes humidified with a moist filter paper for 7 days. Leaves were stained with lactophenol cotton blue and observed under the microscope as described in Chapter 2.

RESULTS

1. *Construction of the plasmid pGA482aglu1*

Aglu1 cDNA was originally cloned into pGEM3Zf(+) (Promega) by insertion into the *Eco*RI site. The *Eco*RI fragment was subcloned into

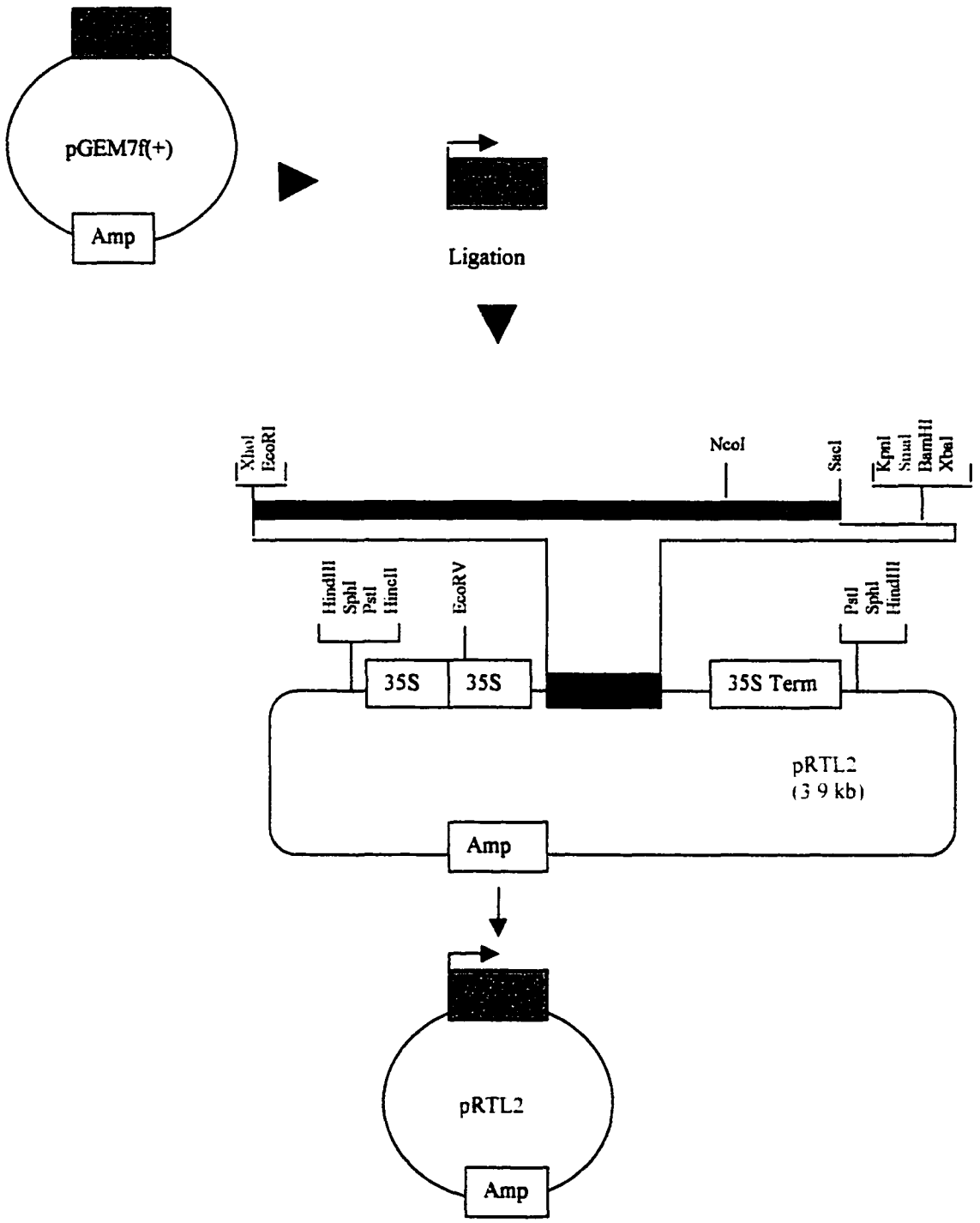


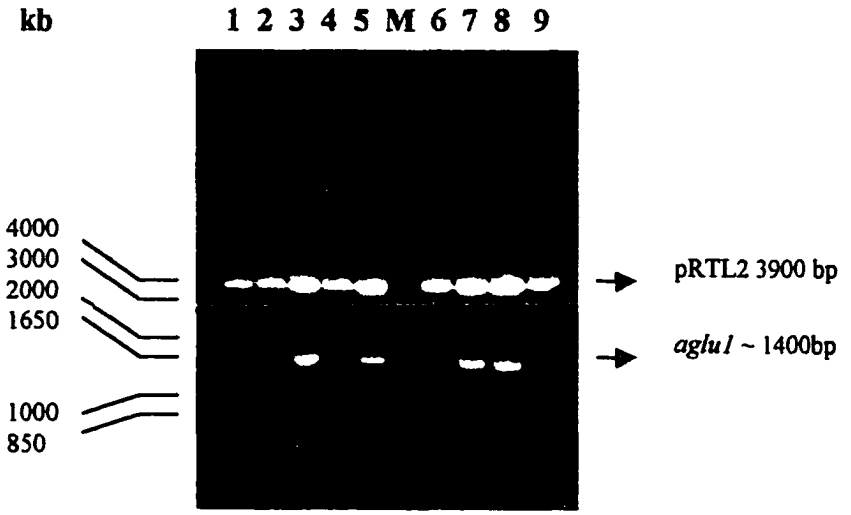
Figure 5.1 : Subcloning of alfalfa glucanase into pRTL2. The cDNA and vector were digested with *XhoI/SmaI*, ligated and transformed into *E. coli* DH5α. ▬ TEV (tobacco etch potyvirus) leader: 5' polyprotein non-translated region. ▬ TEV polyprotein initial coding sequence.

another cloning vector pGEM7Zf(+) (Promega) harboring enzymes that could be used to introduce the cDNA into pRTL2 (Figure 5.1). Recombinant plasmids with *aglu1* cDNA and their orientation were identified by double restriction enzyme digestion. Out of ten colonies tested 9 were recombinants. Five of these recombinants had inserted the *aglu1* cDNA in the correct orientation: the cDNA needs to be inserted in the 5'-3' direction between the CaMV 35S promoter and 35S terminator to be expressed in the plant. *Xho*I and *Stu*I were chosen to check the orientation of the cDNA in the plasmid. *Xho*I does not cut the insert and has a single restriction site in pGEM7Zf(+). *Stu*I has a single cutting site in the *aglu1* sequence at +969 bp, and does not cut the plasmid. Recombinants in the correct orientation were digested by *XhoI/Sma*I or *SmaI/Xba*I and ligated into digested pRTL2 (Figure 5.1).

Ten different recombinants from the two different ligations were analyzed. No colonies were isolated with the pRTL2 vector containing the *aglu1* cDNA inserted between the *Sma*I and *Xba*I restriction sites. Out of ten colonies, five harbored pRTL2 with the 1.4 kb *aglu1* fragment (*XhoI/Sma*I digested) as illustrated in Figure 5.2. All five recombinants had the *aglu1* cDNA inserted into pRTL2 in the correct orientation (Figure 5.2).

The digestion of the recombinants by *Hin*dIII resulted in a 2.8 kb

A-



B-

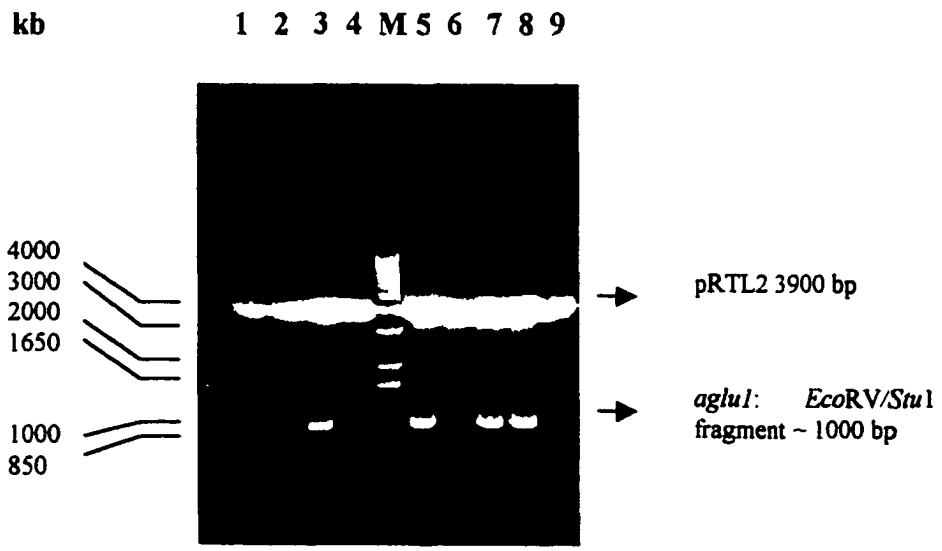


Figure 5.2: Restriction enzyme digestions of 9 putative pRTL2/*aglu1* clones. (A) Agarose gel (1%) with *Xho*I/*Sma*I digestions. (B) Agarose gel (1%) with *Eco*RV/*Stu*I digestions. Lane M, 1kb plus Gibco BRL DNA ladder.

fragment that was inserted into pGA482 and pGA482bar vectors in the same restriction site (Figure 5.3). Four out of four colonies selected were recombinant pGA482 plasmids, and ten out of ten were recombinant pGA482bar plasmids. One pGA482 recombinant plasmid (pGA482-I2) and one pGA482bar plasmid (pGA482bar-I10) were sequenced (Figure 5.3).

Sequence analysis of pGA482-I2 showed 98 % identity with alfalfa *aglu 1* glucanase (GenBank accession number U27179) from nucleotide 12 to 592 in the 3' region and 94 % with the same gene from nucleotide 782 to 1355. Similar results were found with pGA482bar-I10. The open reading frame region of *aglu 1* consists of 1104 nucleotides (Maher *et al.*, 1993) which were present in both constructions.

2. *Transformation and plant regeneration*

Leaves of alfalfa cultivar "Regen SY" were infected with *Agrobacterium tumefaciens* carrying pGA482-I2 or pGA482bar-I10. The process of regeneration through somatic embryogenesis took approximately six months. The different steps are illustrated in Figure 5.4. Kanamycin was included throughout the procedure for selecting positive alfalfa transformants. Calli with proembryos were generated on A2 medium (Appendix 4) after 4 to 8 weeks (Figure 5.4, A). No calli developed on non-

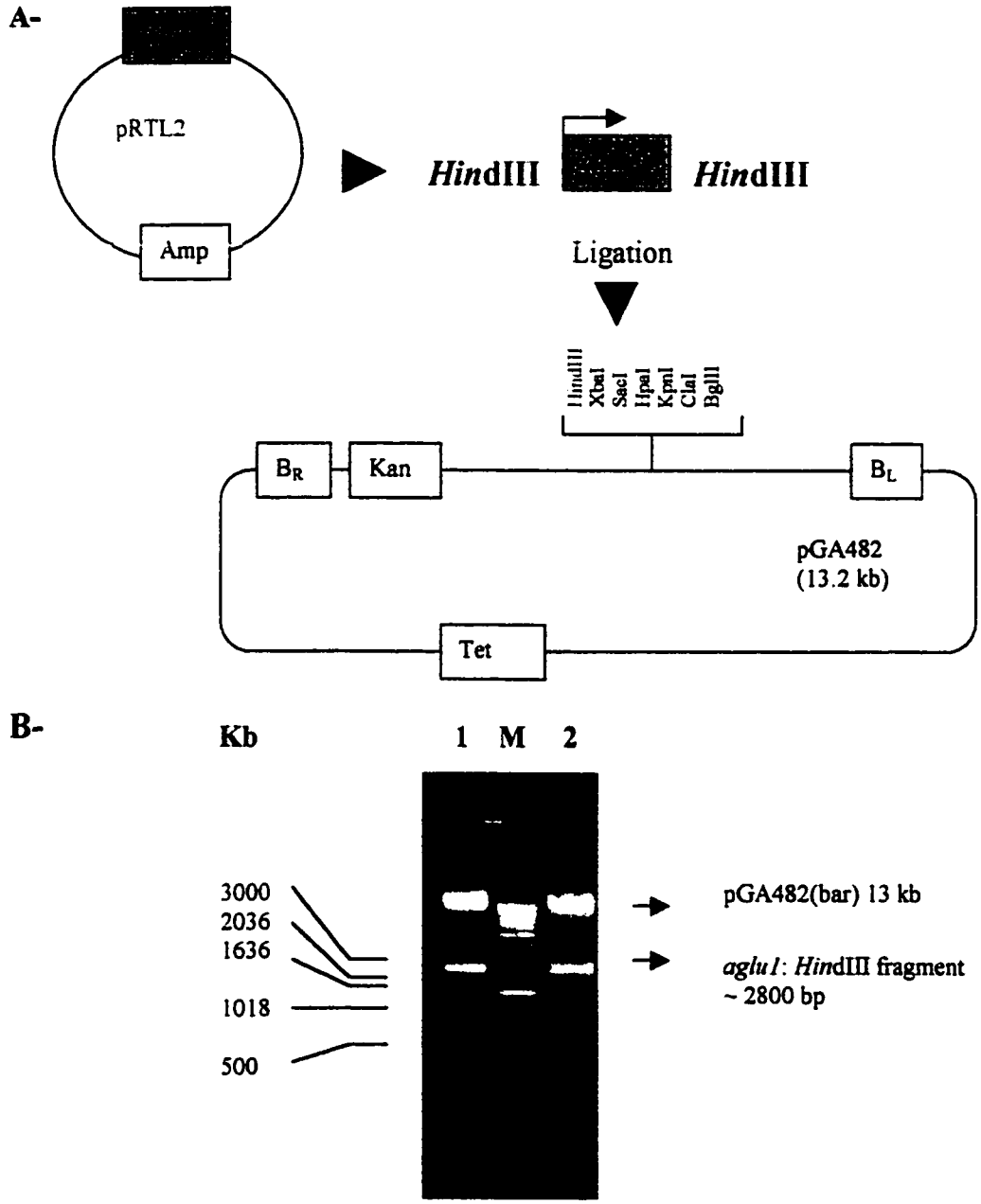


Figure 5.3: (A) Cloning of alfalfa glucanase into pGA482. The cDNA and vector were digested with *Hin*dIII, ligated and transformed into *E. coli* DH5α. B_R, T-DNA right border ; B_L, T-DNA left border. (B) *Hin*dIII digestion of the two recombinant plasmids used for plant tranformation. Lane 1, pGA482-I2 ; Lane M, 1kb Gibco BRL DNA ladder ; Lane 2, pGA482bar-I10.

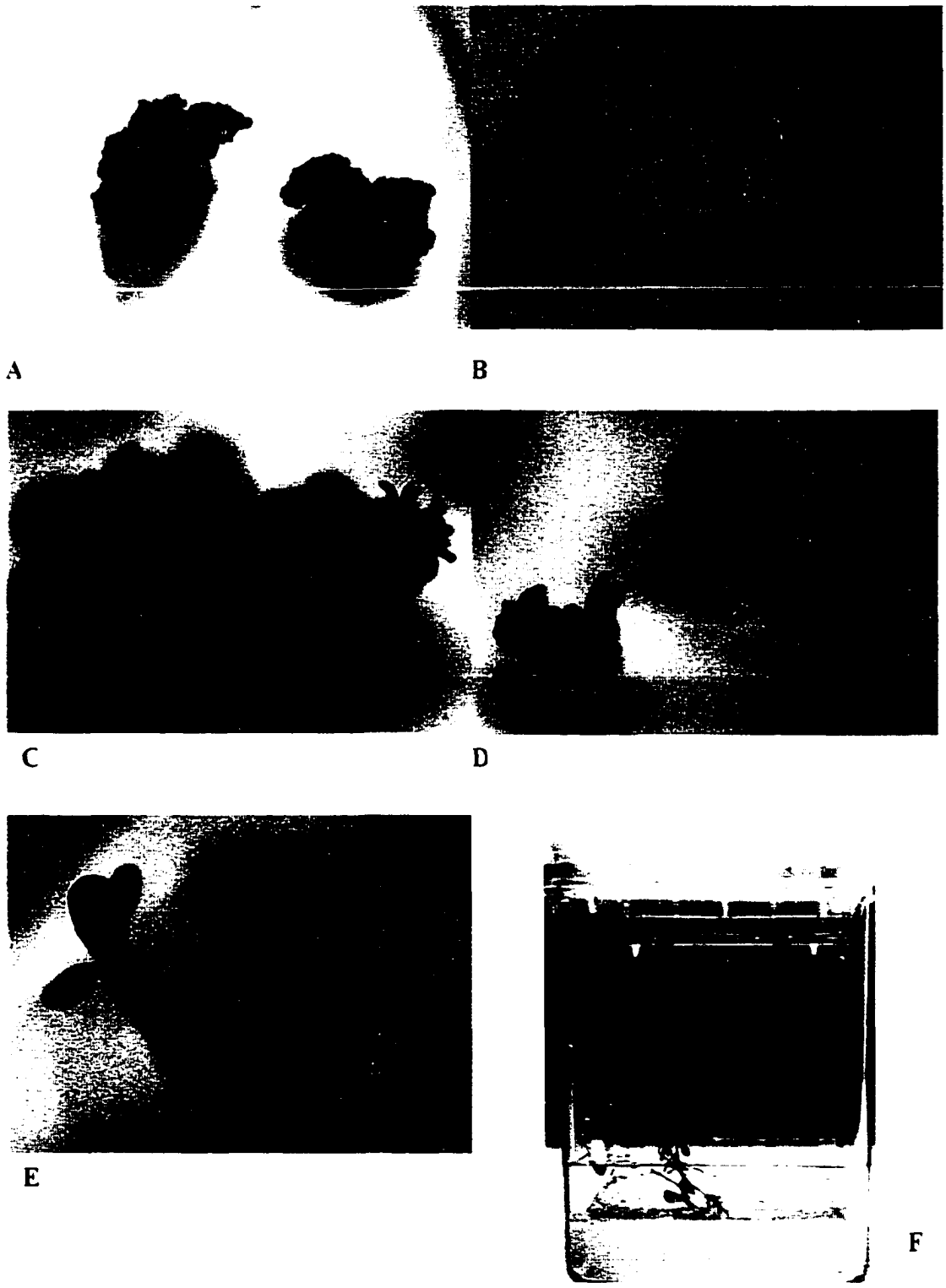


Figure 5.4: Plant regeneration from *Agrobacterium*-infected leaves of alfalfa. (A) and (B), callus induction; (C) and (D), embryo development; (E), trifoliate development; (F), regenerated alfalfa plants growing on kanamycin rooting medium.

infected plants when transferred to A2 medium with kanamycin (Figure 5.4, B). Embryos were obtained on A3 medium (Appendix 4) after 4 to 5 weeks (Figure 5.4, C and D). Another few weeks were needed for embryos to develop their first trifoliate leaves (Figure 5.4, E) on A4 medium (Appendix 4). Explants were retained on this medium until roots developed to about 2 to 5 mm in length. The agar content of the medium was increased from 8 g/l liter of medium to 10 g/l to accelerate the process (Dr. R. Chen, personnal communication). Finally, trifoliate leaves with small roots were transferred to A5 rooting medium (Appendix 5) for the plants to develop (Figure 5.4, F). Overall, around fifty kanamycin-resistant plants were obtained.

3. *Transgene expression in the alfalfa transformants*

After regeneration, alfalfa transformants were analyzed by PCR for the presence of the *aglu1* transgene in their genomic DNA. PCR was performed with 35S promoter and 35S terminator primers respectively to amplify from the 5' and 3' ends of the *aglu1* cDNA. PCR carried out on pRTL2*aglu1*, pGA482-I2, and pGA482-I10, all carrying the *aglu1* cDNA, gave an amplification product of approximately 1.5 kb which is 100 bases in excess of the length of the *aglu1* cDNA due to the parts of the 35S promoter and the 35S terminator amplified with the *aglu1* cDNA. Twenty-eight transgenic

alfalfa lines were tested and at least 75% were transformed with the *aglu1* transgene (Figure 5.5, Table 5.1).

The a*glu 1* transgene was expressed in 74% of the transformed alfalfa plants as determined by in gel enzyme assays, as illustrated in Figure 5.6. Some alfalfa transformants were strongly expressing the transgene (22 %), while others had moderate to low Aglu 1 activity (52% and 26%, respectively). Up to seven different glucanases were detected in some of the alfalfa transgenic plants. The same extracts loaded in gel 1 loaded two days later (gel 2) displayed lower levels of glucanase activity (Figure 5.6). The activity of Aglu 1 and Ga2 was affected to a great extent, and the other isoforms to a lesser extent.

Only two isoforms (Ga4 and Ga6) were expressed in the non-transformed plants and these were also present in most of the transgenic plants. The presence of glucanases in transgenic alfalfa plants that were not present in control lines suggests that the introduction of the *aglu1* transgene can induce the production of several other glucanase isoforms. Indeed, seventeen plants out of twenty expressing the *aglu1* transgene were also expressing Ga1 and Ga2, and fifteen out of twenty plants were expressing Ga7 and/or Ga8.

Low activity was detected for parental glucanase isoforms Ga4 and

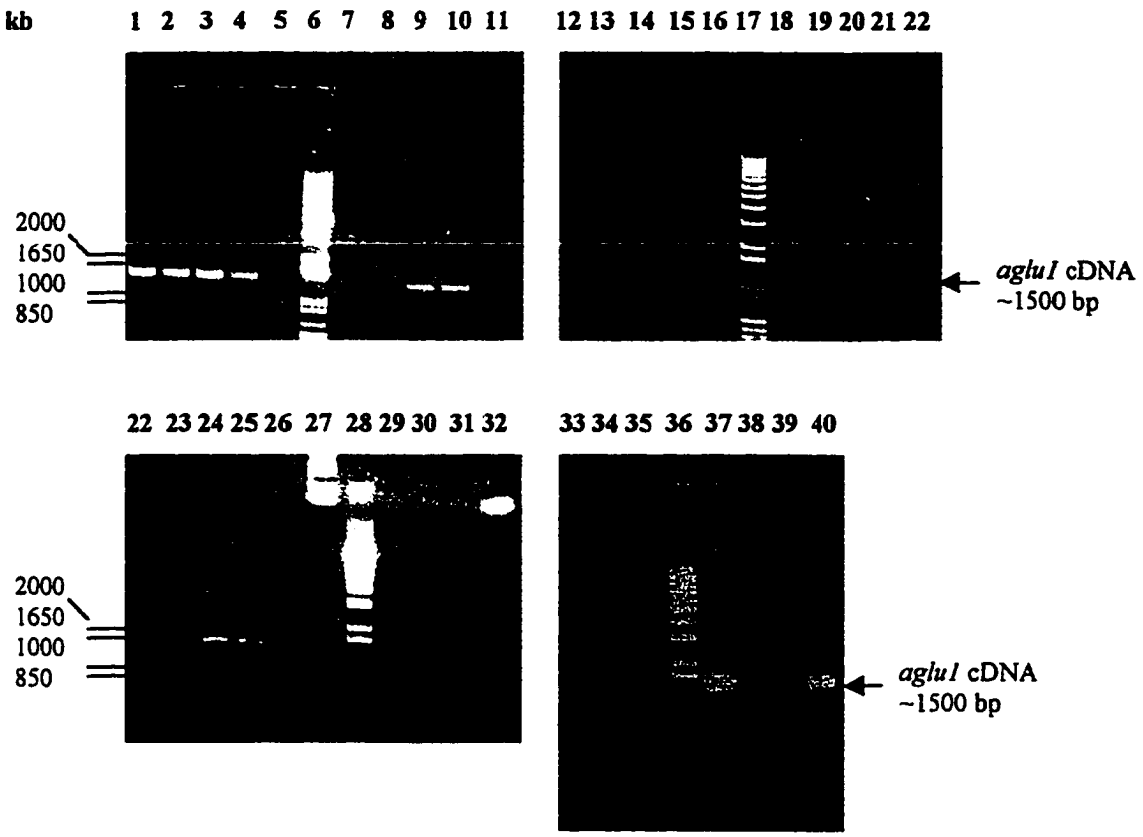


Figure 5.5: PCR amplification of *aglu1* cDNA from alfalfa transformant genomic DNA. Lane 6, 17, 28 and 36: 1kb plus Gibco BRL DNA ladder; Lanes 37: pGA482-I2; Lane 40: pGA482-I10; Lanes 5, 7, 8, and 13: non transformed plants 3f, 2b, 4b and 2a, respectively. Lanes 1, 2, 3, 4, 9, 10, 11, 12, 14, 15, 16, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 29, 30, 31, 32, 33, 34, 35, 38 and 39: transformed plants 1-1-1, 1-1-1b1, 3-2-1, 3-1-1, 4-2-a, 4-1-a, 4-2-1, 1-1-2, 3-1-a, 3-3-1, 5-1-a, 6-1-a2, 6-2-a, 7-2-b, 9-1-b, 9-3-b1, 5-1-1, 17-1-a, 19-1-b1, 1-1-2, 6-1-a2, 9-1-b, 13-1-a1, 17-4-a2, 18-1-a2, 18-2-b, 18-3-b, 20-1-a, 21-1-b1, 13-2-a2, 19-1-2, respectively. All lanes contain 20 µl of each of the 50 µl PCR reaction.

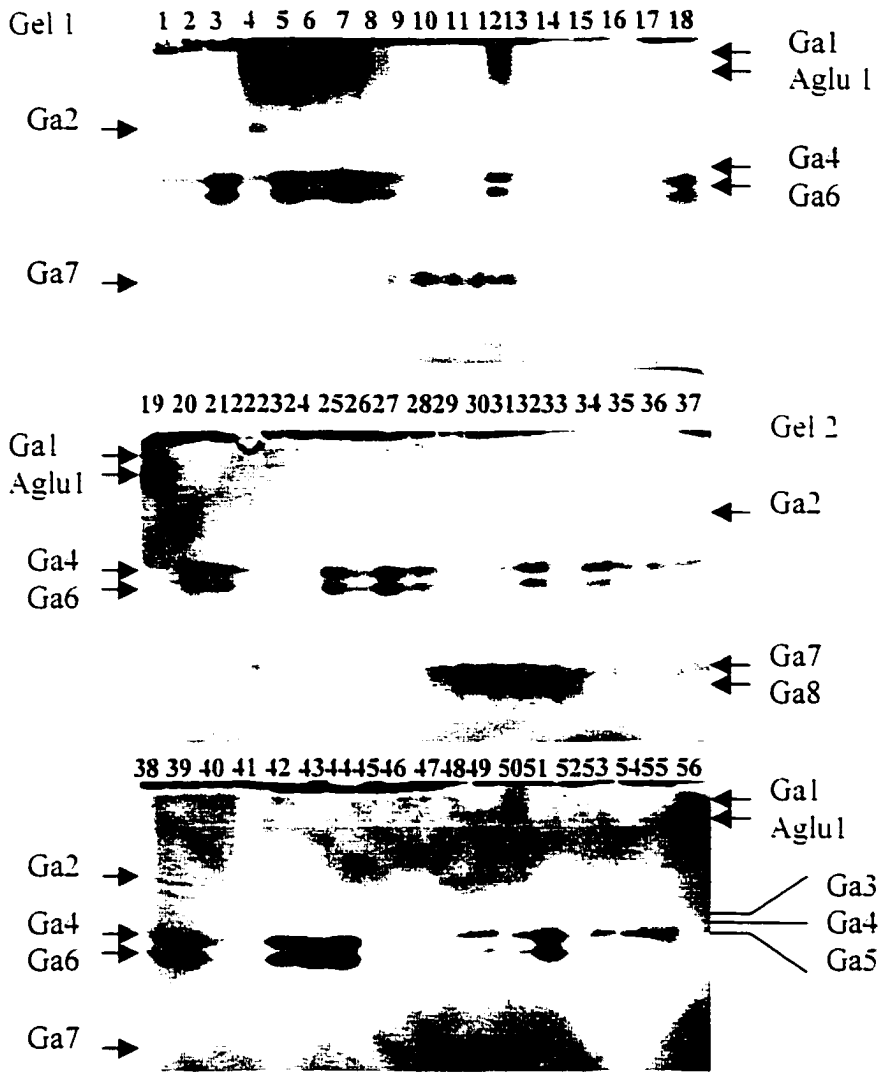


Figure 5.6: Acidic glucanase isoforms separated after native PAGE of leaf extracts of alfalfa transformants. Crude enzyme extracts (150 µg of total protein, except for root extracts, which were 20 µg) were loaded on 12 % PAGE gels, which were then incubated in laminarin solution for 45 min at 37°C and developed for glucanase activity. An extract from roots containing Aglu1 was used for comparison: lanes 19 and 56. Lanes 3 and 20,21,51,52: non-transformed plants 2a, 4b, 2b and 3f, respectively. *Aglu1* transformed plants: lanes 1 and 22 (1-1-2), 2 and 23 (1-3-b), 4 and 24 (3-1-a), 5 and 25 (3-3-1), 6 and 26 (5-1-a), 7 and 27 (6-2-a), 8 and 28 (7-2-b), 9 and 29 (9-1-b), 10 and 30 (13-1-a1), 11 and 31 (13-2-a2), 12 and 32 (17-4-a2), 13 and 33 (18-1-a2), 14 and 34 (18-2-b), 15 and 35 (18-3-b), 16, 36 and 48 (19-1-2), 17,37 and 49 (20-1-a), 18 and 50 (21-1-b1), 38 (1-1-1), 39 (1-1-b1), 40 (3-1-1), 41 (3-2-1), 42 (4-1-a), 43 (4-2-1), 44 (4-2-a), 45 (5-1-1), 46 (17-1-a), 47 (19-1-b1). Lanes 53, 54, 55: Arc, SarAR and Sar, respectively.

Ga6 of four plants (9-1-b, 13-1-a1, 5-1-1, and 17-1-a). In two of them (5-1-1 and 17-1-a), low Aglu1 activity and no induction of any other isoforms were observed suggesting that silencing might be occuring.

Arc, SarAR and Sar extracts were run in the same gels to use as a comparison. Arc and Sar cultivars had one common isoform (Ga4) with the transgenic lines whereas SarAR expressed two different isoforms.

A summary of the alfalfa transformants analysis is given in Table 5.1.

4. *Disease evaluation in transgenic plants*

Colletotrichum trifolii successfully infected stems and leaves of the parent cultivar Regen SY. Black setae produced by acervuli could be seen by eye especially on stems. Acervuli were also detected in leaves under the microscope (Figure 5.7, E, F). Transgenic plants 3-1-a and 17-4-a2 highly expressing Aglu1 were selected to test if the expression of Aglu1 could not prevent the symptoms caused by races 1 and 2 of *C. trifolii.* The transgenic plants (Figure 5.7, K, L, N, O) had the same disease severity as Regen SY and the non-transformed plant 2a (Figure 5.7, H, I). The Arc cultivar was infected by the same fungal inocula and was resistant to race 1 and susceptible to race 2 (Figure 5.7, B, C) as shown previously (Chapter 2).

Table 5.1: DNA and protein detection of the *aglu 1* transgene in alfalfa transformants. 2a, 4b, 2b and 3f: non-transformed plants. - : non detected; + : presence of *aglu1* or Aglu1.

Plants	*aglu 1* (PCR)	Ga1	Aglu 1	Ga2	Ga3	Ga4	Ga5	Ga6	Ga7	Ga8
2a	-					++		++		
4b	-					++		++		
2b	-					++		++		
3f	-					+		+		
1-1-2	+	+	+	+		+		+	+	
1-3-b	?	+	+	+		+		+	+	
3-1-a	+	+	++	+		+		+	+	
3-3-1	+	+	++	+		++		++		
5-1-a	+	+	++	+		++		++		
6-2-a	+	+	++	+		++		++		
7-2-b	+	+	++	+		++		++	+	
9-1-b	-	+	+	+		+/-		+/-	+	+
13-1-a1	-	+	+	+		+/-		+/-	+	+
13-2-a2	-	+	+	+		+		+	+	+
17-4-a2	+	+	++	+		++		++	+	+
18-1-a2	-	+	+	+		+		+	+	+
18-2-b	-	+	+	+		++		++	+	
18-3-b	+	+/-	+	+		+		+	+	
19-1-2	-	+	+	+		+		+	+	
20-1-a	+	+	+	+		+		+	+	+
1-1-1	+	+/-	+/-			+		+		
1-1-b1	+	+	+			++		++		
3-1-1	+	+	+			+		+	+	
3-2-1	+	+/-	+/-			+		+		
4-1-a	+	+	+			++		++	+	
4-2-1	-		+/-			++		++		
4-2-a	+		+/-			++		++		
5-1-1	+		+/-							
17-1-a	+		+/-						+	
19-1-b1	+	+/-	+/-			+		+	+	
21-1-b1	+	+	+	+		+		+	+	+
Arc					+	+				
SarAR					+		+			
Sar						+	+			
Root			+							

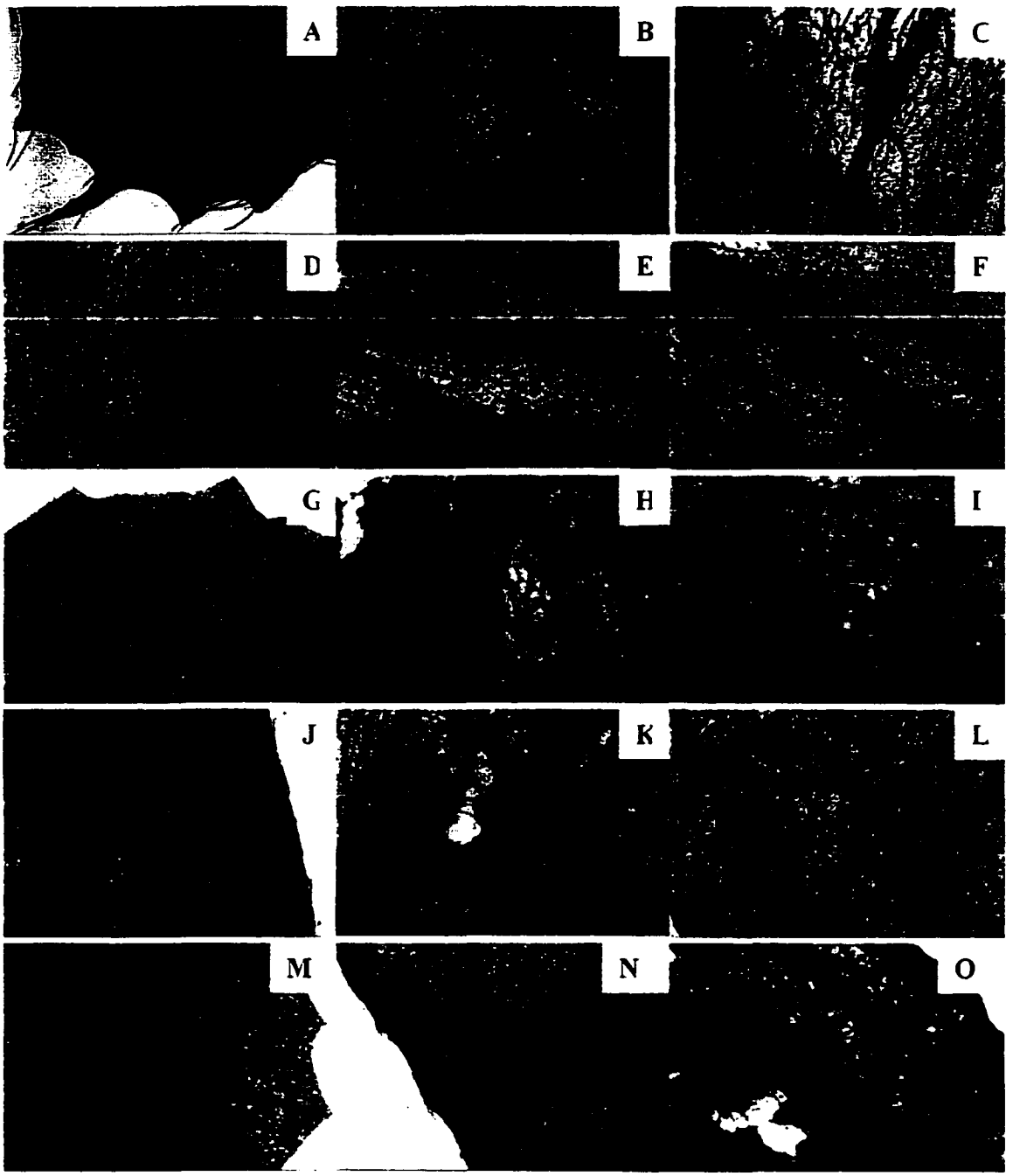


Figure 5.7: Evaluation of disease symptoms in alfalfa leaves caused by race 1 and race 2 of *Colletotrichum trifolii,* 7 days after infection. A,B,C, Arc cultivar; D,E,F, Regen SY SY; G,H,I, 2a non transformed alfalfa; J,K,L, 3-1-a *aglu 1* transformed alfalfa; M,N,O, 17-4-a2 *aglu 1* transformed alfalfa. Column A-M, non-infected alfalfa plants; column B-N, race 1 infected alfalfa plants, column C-O, race 2 infected alfalfa plants.

5. *Phytoalexin response in transgenic alfalfa after infection by C. trifolii*

To test the hypothesis that overexpressing the glucanase gene *aglu1* in alfalfa might release elicitors, thus inducing other plant defenses, isoflavonoid levels were analyzed in the two transformants 3-1-a and 17-4-a2 and compared with the control lines SY and 2a, as well as Arc. Arc leaves accumulated medicarpin and medicarpin conjugates faster during an incompatible interaction compared to a compatible interaction when infected by *C. trifolii.* The accumulation occurs from 24 to 48 h after inoculation (Chapter 2). These two time points were therefore chosen for our study with the transgenic plants. Figure 5.8 shows HPLC profiles of leaf extracts from Arc and one of the *aglu1* overexpressing lines (17-4-a2) 48 h after inoculation with race 1 of the fungus. No medicarpin, formononetin or their conjugates were detected in any of the transgenic alfalfa plants or the control lines infected by race 1 or race 2 of *C. trifolii* or in non-infected plants (Figure 5.8, A). As previously observed, a peak of medicarpin was detected in race 1 infected Arc leaves 48 hours after inoculation (Figure 5.8, B).

Taken together these data suggest that overexpressing *aglu 1* in alfalfa does not potentiate a phytoalexin response after infection by the fungal pathogen *C. trifolii,* but provide further evidence for the importance of phytoalexins in the alfalfa/*C. trifolii* interaction. In the experiments

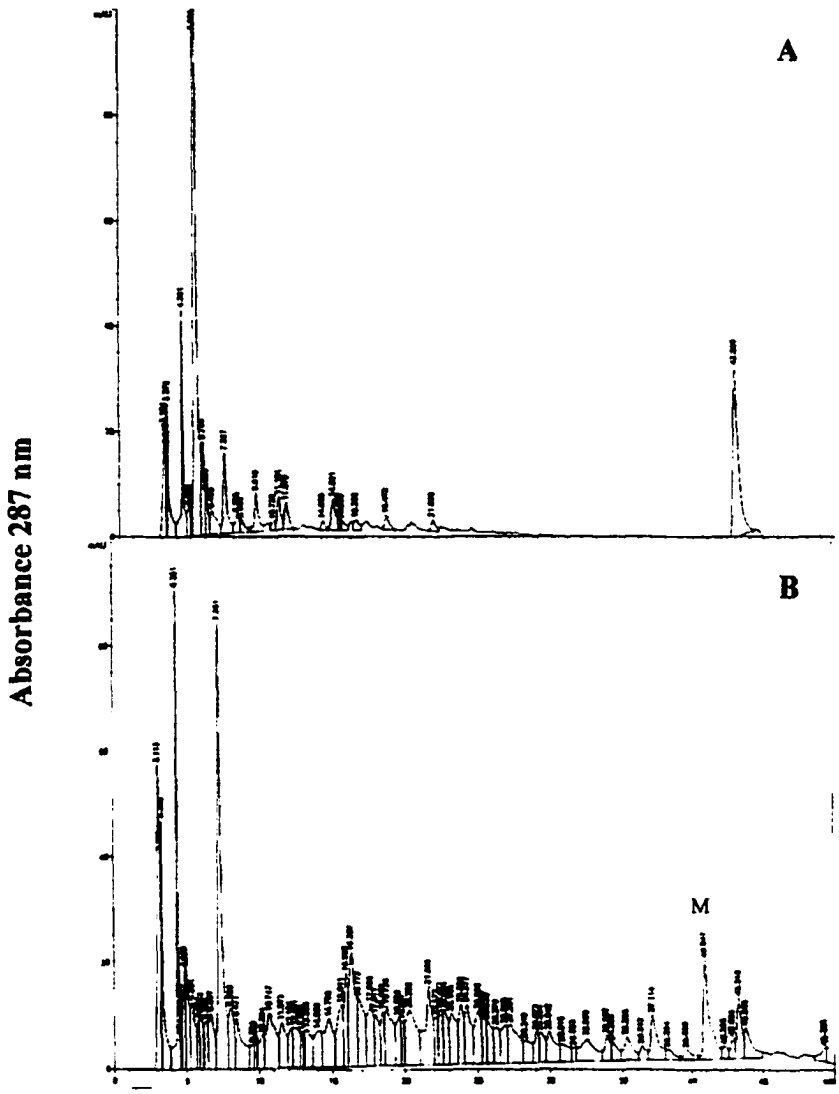


Figure 5.8: HPLC analysis of alfalfa leaves infected with Race 1 *C. trifolii* 48 hours after inoculation, with the eluant monitored at 287 nm. (A) 17-4-a2 *aglu 1* transformed alfalfa. (B) Arc cultivar. Metabolites were identified as: M = medicarpin.

described above, Arc which is resistant to race 1 of the fungus produced medicarpin only when infected by race 1 of *C. trifolii* but not when infected by race 2 (Figure 5.7, B and Figure 5.8, B). The importance of the medicarpin response is also strongly suggested by the fact that non-transformed and transformed Regen SY leaves do not produce any medicarpin during an infection by either race of *C. trifolii* and are all susceptible to the fungus.

DISCUSSION

Fungal diseases are of great economic importance because of the yield losses they cause on crops and forage plants. The extensive application of chemicals to control them has become alarming for the environment and less efficient due to the development of resistance in fungal pathogens. Therefore, much effort has been given to develop alternatives to time consuming classical breeding and the use of chemicals. One of them, genetic engineering that involves direct transfer of defense genes into plants of interest has been successful in enhancing fungal resistance. Examples of defense genes include genes encoding PR-1a (Alexander *et al.*, 1993), chitinases and glucanases (Broglie *et al.*, 1991; Vierheiling *et al.*, 1993; Zhu *et al.*, 1994; Masoud *et al.*, 1996; Yoshikawa *et al.*, 1993; Grison *et al.*,

1996), osmotin (Liu *et al.*, 1994) and lipid transfer proteins (Molina and Garcia-Olmedo, 1997). Other approaches to engineer fungal resistance have been based on overexpressing genes encoding enzymes involved in the biosynthesis of phytoalexins (Hain *et al.*, 1993, Thomzik *et al.*, 1997, Stark-Lorenzen *et al.*, 1997; Hipskind and Paiva, 2000; He and Dixon, 2000). Finally, another possibility involves the production of proteins that activate signaling pathways and thus trigger several plant defenses (Wu *et al.*, 1995; Keller *et al.*, 1999).

In that last context, a glucanase gene (*aglu1)* was introduced into alfalfa to determine if its constitutive expression would provide fungal resistance by inducing other plant defenses as a result of its potential elicitor releasing ability. Most of the plants had successfully integrated the *aglu1* transgene in their genome. Activity gel analysis indicated that the gene was successfully expressed in the majority of the trangenic alfalfa plants although with some variation. This observation, in addition to the suppression of endogenous glucanases Ga4 and Ga6 in two of the plants that were weakly expressing the transgene, suggest that the introduced gene and its homologous endogenous genes could be suppressed by silencing. This mechanism has already been described in previously generated alfalfa plants overexpressing the same glucanase gene (Masoud *et al.*, 1996; Chapter 4)

and in other plants overexpressing chitinase and glucanase genes (De Carvalho *et al.*, 1992; Neuhaus *et al.*, 1991; Hart *et al.*, 1992).

Two transgenic plants highly expressing the *aglu1* transgene were tested for resistance against *C. trifolii*. No reduction of the symptoms was detected on the plants. Susceptibility to the fungal pathogen after overexpressing a defense gene has been reported in the literature (Neuhaus *et al.*, 1991a; Masoud *et al.*, 1996; Kobayashi *et al.*, 2000). However, more plants need to be tested for disease resistance to *C. trifolii* to confirm that the engineered plants are always susceptible to the fungus. In addition, other alfalfa fungal pathogens need to be inoculated on transgenic plants to determine if fungal resistance to alternative pathogens may have been successfully engineered.

The possibility that *aglu1* could release elicitors from *C. trifolii* during an infection was indirectly investigated by analyzing the content of isoflavonoids in two *aglu1* overexpressing alfalfa plants. Numerous reports attest the induction of phytoalexins after elicitor treatment (Ayers *et al.*, 1976; Yoshikawa *et al.*, 1981; Keen *et al.* 1983; Ren and West, 1992; Milat *et al.*, 1991; Kessmann *et al.*, 1990a,b). No accumulation of medicarpin was detected in leaf extracts from the transgenic plants infected with either race of the fungus. Most likely *aglu1* does not release elicitors from the cell wall

of *C. trifolii* as no medicarpin response was detected, and thus glucanase is not a factor greatly influencing the outcome of the interaction as plants did not show any signs of resistance.

Previous results with *C. trifolii* provided evidence for the importance of the accumulation of phytoalexins in alfalfa in conferring resistance to the fungus (Chapter 2). The data presented here on phytoalexin responses after *C. trifolii* infections (race 1 and 2) in *aglu1* overexpressing plants give more evidence for the important role of phytoalexins in resistance against this fungus. When no medicarpin was induced upon infection, all plants were susceptible to the fungus. On the other hand, the only plant (Arc) accumulating medicarpin was resistant to the fungus.

An interesting observation in plants expressing the *aglu1* transgene was the presence of four other glucanases (Ga1, Ga2, Ga7 and Ga8) and possibly a fifth one (data not shown). Indeed, 95% of the transgenic plants expressing *aglu1* had at least two additional glucanase isoforms compared to the trangenic plants not expressing the transgene or non-transformed lines. The Ga2 isoform observed in this study has similar electrophoretic mobility in native PAGE compared to the Ga2 isoforms detected in two alfalfa plants overexpressing the *Rch10* rice chitinase gene (Table 4.1, Chapter 4). This information suggests that hydrolytic enzymes in alfalfa are closely regulated

and the dose of each of them may be important for the plant. Plants expressing glucanase or chitinase transgenes may generate signals that induce genes of the same family, or related genes encoding for proteins having the same biological functions. As these glucanases have not been isolated and their corresponding genes cloned, it may be possible that these isoforms are generated from glucanases present in the plant (Aglu1, Ga4 and Ga6) after splicing or post-translational modifications.

Chitinases and glucanases have been shown to have synergistic anti-fungal properties (Mauch *et al.*, 1988a,b; Sela-Buurlage *et al.*, 1993; Ji and Kúc, 1996). It would be of interest to purify these proteins and test their anti-fungal activity with several fungi *in vitro*. If they were acting synergistically, that would explain their concomitant induction *in vivo*.

The hypothesis that overexpressing a glucanase in alfalfa would release elicitors and therefore induce other plant defenses can not be totally ruled out before testing more transgenic plants and more fungi. Aglu1 may not release elicitors from *C. trifolii* cell walls but may do so from another fungal pathogen. Furthermore, the additional glucanase isoforms produced in transgenic plants could release active elicitors themselves or have anti-fungal activities against other fungi than *C. trifolii*. Phytoalexins definitely appears to play an important role in plant defense for the alfalfa/*C. trifolii*

interaction. Direct evidence for the involvement of phytoalexins in fungal resistance has been demonstrated through genetic engineering of alfalfa against *Phoma medicaginis* (Hipskind and Paiva, 2000; He and Dixon, 2000). Clearly, the optimum strategy to engineer fungal resistance in alfalfa is to introduce genes that lead to an increased phytoalexin response but that are only induced upon fungal attack to avoid the phytotoxic effect of medicarpin and the silencing of the transgene.

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Appendix 1

1-Glucanase colorimetric assay

- Copper solution (1 l): a-160 mM Copper Sulfate
  b-1.3 M Sodium Sulfate
  c-226 mM Sodium Carbonate
  d-190 mM Sodium Bicarbonate
  e-43 mM Sodium Potassium Tartrate

Dissolve b, c, and e in appoximately 500 ml of deinonized water, dissolve a in approximately 100 ml of deionized water and slowly add a to the above solution. First dissolve the sodium bicarbonate in deionized water then into the above solution. Add deionized water up to 1 l. Store the solution in the dark at room temprature.

- Arsenic-Molybdic solution (1 l): 40mM Molybdic acid
  19 mM Arsenic acid
  756 mM Sulfuric acid

Dissolve molybdic acid in 300 ml of deionized water, then add sulfuric acid slowly. First dissolve arsenic acid in water and add it to the above solution. Bring the solution to 1 l by addition of deionized water and incubate at 37°C for 72 H. Store the solution in the dark at room temperature.

2-Chitinase colorimetric assay

- DMAB reagent: Dissolve 8 g of dimethylaminobenzalhedyde in 70 ml of acetic acid and mix it with 10 ml of HCl. Add 1 volume of this reagent to 9 volumes of acetic acid just before experiment.

3-HPLC analysis

- Citrate-Phosphate buffer: Dissolve 0.467 g of citric acid and 1.378 g Na_2HPO_4 in about 80 ml of water, adjust the pH to 5.2, and bring up the volume to 100 ml with deionized water.

4-Northern-blot

1. Formaldehyde gel electrophoresis:

- 10 X MOPS: 200 mM MOPS
  50 mM Sodium acetate
  10 mM EDTA
  pH 8.0 with acetic acid or NaOH.
  Up to volume with DEPC water
  Do not autoclave.
- 5 X RNA loading buffer: 0.4 % saturated bromophenol blue
  80 µl 500 mM EDTA, pH 8.0
  720 µl 37 % formaldehyde (12.3 M)
  2 ml glycerol
  3084 µl formamide
  4 ml 10 X MOPS
  Add DEPC water up to 10 ml.
  Stability: 3 months at 4°C.

2. RNA hybridization

- Prehybridization buffer: 5 X SSC
  0.1 % SDS
  40 % deionized formamide
  5 X denharts solution (appendix 1)
  0.5 mg/ml sonicated Salmon sperm DNA (Stratagene) (10 mg/ml)

First denature salmon sperm DNA by incubating in boiling waterbath for 3 min and then add it to the prehybridization buffer.

- Hybridization buffer: 5 X SSC
  0.2 % SDS
  40 % deionized formamide
  5 X denharts solution (appendix 1)
  Radiolabelled probe (50 µl) from kit "ready to go"

First denature the probe for 3 min and then add it to the hybridization buffer.

- Labelling of cDNA probe (Ready to go kit, Pharmacia):

Denature the DNA to be labelled by heating at 95-100°C for 3 min, and place it on ice. Add the DNA (≤45 µl) to the reaction mix bead tube, then 5 µl of α32P dCTP (3000 Ci/mmol) and water up to 50 µl. Gently mix the components and incubate the reaction out for 1 H at 37°C.

Appendix 2

1-Media

- Liquid medium for fungal growth:

  15 g sucrose
  2 g asparagine
  0.2 g $MgSO_4$ 7 H_2O
  1 mg $FeSO_4$ 7 H_2O
  10 mg $CaCl_2$ 2 H_2O
  1 mg Thiamin-Cl
  1.04 g K_2HPO_4
  1.9 g KH_2PO_4
  1 mg $ZnSO_4$ 7 H_2O
  3 g Ca Nitrate
  Water up to 1 liter
  Sterilize 30 minutes.

- Medium for alfalfa cell cultures (1 l):

  1 X SH medium (Dixon and Fuller, 1976)
  30 g sucrose
  pH 5.8
  Sterilize 30 minutes.

Appendix 3

Southern-blot

1. Total DNA extraction:
    - DNA extraction buffer (CTAB 2 X): 100 mM TrisCl
      1.4 M NaCl
      20 mM EDTA
      2 % CTAB
      0.2 % 2-mercaptoethanol
      pH 8.0
    - DNA precipitating buffer: 1 % CTAB
      50 mM TrisCl
      10 mM EDTA
      pH 8.0
2. Restriction enzyme digestion and agarose gel electrophoresis
    - 40 X TAE: 1.6 M Tris
      0.8 M Sodium acetate-$3H_2O$
      40 mM $Na_2EDTA\text{-}2H_2O$
      pH7.2 with glacial acetic acid
    - 10 X Agarose loading buffer: 50 % glycerol
      100 mM $Na_2EDTA\text{-}2H_2O$
      1 % SDS
      0.1 % Bromophenol Blue
      Add deionized water up to 10 ml.
3. DNA transfer to nylon membrane
    - 20 X SSC: 3 M NaCl
      0.3 M Sodium citrate dihydrate
      pH 7.0
4. DNA hybridization
    - Prehybridization buffer: 4 X SSC
      0.1 % SDS
      5 X Denharts solution

Salmon sperm DNA (10 mg/ml) add 100 µl for every 10 ml of buffer.

First denature salmon sperm DNA by incubating in boiling waterbath for 3 min and then added to the prehybridization buffer.

- 100 X Denharts solution: PVP (MW 40,000) 1 g
  Bovine serum albumin 1 g
  Ficoll 400 1 g
  Add deionized water to make 50 ml and filter-sterilize. Store at -20°C.
- Hybridization buffer: 4 X SSC
  0.1 % SDS
  5 X Denharts solution
  Radiolabelled probe (50 µl) from kit "ready to go"

First denature the probe for 3 min and then add it to the hybridization buffer.

- Labelling of cDNA probe (Ready to go kit, Pharmacia):

Denature the DNA to be labelled by heating at 95-100°C for 3 min, and place it on ice. Add DNA (≤45 µl) to the reaction mix bead tube, then 5 µl of α32P dCTP (3000 Ci/mmol) and water up to 50 µl. Gently mix the components and incubate the reaction for 1 H at 37°C.

Appendix 4

1-Plant transformation

- YEP (1 l): 10 g Bacto peptone
  10 g Yeast extract
  5 g NaCl
- Chlorox solution: 80 ml sterile deionized water
  20 ml of chlorox
  4 drops of Liqui-Nox detergent
- A1 medium (1 l): 4.3 g MS Salt mix
  10 ml Vitamin B5 100 X Gamborg's solution
  0.25 g Casein hydrolysate
  30 g sucrose
  1 ml 10 mM 2,4-D (Dichlorophenoxy acetic acid)
  1 ml 1 mg/ml BAP (6-Benzylaminopurine)
  (8 g Phytagar)
  pH 5.7

When medium is ≤55°C add 0.1 ml of 1 M sterile acetosyringone.

- A2 medium (1 l): 4.3 g MS Salt mix
  10 ml Vitamin B5 100 X Gamborg's solution
  0.25 g Casein hydrolysate
  30 g Sucrose
  1 ml 10 mM 2,4-D
  1 ml 1 mg/ml BAP
  pH 5.7

When medium is ≤55°C add filter sterilized antibiotics:

1 ml 400 mg/ml Carbenicillin

0.5 ml 50 mg/ml Kanamycin

- Alfalfa stock solution: Carbenicillin 400 mg/ml in ddH_2O, filter sterilized.
  Kanamycin 50 mg/ml ddH_2O, filter sterilized.

2,4-D 10 mM in 95 % ethanol.

Acetosyringone 1 M in DMSO, filter sterilized.