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THE PATHOGENESIS RELATED PROTEIN, CHITINASE, AND ITS
ROLE IN THE SYSTEMIC ACQUIRED RESISTANCE PHENOTYPE
IN CUCUMBER PLANTS (*Cucumis sativus* L.)

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Luis Velasquez

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**THOGENESIS RELATED PROTEIN, CHITINASE, AND ITS ROLE IN THE
SYSTEMIC ACQUIRED RESISTANCE PHENOTYPE IN CUCUMBER PLANTS
(*Cucumis sativus* L.)**

By

LUIS A VELASQUEZ

A DISSERTATION

**Submitted to
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ABSTRACT

THE PATHOGENESIS RELATED PROTEIN, CHITINASE, AND ITS ROLE IN THE SYSTEMIC ACQUIRED RESISTANCE PHENOTYPE IN CUCUMBER PLANTS (*Cucumis sativus* L.)

By

Luis A Velasquez

Systemic Acquired Resistance (SAR) refers to a distinct plant response that systemically confers broad-spectrum disease resistance after an induction of biotic or abiotic nature. Accumulation of Pathogenesis-Related (PR) proteins such as chitinase (PR-8) takes place during the induction of SAR.

In order to study chitinase activity in cucumbers, a new method for chitinase detection and quantification was developed. In addition to determine if SAR requires active metabolism after challenge, leaf disks of cucumber plants expressing SAR were treated with the protein synthesis inhibitor cycloheximide and then challenged with the fungus *Colletotrichum orbiculare*. Cyclohexamide treatment in SAR expressing plants caused an increase in penetration of the fungus and symptom development was comparable to non-induced plants. This result suggests that the induced state of cucumber plants is dependant of protein synthesis.

In field and greenhouse experiments, no correlation between the amount of chitinase induction and the degree of resistance acquired through SAR was found. In addition, a differential cultigen response to two pathogens with different infections strategy was found. Cucumber cultivars expressing SAR are generally resistant to *Colletotrichum . orbiculare* but are not always resistant to *Didimella*

bryoniae. Several cultivars expressing SAR developed more severe symptoms to *D. bryoniae* despite the high chitinase activity detected. This suggests a differential resistance pathway and questions the ability of chitinase to decrease pathogen growth within the tissue.

Transgenic cucumber plants bearing the cDNA sequence of chitinase type III in the sense and antisense directions were generated. Treatment of the transformed plants with the resistance activator acybenzolar-S-Methyl (ASM) demonstrated no significant differences in their ability to withstand pathogen attack or express induced resistance. Results obtained suggest that the acidic chitinase is important for the basal levels of resistance prior to induction. The amount of acidic chitinase activity was suppressed in plants bearing the sense and antisense constructs possible due to gene silencing.

The NPR1 gene regulates SAR and ISR and could be a good molecular SAR marker. Constitutive levels of expression can be correlated with resistance due to SAR. A partial sequence of a cDNA fragment of the NPR1 of cucumber plants was isolated.

The present study indicates that chitinase does not play a significant role in the SAR phenotype. It is possible that the timely activation of the entire set of SAR genes is more important than the effect of a single gene even if it is over expressed.

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DEDICATION

A mis padres, Don Obdulio Velasquez y doña Bertita de Velasquez por darme vida y darme la oportunidad de seguir mis sueños. A mis hermanos y hermanas por su apoyo moral. A mi adorada esposa, Sara, por su apoyo, su amor y su comprension. A mi pueblo, San Pedro Sacatepequez, San Marcos, en la tierra de la eterna primavera, Guatemala, Centro America. Pedacito de mi corazon que jamas olvido.

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CHAPTER I
GENERAL INTRODUCTION

INTRODUCTION

Plants are subjected to various adverse conditions throughout their life from stresses caused by both, abiotic and biotic agents such as plant pathogenic microorganisms (Agrios 1997). The challenge of producing an adequate supply of food becomes harder as the world population grows, the amount of land for agriculture shrinks and the pressure of new or emerging and pesticide resistant plant pathogens intensifies.

Resistance, as defined by Agrios (Agrios 1997), is the ability of an organism to exclude or overcome, completely or in some degree the effect of a pathogen or other damaging factor. Disease resistance in plants is expressed in two forms; a constitutively expressed resistance and resistance that is induced to be expressed after initial infection. The preexisting structural and chemical structure is the first line of defense, which the pathogen must penetrate to cause disease. Such structures include, amount and quality of wax and cuticle over the epidermal cells, the size, location and shapes of stomata and lenticels, and the presence on the plant tissue made of thick-walled cells that hinder the advance of the pathogen. In addition there are also the preexisting chemical defenses. These include among others, fungi-toxic exudates, inhibitors present in the cell before infection such as phenolic compounds, tannins and some fatty acid-like substances.

The Induced form of resistance must have a recognition event of the pathogen by plants. The speed by which the plant recognizes the pathogen, sends out alarm message(s), and mobilize its defenses will determine whether

an infection will be successful or not, and/or how severe the disease will be. The induced form of resistance can be segregated into two different groups, induced structural defenses and induced biochemical defenses. It has been accepted that plants develop a broad type of resistance known as systemic acquired resistance (SAR) (Kuc 1982). The SAR phenomenon has been marked by the systemic protection of plants against pathogens. SAR activation starts with a local infection of plants by pathogens, which result in the spreading of the resistance to the vicinity of the lesion and to distant plant organs (Kuc 1995; Ryals et al. 1996).

Historical perspective of SAR

SAR was recognized by the description of the initial observations since the turn of the century compiled by Chester in (Chester 1933). Such observations described the heightened resistance to pathogen attack that developed by plants surviving an initial infection. Ross (Ross 1961) coined the term "Systemic Acquired Resistance" and made extensive characterizations of the phenomenon. After inoculating *Ngene* tobacco with TMV, Ross observed that the area around the inoculation became more resistant to TMV, a phenomenon now recognized as localized acquired resistance. In addition, other plant parts remote from the initial inoculation site became more resistant, as expressed by the fewer number of lesions developed after a challenge inoculation to TMV. In the 1970's, Kuć and coworkers, demonstrated that induction of the first leaf of cucumber plants with *Colletotrichum orbiculare* primed the rest of the plant to become more

resistant against *Colletotrichum orbiculare*, and other fungi, bacteria and viruses (Kuc 1982). Overall, the symptom severity caused by the challenging pathogen was substantially reduced (Hammerschmidt 1999). These observations indicate that SAR is a mechanism through which the level of general resistance to pathogens is greatly increased (Van Loon 1997).

Biochemistry of SAR

Induction of SAR

The induction of systemic acquired resistance can be accomplished by an array of biological and chemical inducers (Oostendorp et al. 2001). In all cases, the induction is characterized by an overall decrease of pathogen damage in parts remote from the site of primary inoculation/induction.

The activation of SAR can be achieved by several means. This usually refers to macromolecules, originating either from the host plant or from the plant pathogens, which are capable of inducing SAR such as chitosan and chitin oligomers (Barber et al. 1989; Barber and Ride 1994; Benhamou and Nicole 1999). Microbial-mediated SAR as in the case of fungi (Roby et al. 1988 Hammerschmidt, 1981 #169) bacteria (Kuc and Hammerschmidt 1978), viruses (Ross 1961). In addition to the biotic inducers, the chemically induction of SAR is also possible by the application of plant disease resistance activators (Friedrich et al. 1996; Kastner et al. 1998; Narusaka et al. 1999). Among these plant disease resistance activators are exogenous applications of salicylic acid, 2,6-dichloroisonicotinic acid (DCIA) (Kessmann et al. 1994), the novel plant protection substance acibenzolar S-methyl (ASM) formerly known as BTH

(Friedrich et al. 1996; Lawton et al. 1996); (Dann et al. 1998) and ethylene (Boller et al., 1983).

The Signaling nature of SAR

Evidence indicates that the primary inoculation/induction results in the translocation of a still unknown signal produced at the site of the initial inoculation (Metraux 2001). This signal seems to trigger a complex array of defense mechanisms that in turn make the plant more resistant to pathogen attack. The nature of the signal triggering this response has been object of intense review and research. Early experimental data indicated that exogenous application of Salicylic acid (SA) might play a role in the establishment of SAR (White 1979). Moreover, in cucumber, it was showed that SA concentrations become elevated after pathogen infection of leaves (Metraux et al. 1990) providing further evidence of the possible role of SA in SAR expression and establishment. SA was then, regarded as the direct signaling mechanism to trigger SAR . However, the findings of Vernooij *et al* (Vernooij et al. 1994) employing *NahG* tobacco in grafting experiments showed that SA might not be the systemic signal. In addition, the analysis of phloem exudates from cucumber petioles prior during and after the setting of SAR suggest that SA and is synthesized *de novo* in stems and petioles in response to a mobile signal from the inoculated leaves (Smith Becker et al. 1998). This supports the findings of Rasmussen and Hammerschmidt (Rasmussen et al. 1991). Their work endorse a role for salicylic acid as an endogenous inducer of resistance, but their data also suggest that

salicylic acid is not the primary systemic signal of induced resistance in cucumber.

Changes in planta after SAR induction

Once the SAR signal is produced, an array of responses are generated in the areas surrounding the original inoculation as well as in areas remote from the initial inoculation point (Sticher et al. 1997). Activation of several gene families and changes in the biochemistry of the plant is usually the norm. Among the initial responses, Salicylic acid is synthesized (Smith Becker et al. 1998), lignin deposition is increased (Hammerschmidt and Kuc 1982; Stein et al. 1993). In addition, an enhanced peroxidase activity that has been associated with systemic acquired resistance of cucumber against *Colletotrichum orbiculare*, also takes place (Hammerschmidt et al. 1982; Rasmussen et al. 1995). After infection, strengthening of the cell wall can also occur by the action of peroxidase catalyzed cross-linking of hydroproline-rich structural cell wall glycoproteins (Bradley et al. 1992). In addition to cell wall depositions, the accumulation of the proteins commonly known as Pathogenesis-Related (PR) proteins also takes place during the induction of systemic acquired resistance (van Loon and van Kammen 1970). Among these PR proteins, the presence of β -1,3-glucanase (PR-2) and chitinase (PR-8) among others are a characteristic of cucumber plants expressing SAR. The expression of the SAR genes and the changes in the biochemistry of the plant after induction may contribute to the enhanced resistance of the plant against pathogens. This resistance might be expressed by either stopping the pathogen from infecting the plant and or by limiting the

spread of the disease, which would allow the plant to respond to the disease more effectively by activating other defense mechanisms (Mettraux 2001). Among the most studied responses of the SAR phenomenon, is the expression increase of the pathogenesis related (PR) proteins after induction and their possible role in plant resistance.

The Pathogenesis Related (PR) Proteins

Definition:

PR- proteins were originally described in the 1970's independently by Van Loon and Gianinazzi (Gianinazzi et al. 1970; van Loon and van Kammen 1970). These proteins were first observed as new protein components induced by the interaction between tobacco mosaic virus (TMV) in hypersensitivity reacting tobacco (van Loon and Cornelis 1999) detected by protein electrophoresis analysis. Originally they were defined as acid-soluble protease-resistant, acidic proteins localized in the extracellular space (Sticher et al. 1997). Later, the name PR-proteins was coined to refer to proteins coded by the host plant, but induced only in pathological related situations (van Loon and Cornelis 1999). These pathological related situations were not only limited to plant pathogen interaction, but also includes nematodes, insects or herbivores, treatments with certain chemicals, and other types of stress (Sticher et al. 1997).

Classification of the PR proteins:

As research on the PR proteins continued, the proteins have been grouped into families. Initially, the 10 major acidic PRs of tobacco were grouped

into five families, designated PR-1 to PR-5 (van Loon et al. 1994; van Loon and Cornelis 1999). This classification sets a convenient protocol to follow for classification of other PR proteins from other species. Eleven families are now known in tobacco, and the existing nomenclature is being used to classify PR proteins in other plant species such as maize, cucumber, *Arabidopsis* (van Loon et al. 1994). The proposed nomenclature calls for the families to be numbered, and the different members within each family are assigned letters according of the order in which they are described. They must belong to the same PR famil, but the lettering only reflects how many proteins of that family had been identified within the plant species previous to their discovery (van Loon and Cornelis 1999; Van Loon and Van Strien 1999).

Possible Functions:

Phytoalexin accumulation and cell wall modifications are local reactions, accumulation of PR proteins extends into non-inoculated plant parts, that upon challenge, exhibit SAR (Ryals et al. 1996). The demonstration that some PR proteins such as β -1,3-glucanase and chitinases have lysozyme activity (Kauffmann et al. 1987; Legrand et al. 1987) and anti-fungal activity *in vitro* (Ji and Kuc 1996) immediately suggest that these enzymes might have a direct role against cell walls of bacteria and fungi. In addition, their localization, apoplast, (Boller and Metraux 1988) seems to guarantee contact with invading pathogens before these are able to colonize plant tissue. This fact suggests that these PR proteins are indeed playing a direct role in the defense mechanism of the SAR expressing plants (Boller and Metraux 1988; Lee and Hwang 1996). Another

possible role suggested is the release of elicitors from the walls of the invading pathogens (van Loon and Cornelis 1999). These elicitors might trigger or enhance the expression other defense mechanisms. This role might be possible since chitinases and glucanases do release chitin and glucan oligomers from the cell wall of invading pathogens and their effects on the defense of plants has been somewhat assessed (Roby et al. 1987; Roby et al. 1988). In addition to their possible antimicrobial characteristics, most PR proteins are also found in various floral tissues suggesting specific physiological functions during flower development rather than a role in general defense against pathogen infection (van Loon and Cornelis 1999).

Chitinase:

Chitinase occurs naturally in a wide variety of higher plants and catalyzes the hydrolysis of chitin, a linear polymer of $\beta(1,4)$ -N-acetylglucosamine (Muzzarelli 1977), a constituent of fungal cell walls and insect cuticles. Chitinase from plant tissue has been found to be partially responsible in the process of dissolution of hyphal walls in plants *in vitro* (Skujins et al. 1965; Ji and Kuc 1996). Increase in chitinase activity has been correlated with the increase in disease resistance observed in plants expressing induced resistance (Roby and Esquerre Tugaye 1987; Roby et al. 1988; Dalisay and Kuc 1995; Hwang et al. 1997; Xue et al. 1998). SAR inducible acidic chitinase is found in very low amounts in healthy tissue, but its quantity and activity is increased in response to pathogen attack by fungi (Roby and Esquerre Tugaye 1987; Dalisay and Kuc 1995; Dann et al. 1996; Kastner et al. 1998), bacteria, (Lee and Hwang 1996; Gerhardt et al.

1997) viruses, nematodes (Rahimi et al. 1998) and even insects (van der Westhuizen et al. 1998). The enzyme can be induced by wounding (Cabello et al. 1994; Zhang and Punja 1994), heat shock (Margispinheiro et al. 1994) and the application of plant disease resistance activators (Friedrich et al. 1996; Kastner et al. 1998; Narusaka et al. 1999) or ethylene (Boller et al. 1983). The systemic induction of PR proteins in SAR is associated with an overall decrease of pathogen damage in parts remote from the site of primary inoculation/induction. In addition, their localization within the tissue in association with fungal structures in resistant cultivars has been also demonstrated (Boller and Metraux 1988).

Classification of chitinases:

As of today a total of 189 chitinases have been purified and/or cloned from many plants and reported in the EMBL database. Originally chitinases were classified: as glycosyl hydrolases, as pathogenesis-related proteins, chitinases classes, and gene families. A classification was introduced which was composed of three classes, later it was extended to six classes based on sequence comparisons. To better understand this nomenclature the following chart shows the current classification of plant chitinases (Neuhaus 1999)

PR Proteins	Family of glycosyl hydrolases	Gene name	Class	Chitin-binding domain	Catalytic domain	Number of known sequences
PR-3	19	<i>Chia1</i>	I	1	I	50
		<i>Chia2</i>	IIb	-	I	11
		<i>Chia3</i>	IIa	-	II	16
		<i>Chia4</i>	IV	1	IV	24
		<i>Chia5</i>	V	2	C	1
		<i>Chia6</i>	VI	1/2 + Pro		1

		<i>Chia7</i>	VII	-	IV	1
PR-8	18	<i>Chib1</i>	III	-		19
PR-11	18	<i>Chic 1</i>	I	-		3
PR-4	Unassigned	<i>Chid1</i>	I	-		7
		<i>Chid2</i>	II			5

PR-8/Class III chitinases

These chitinases were originally purified and cloned from cucumber (Metraux et al. 1989) and soon many others follow. The chitinases in this class have similar amino acid sequences. However, they vary in isoelectric points (Boller and Metraux 1988). The cucumber class III chitinase is acidic, and is localized in the intercellular spaces of plant expressing SAR. (Boller and Metraux 1988). Chitinases from cucumber of this class has been found to have antimicrobial activity *in vitro* (Ji and Kuc 1996).

The Connection of Chitinase and SAR

During the last years of chitinase research in cucumber, most of the data has come from the use of a limited number of varieties. In addition to SMR-58, which has been the primary cultivar used for induced resistance research in cucumbers, two other cucumber cultivars had been examined for this enzyme. In all cases, chitinase activity has been correlated with the amount of enhanced resistance after SAR induction. In other words, the activation of SAR in cucumbers via biotic or abiotic inducers follows an activation of SAR genes including acidic chitinases and an enhanced resistance against a series of pathogens. However, these correlations present several problems. First, as

mentioned before, the majority of the experiments had been done with a limited amount of cucumber genotypes, and most of the experiments had been done in controlled greenhouse conditions. Therefore, the lack of diversity in the genotypes used for SAR activation and correlation studies linking chitinase expression with the enhanced state of resistance after SAR activation questions the validity of these correlations. In addition experiments have also been done in which the state of resistance is long gone, however the chitinase activity still high in induced plants.

Chitinase as a biochemical marker for SAR

The chitinase in cucumber, and in many other species has been frequently used as a marker for systemic acquired resistance, and other forms of resistance against pathogens (Roby et al. 1988; Roberts et al. 1994; Salzer et al. 2000). In addition, the localization of the chitinase in the apoplast (Boller and Metraux 1988), and its *in vitro* antifungal activity (Skujins et al. 1965) has provided additional arguments to support the role for the acidic chitinases in plant defense. However, there is inconclusive evidence *in vivo* for the role of the induced chitinase in stopping the spread of the pathogen in host tissue. As for the marker theory, it is limited to speculations since no many experiments had been done in order to quantify the induction of chitinase and its possible use as marker for plant resistance. It was not until recently, when it was reported that the basal amounts of PR proteins might serve as markers for resistance (Vleeshouwers et al. 2000). Finally, there is a lack of information on the interaction of the chitinase and the pathogen *in vivo*.

Over expression experiments

Transgenic plants overexpressing chitinase had been used to generate evidence for the role of chitinase in resistance (Sticher et al. 1997); (Bauer et al. 1998; Tabei et al. 1998; Tabaeizadeh et al. 1999). However, the results have not been as clear as expected. There are several reasons why these experiments might have shown inconsistent results. For example, once the chitinase gene is being overexpressed, there are reports where this overexpression might undergo plant host gene silencing (Chareonpornwattana et al. 1999). The gene silencing mechanism of the plant avoids the accumulation/over expression of the chitinase, which would be limited to perform its function. Despite the success in the generation of positive transformants, the chitinase might not be expressed fully.

NPR1: A regulator gene for the expression of Induced Resistance

The *Arabidopsis* NPR1 gene controls the onset of systemic acquired resistance (Cao et al. 1994). The *npr1* (nonexpresser of PR genes) mutant failed to respond to various SAR-inducing treatments (Cao et al. 1997), displaying little expression of pathogenesis-related (PR) genes and exhibiting increased susceptibility to infection (van Loon et al. 1998). NPR1 was cloned using a map-based approach and was found to encode a novel protein containing ankyrin repeats (Cao et al. 1997). The lesion in one *npr1* mutant allele disrupted the ankyrin consensus sequence, suggesting that these repeats are important for NPR1 function. Furthermore, transformation of the cloned wild-type NPR1 gene into *npr1* mutants not only complemented the mutations, restoring the responsiveness to SAR induction with respect to PR-gene expression and resistance to infections, but also rendered the transgenic plants more resistant to infection by *P. syringae* in the absence of SAR induction (Cao et al. 1997; Cao et al. 1998). During an experiment in which enhancement of induced resistance by simultaneously activation of salicylate and jasmonate-dependent defense pathways in *Arabidopsis* was found several key findings were reported (fig 1.1). First, plants expressing both types of induced resistance (SAR, ISR) did not show elevated *Npr1* transcript levels indicating that the constitutive level of *NPR1* is sufficient to facilitate the expression of both SAR and ISR (Saskia et al 2000). The finding is interesting because the basal levels of *NPR1* transcription might not be the same across species or cultivars expressing different levels of SAR potentiation. If this is the case, the basal *NPR1* expression can be investigated

and associated/correlated with the ability of the cultivar to express disease resistance.

Npr1 gene regulation

Arabidopsis mutant *npr1* does not express PR-genes and does not exhibit SAR (Cao et al. 1994) Because rhizobacteria-mediated ISR was found to be independent of SA and not associated with PRs (Pieterse et al. 1998), it was expected that ISR would still be expressed in this mutant. However, the *npr1* mutant of *Arabidopsis* did not express ISR mediated by the rhizobacterium WCS417 (Pieterse et al. 1998). This finding implies that NPR1 functions beyond the expression of PR-genes and is required for both pathogen and rhizobacteria-mediated systemic induced resistance. The theory in that Salicylic or Jasmonic acid pathways might be controlled or regulated by one gene implies that SAR and ISR converge at the last part of the signaling pathway. This can explain why SAR and ISR are phenotypically similar. In both the defensive capacity of the plant against a broad spectrum of pathogens is enhanced after an initial induction. Yet, PRs are induced concomitant with SAR expression, whereas activation of PR genes is not part of the pathway leading to ISR in *Arabidopsis*. Apparently, NPR1 differentially regulates defense responses mediated by different signaling pathways. Despite the source of induction, the *NPR1* gene seems to potentiate the defense mechanisms typical of SAR and ISR. However, the study of the NPR1 basal levels and its correlation with SAR expression ability is worthwhile aiming to provide a marker for SAR potentiation since SAR levels positively correlate with pathogen resistance. This hypothesis is supported by

the overexpression of the NIM1 (NPR1) gene in *Arabidopsis*. In this study, transgenic lines expressing the NPR1 gene exhibited heightened responsiveness to SAR-inducing compounds. Activation of SAR in transgenic plants was detected when treated with SA concentrations that would not activate SAR in wild plants (Friedrich et al 2001).

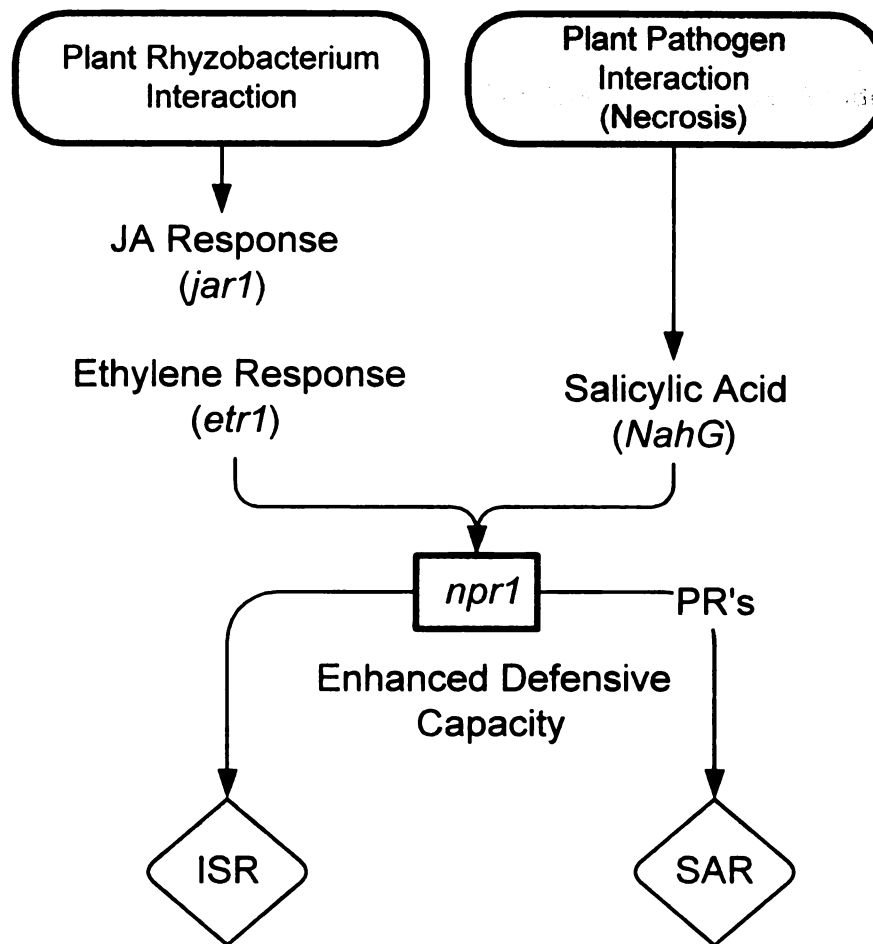


Figure 1.1: Signal-transduction pathways leading to pathogen-induced systemic acquired resistance (SAR) and rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis thaliana*. In plant species other than *Arabidopsis*, rhizobacterially-produced salicylic acid can trigger the SAR pathway as well as ISR. In parenthesis, mutations that compromise the normal pathway. (Modified from L. C. van Loon *et al* Annu. Rev. Phytopathol. 1998. 36:453-483)

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Chapter II

DEVELOPMENT OF A METHOD FOR THE DETECTION AND QUANTIFICATION OF TOTAL CHITINASE ACTIVITY FROM PLANT EXTRACTS.

ABSTRACT

A method for the quantitative assessment of chitinase activity from crude plant extracts has been developed. Dilution series of commercial chitinase extracts were assayed under different conditions using glycochitin as enzyme substrate. The assay is based on the affinity of fluorescent brightener 28 with undigested glycochitin. An agarose plate is used to support the substrate and the reaction plate is viewed under UV transillumination. Results obtained revealed that the linearity between chitinase activity values measured using this method and enzyme concentration was reproducible and reliable and most importantly faster allowing analysis of large number of samples.

INTRODUCTION

Chitinase occurs naturally in a wide variety of higher plants and catalyzes the hydrolysis of chitin, a linear polymer of $\beta(1, 4)$ -N-acetylglucosamine (Muzzarelli 1977), found in fungal cell walls and insect cuticles. Chitinase from plant tissue has been reported to be antifungal and cause partial dissolution of hyphal walls. (Skujins et al. 1965). These results suggest a role for chitinase in plant defense. Some chitinases function as pathogenesis related (PR) proteins. PR proteins are found in very low concentration in plant tissues until after pathogen attack. (Roby and Esquerre Tugaye 1987; Dalisay and Kuc 1995; Dann et al. 1996; Kastner et al. 1998) bacteria, (Lee and Hwang 1996; Gerhardt et al. 1997), viruses, nematodes (Rahimi et al. 1998) and insects (van der Westhuizen et al. 1998). The enzyme can also be induced by wounding (Cabello et al. 1994; Zhang and Punja 1994), heat shock (Margispinheiro et al. 1994), and the application of plant disease resistance activators (Friedrich et al. 1996; Kastner et al. 1998; Narusaka et al. 1999) or ethylene (Boller et al. 1983). The induction of chitinase after pathogen or pest attack provided further correlative evidence for the role of this enzyme in pathogen resistance.

Because of their potential role in plant defense, chitinases have received wide research attention. Increase in chitinase activity has been correlated with an increase in disease resistance observed in plants expressing induced resistance (Roby and Esquerre Tugaye 1987; Roby et al. 1988; Dalisay and Kuc

1995; Hwang et al. 1997; Xue et al. 1998). In addition, their localization within the tissue in association with fungal structures in resistant cultivars has also been demonstrated (Manocha and Zhonghua 1997). Therefore, chitinase has been used as a molecular marker for Systemic Acquired Resistance (SAR), and in experiments involving plant microbe interactions at the molecular and biochemical level. Over the last few years much research effort has been made in finding improved methods of identifying promising germplasm for disease resistance breeding. The success of plant breeding depends on the ability to identify this promising germplasm and to recognize and select effectively between segregating populations. Among the traits that are highly desirable is the ability of a plant to withstand a pathogen attack by rapidly deploying its defense mechanisms. In addition to being useful as a marker for such deployment, chitinases have been shown to have antifungal activity in vitro. The possible correlation between their expression and increased plant resistance makes chitinase a potential biochemical marker to screen for resistant cultivars (Roberts et al. 1994). PR protein basal expression was found to be correlated to non-specific resistance in *Solanum* species to *Phytophthora infestans* and it has been suggested that these levels might be used as biochemical markers for germplasm selection for resistance (Vleeshouwers et al. 2000)

The potential use of chitinase as a biochemical marker for resistance would involve screening large population samples in an effective and efficient way. The current detection methods for activity include colorimetric assays

(Reissig et al. 1955), radioactive assays (Molano et al. 1977), direct detection of activity after polyacrylamide gel electrophoresis (Trudel and Asselin 1989; Pan et al. 1991), HPLC (Koga et al. 1998), and even reflectance spectroscopy (Roberts et al. 1994). These methods albeit satisfactory, require large sample sizes, use of hazardous chemicals, and expensive equipment and can be very time consuming. In addition, some of these methods use colloidal chitin as a substrate with one serious disadvantage; colloidal chitin is not always dispersed homogeneously in the substrate. This decreases sensitivity and reliability for some of the techniques (Nitoda et al. 1999). In this chapter, a rapid and simple method to detect and quantify chitinase activity in plant extracts is described. This method is based on the affinity of Calcoflour white M2R for chitin (Maeda et al., 1967) using glycol chitin as a substrate for endochitinases (Pan et al. 1991). Glycol chitin is embedded in an agarose gel providing a homogeneous substrate for the reaction to take place. The principle of the detection is as follows; glycol chitin, which serves as a substrate for chitinases (Koga and J. 1983), binds to fluorescent brightener 28 by affinity (Maeda and Ishida 1967). After proper incubation and enzymatic activity, the brightener is bound only to undigested glycol chitin (Trudel and Asselin 1989). The result is a well defined dark area on a fluorescent background viewed under UV light transillumination.

MATERIALS AND METHODS

Glycol chitin, deacetylated (C7753), pure chitinase enzyme from *Streptomyces griseus* (C6137), and Calcoflour white M2R (F3543) were obtained from Sigma (St Louis, MO). High melting point, analytical grade agarose was obtained from Gibco Corp. The benzothiadiazole derivative, CGA-245704 (BTH) was obtained from Syngenta Corporation. All other chemicals used for buffers were obtained from Sigma (St. Louis, MO) unless otherwise specified. Glycol chitin, a soluble modified form of chitin was prepared as previously described (Trudel and Asselin 1989). Protein extracts were obtained from cucumber plants expressing Systemic Acquired Resistance (SAR) and from control plants.

Cucumber plants (*Cucumis sativus* L.) cv. Wisconsin SMR58 were grown under standard greenhouse conditions. The cucumber plants were induced to express SAR with the benzothiadiazole derivative, acibenzolar-S-methyl (CGA-245704, ASM) (Friedrich et al. 1996) at a rate of 20 ppm of active ingredient in water. Treatments were applied to plants when the first true leaf (leaf 1) was fully expanded and the leaf above (leaf 2) was one-third to one-half expanded.

Protein Extraction

Leaf tissues from cucumber plants expressing SAR and control plants were collected seven days after induction with ASM, immediately weighed and frozen in liquid nitrogen and stored at -20 °C. Frozen leaf tissues were ground using a mortar and pestle and homogenized in 0.01 M sodium acetate (pH 5.0) in

a ratio of 3 ml of buffer for 1 g of fresh tissue. The homogenate was filtered through 2 layers of cheesecloth, centrifuged at 12000xg for 15 min at 4 °C and the supernatant was decanted into a clean tube. The protein content of the supernatant was measured by the method of Bradford (Bradford 1976) and was used as crude enzyme extract.

Preparation of the substrate and enzyme activity assay

A 1% (w/v) agarose solution was prepared in sodium phosphate (0.01M pH 5.5). The solution was melted and cooled to a temperature of 56 °C. When the agarose gel reached the desired temperature, 1 ml of a 1% glycol chitin solution was added to 100 ml of the agarose solution. The resulting suspension was stirred to ensure homogeneous distribution of the substrate and, 30 ml aliquots were poured into polypropylene petri dishes (15 cm diameter). The agarose was allowed to cool and solidify for 20-25 min. Small wells (3mm diameter) were carved in the agarose gels at a distance of 1.5 cm from each other to form a grid. The agarose plugs were removed by suction with a small Pasteur pipette attached to a vacuum. Five µl samples of the enzyme solution were loaded into each well. The plate was incubated for different times at 37 °C by floating the petri dishes in a water bath. After incubation, 50 ml of 0.5 M solution of Tris-HCl (pH 8.9) with 0.01% calcoflour brightener 28 were added to the plate and incubated for 10 min to the petri dish to stop the reaction and stain

the plate. The gel was rinsed twice and flooded with distilled water followed by overnight color development in the dark.

Chitinase Detection and Standard Curve Production

Dilutions of commercial chitinase were prepared to contain 12.5, 25, 50, 100 and 200 $\mu\text{units ml}^{-1}$ in sodium phosphate buffer (0.01M pH 7.0). Five μl of each concentration were placed in the wells of the reaction plate. Each concentration was assessed five times per experiment. Total protein extracts from cucumber plants expressing SAR as well as control plants were measured using Biorad's protein quantification kit. The protein concentration was equalized among all samples and 5 μl of each sample were loaded in the wells of the reaction plate. After the wells were loaded with the protein extracts, the reaction plates were placed in a water bath at 37 $^{\circ}\text{C}$ for different lengths of time to optimize the assay as described above.

Image Analysis

The day after the assay was performed, the gel was photographed under long wave UV light (356 nm) and the resulting image was analyzed using a personal computer with software for image processing developed by Jandel Scientific (Sigma Scan). The area of activity, observed as a dark area against a white background was measured in number of pixels. The ability of the software to discriminate areas of different color intensities made the analysis very easy to perform. The data was subjected to statistical analysis. Each sample in every test was measured at least five times, and the average area of activity expressed

in number of pixels was statistically analyzed. In addition, similar results were obtained when the area of activity was measured by other means, such as diameter of the area of activity or total area of activity expressed in mm².

RESULTS

Standard Assay.

The solubility of the chitinase substrate glycol chitin allowed us to devise a simple assay for total chitinase activity by uniformly distributing the substrate in an agarose matrix. Because glycol chitin binds the fluorescent brightener calcofluor 28, glycol chitin that is hydrolyzed by chitinase will lose the ability to bind calcofluor and thus not fluoresce. The reaction mixture contained, in a total volume of 30 ml per 15 cm diameter petri dish, a 1% (w/v) agarose prepared in buffer, and 1% (v/v) of the prepared glycolchitin. Areas of activity were easily observed under UV transillumination as non-fluorescent dark regions in a fluorescent background (Fig 2.1). The reaction gel as observed under transillumination revealed the potential to detect as little as 12.5 $\mu\text{Units ml}^{-1}$ of commercial chitinase.

Detecting commercial chitinase.

The generation of a linear relationship for the quantification of chitinase through this method depends on two variables: the time of incubation and the amount of enzyme added. The data collected (square pixels) were plotted as a function of time (Fig 2.2) and as a function of enzyme added (Fig 2.3). The regressions analysis in the figures were calculated from at least 3 independent experiments and each experiment contained at least 5 data points per

concentration. In all experiments, there was a slight departure from linearity as a function of time. This deviation was partially explained by Algranati (Algranati 1963) for enzymatic reactions that progress in a non-linear fashion with time in which the product of the reaction might interfere with the ongoing reaction. The statistical analysis for the maximum time of incubation for this experiment, 6 hours, yielded an r^2 of 0.8911, similar results had been used for germplasm screening using a different chitinase activity test (Roberts et al., 1994). The differences between readings from experiment to experiment were not significantly different at any level (Table 1) and the standard errors were near 10% of the mean. After 6 hours of incubation, the edges of the areas of activity of the higher concentrations started to merge thus making the analysis of the gels difficult, nevertheless, the r^2 was 0.8911. The highest linearity was observed at 1 hour of incubation, ($r^2 = 0.94$), however the area of activity for the lower chitinase concentrations were difficult to analyzed due to its small size. Incubation for 4 hours yielded the best resolution and size of areas of activity for easy analysis yielding an r^2 of 0.8990.

Detection of Cucumber Chitinases

The treatment of cucumber plants with ASM induces accumulation of chitinases. Chitinase has been correlated with the activation of SAR thus; a large increase in chitinase activity throughout the whole plant was expected. When assessed, using this method, chitinase activity from induced plants versus control

plants was always significantly higher than extracts from control plants (Fig 2.4). In addition, the degree of chitinase induction measured using this method is also consistent with results obtained elsewhere.

The preparation of the substrate gels with agarose and glycol chitin is relatively easy, and can be accomplished in a very short time. However, due to thinness, the gels are fragile and must be handled carefully. In addition when preparing the wells, care must be taken when removing the plugs to ensure uniform well size otherwise skewed data might be produced. A Pasteur pipette attached to a vacuum hose gave the best results to remove the previously carved agarose plugs. The casting of the gels must also be done in level surfaces to avoid areas of different gel thickness which also could distort the results since the wells in areas of uneven thickness would be of different volumes. After the proper incubation time, the agarose gel once attached to the plate becomes loose in the plate and if not handled carefully would break easily. In addition, it is important to mark the gel according to the direction in which the samples were loaded in order to avoid confusion, since the gel becomes detached from the plate and easily misread. The area of activity, a black circular area on a fluorescent blue background can easily be seen immediately after the removal of the Tris-HCl + Calcoflour solution and the first rinse with distilled water. However, the maximum color development was attained after a minimum of 6 hours of incubation at room temperature in the dark.

DISCUSSION

We have developed an assay to detect and quantify total chitinase enzyme activity, which has the potential to simplify and speed its analysis. Because very few chitinases seem unable to digest glycol chitin (Molano et al 1979) this method of detection and quantification of total chitinase activity from plant extracts can be used widely. A basal expression of PR proteins has been identified as a possible marker for resistance in *Solanum* sp against *Phytophthora infestans* (Vleeshouwers et al. 2000). Therefore, plant breeders, for example, could potentially screen germplasm using this method. It can also be utilized in the investigation of the induction of the enzyme in plant-pathogen interactions, SAR and plant defense deployment.

It is important to mention that total protein extracts include in addition to the acidic inducible chitinase, basic chitinases and other enzymes that might express partial endochitinolytic activity. However, it is likely that the increase of activity due to SAR is directly linked to the newly synthesized inducible PR chitinase. In cucumber plants, the acidic chitinase type III is exported to the apoplast immediately after synthesis. Potentially, the intercellular fluids can be harvested via infiltration/centrifugation and their chitinase activity assessed.

Overall, there are some critical steps to be considered when doing this assay; the time of incubation and the well distance are key factors. Thus, when dealing with samples of unknown chitinase activity, initial tests should be performed in order to optimize the distance between wells as well as the time of

incubation and protein concentration. Moreover, documentation of the gel should be done after the maximum color development has taken place usually the next morning. Failure to do so usually results in the loss of color and contrast in the picture. This method has been used successfully with cucumber, potato, and soybean extracts (data now shown). Other groups are also using it for detection of chitinase activity in root extracts of sugar beet and *Arabidopsis*.

In conclusion, this procedure provides a simple, rapid and reliable method for detection chitinolytic activity from plant protein extracts. It avoids the hassle of handling hazardous chemicals, it saves time and materials and most importantly, it can be performed with small sample quantity.

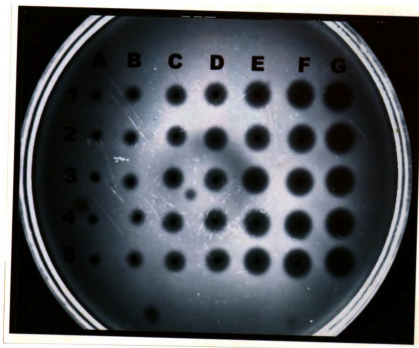


Figure 2.1: Sample of an activity reaction agarose plate viewed and photographed under UV light (365 nm). Increasing amounts of enzyme were placed in the wells A-G (0 - $250 \mu\text{Units ml}^{-1}$), and each concentration was repeated 5 times as indicated in the gel. Notice the consistence of the color, shape and size of the chitinase activity areas.

Figure 2-1: Regression analysis of commercial chitinase activity expressed in number of pixels in relation to time of incubation for 5 different chitinase concentrations. Inserted are the r^2 values of the regression analysis equation, bars represent standard deviation.

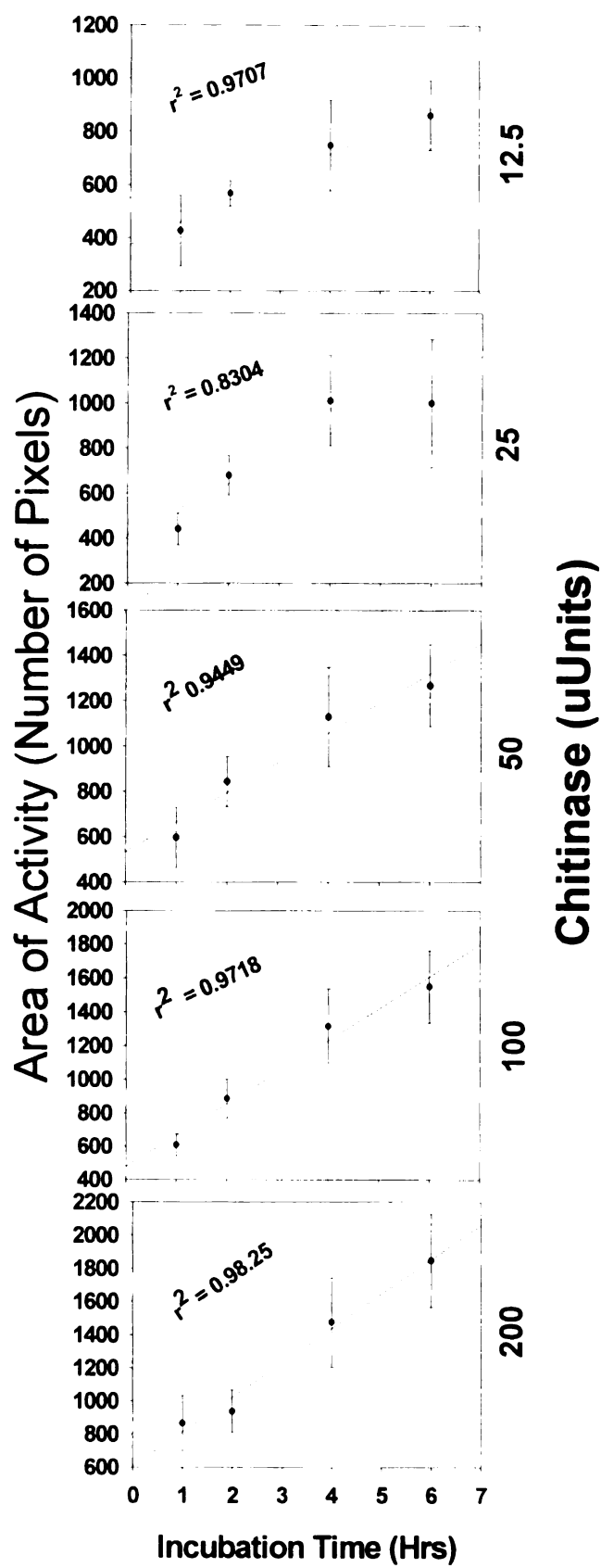
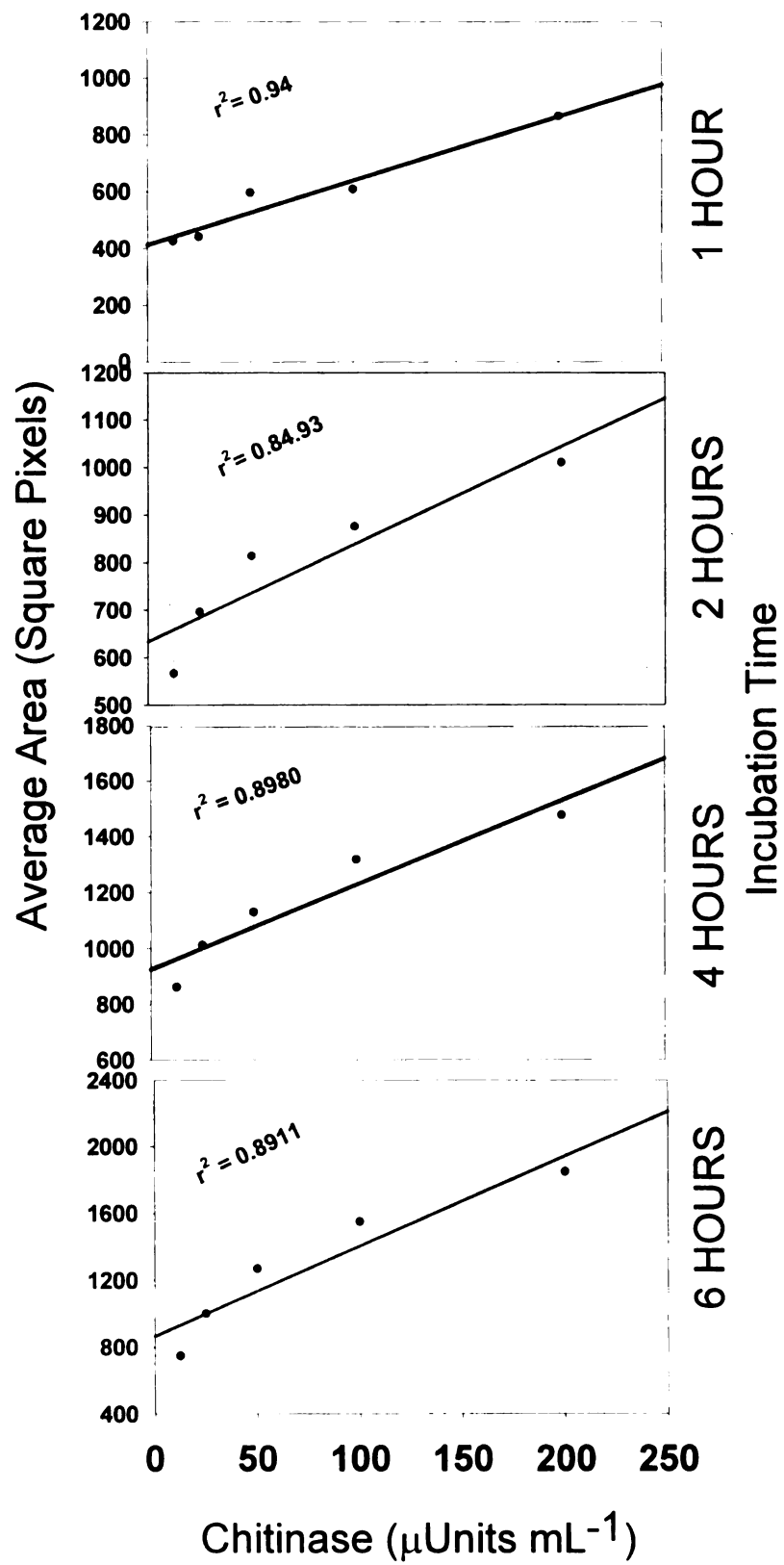


Figure 2-2. Regression analysis of chitinase activity expressed in number of pixels in relation to different chitinase concentrations for different times of incubation. Inserted are the r^2 values of the regression analysis equation, bars represent the standard deviation.



Expression of Chitinase in Control and Induced Plants SMR58

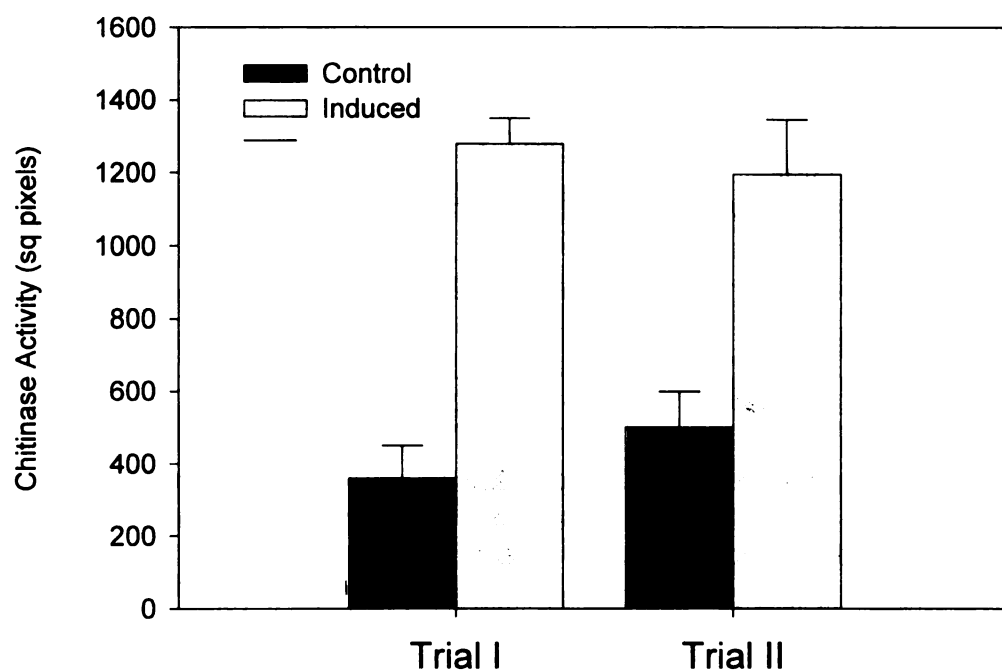


Figure 2-3: Graph shows the resulting chitinase detection from cucumber plants, (control and induced). Two different extractions were made from the same group of plants and detected in different reaction plates labeled Trial I and II. No significant differences were observed between trials ($P < 0.01$).

Table 2.1: Results of three different experiments using commercial chitinase and five different concentrations.

Chitinase μ Units/ml	Average Area of Activity, ** After 4 hours of incubation					St Dev
	run1***	run 2***	run3***	Total Average*		
12.5	844.33	872.14	870.50	862.33	$\pm$	15.60
25	1013.00	1049.57	1111.57	1058.05	$\pm$	49.83
50	1099.86	1147.14	1142.00	1129.67	$\pm$	25.94
100	1269.43	1292.86	1404.67	1322.32	$\pm$	72.27
200	1300.20	1332.71	1688.00	1440.30	$\pm$	215.13

* No significant difference was detected between experimental trials

** Data taken in number of pixels in the area of activity

***Each data point in each trial for every chitinase concentration was the average of 5 data points.

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CHAPTER III
THE EXPRESSION OF INDUCED SYSTEMIC RESISTANCE TO PATHOGEN
CHALLENGE IS DEPENDANT ON PROTEIN SYNTHESIS

ABSTRACT

During the development of SAR in cucumber, there is systemic expression of chitinase, glucanase and peroxidase as well as the enhanced ability to block subsequent challenge infection by fungi through rapidly induced-cell wall changes. However, it is not known if new translation/transcription beyond that expressed during the resistance induction phase are needed to block fungal penetration into SAR-expressing tissues. Leaf disks of cucumber plants expressing SAR were treated with the protein inhibitor cycloheximide and challenged with the fungus *Colletotrichum orbiculare*. The effect of cycloheximide on induced plants was an increase in penetration of the fungus into the tissue, and symptom development was comparable to non-induced plants. The effect of the protein synthesis inhibitor in induced plants suggests that the induced state of cucumber plants is dependant of protein synthesis.

INTRODUCTION

Systemic Acquired Resistance (SAR) generally is associated with a variety of induced defense mechanisms in plants. These defense mechanisms include the production of phytoalexins (Hammerschmidt 1999), hydroxyprolyne-rich glycoproteins (Garcia-Muniz et al. 1998; Raggi 1998), callose and silicon deposition (Stein et al. 1993), lignin (Hammerschmidt and Kuc 1982) and a variety of proteins known as pathogenesis related proteins (PR). Some PR proteins are known to by lytic enzymes such as β -1,3-glucanases and the acidic type III chitinase (van Loon and Cornelis 1999). In cucumber plants, PR protein research in relation to SAR has focus on the acidic type III chitinase (Dalisay and Kuc 1995) and β -1,3-glucanase (Narusaka et al. 1999). Both of these enzymes have been shown to accumulate in plants tissues following resistance-inducing treatments. They have also shown to have antifungal activity *in vitro* (Skujins et al. 1965; Ji and Kuc 1996). These authors purified an acidic β -1,2 glucanase and three isozymes of chitinase isolated from cucumber associated with SAR, and reported antifungal activity. In tomato, these PR proteins have also been found to have lysosome activity (Kauffmann et al. 1987; Legrand et al. 1987). Finally, in cucumber, their extracellular localization places them in the immediate vicinity of tissue penetrated by fungi (Boller and Metraux 1988).

In addition to the characteristics of the PR proteins above mentioned, some have suggested that these PR proteins due to their hydrolytic activity are also capable of releasing elicitors from the invading pathogen in the form of chitin

and glucan oligomers (Roby et al. 1987; Barber and Ride 1994); (Keen and Yoshikawa 1983). Consequently, studies have correlated the increase of chitinase activity to the ability of the plant to inhibit infection.

In cucumber plants, the expression of systemic acquired resistance to *Colletotrichum orbiculare* is characterized by the ability of the plant to block penetration by the formation and rapid deployment of structures such as papillae that contain callose (Kovats et al. 1991), lignin (Hammerschmit and Kuc 1982) and silicon (Stein et al 1993). These structures and their timely deployment are believed to be important for the SAR phenotype to be expressed fully in cucumber plants against pathogens. However, it is not known if new translation/transcription beyond that expressed during the SAR induction phase are needed to block fungal penetration into SAR expressing tissues. In other words, is protein synthesis required to maintain the state of induction? Would PR proteins and their antifungal activities restrict the pathogen from spreading into the induced tissue in the absence of newly synthesized proteins?

Cycloheximide (CHX), a protein synthesis inhibitor, compromises the ability of plants to form papilla after inoculation with fungal pathogens (Vance and Sherwood 1976; Skalamera et al. 1997) Based on these studies, it seemed possible that use of synthetic protein synthesis inhibitor could be a tool to help determine the role of papilla formation in cucumber induced resistance. In this study, we tested the effect of cycloheximide on the expression of induced resistance in cucumber that was induced with the synthetic plant activator acybenzolar-S-methyl ("ASM", "Actigard").

In previous experiments it was found that spraying low levels of cycloheximide induced mRNA expression of PR-related proteins in tomato (Uknes et al. 1993). Why this response to partial protein synthesis inhibition occurred is not clear. It has been suggested that the plant cell could detect some sort of protein imbalance, which would lead to expression of defense genes (Lawton et al. 1994). The treatment of plants with a resistance activator in combination with low levels of cycloheximide yielded a super induction of chitinase in tobacco (Hovarth and Chua 1996). In the present study the super-induction of chitinase, β -1,3-glucanase and peroxidase caused by cycloheximide in combination with the SAR activator and its effect on the expression of resistance in cucumber plants was investigated. Despite the higher induction of chitinase, glucanase and peroxidase in plants induced with the CHX/BTH combination vs. BTH alone, the levels of resistance were not heightened.

MATERIALS AND METHODS

Plant Material and Fungal cultures.

Cucumber plants (*Cucumis sativus* L.) cv. Wisconsin SMR58 were grown under standard greenhouse conditions. The cucumber plants were induced to express SAR when the plants' first true leaf (leaf 1) was fully expanded and the leaf above (leaf 2) one-third to one-half expanded. The induction was achieved by spraying the plants with the benzothiadiazole derivative, CGA-245704 (ASM) (Friedrich et al. 1996) at a rate of 20 PPM of active ingredient in water. Controls were sprayed with water. Seven days after induction, the second leaf of control and induced plants were harvested and brought into the laboratory. Ten mm diameter leaf-disks were cut from the leaves with a cork borer and then floated on water or aqueous solutions of cycloheximide (10 ug/ml) in petri dishes (Vance and Sherwood 1976).

Cultures of *Colletotrichum orbiculare* were maintained on V-8 juice agar at room temperature in the dark. Conidia were harvested from 7-10 days cultures by flooding the dish with sterile water. Following filtration through cheesecloth, spore concentration was adjusted to 10^6 spores ml^{-1} . A 10 μl drop of inoculum suspension was placed on the upper surface of the leaf disks. After inoculation, the petri dishes containing the leaf disks were maintained at room temperature.

Preparation of fungal cell walls

A conidia suspension of *Colletotrichum orbiculare* was added to Czapek-Dox media (Bonnen and Hammerschmidt 1989) and grown for five to seven days. The mycelium was harvested by suction filtration, washed with double distilled water to remove the media and crude fungal cell wall preparations were made as previously reported (Ren and West 1992). The crude cell wall preparations were freeze dried and ground with a mortar and pestle. One preparation was made for all of the experiments described within.

Histological observations

For histological observations, leaf disks were removed from the Petri dishes and cleared in a solution of glacial acetic acid:ethanol (1:3 v/v). After clearing, the disks were hydrated and mounted in glass slides with sterile glycerol. The following stains were used: for callose deposition, lacmoid blue and aniline blue; for lignin, phloroglucinol-HCl (Krishnamurthy 1999); cotton blue was used for fungal hyphae (Nicole and Gianinazzi-Pearson 1996).

Protein Extraction

Leaf disks harvested at different times of the infection process were frozen in liquid nitrogen ground using a small plastic mortar in 1.5 ml eppendorf tube and homogenized in 0.01 M sodium acetate (pH 5.0) in a ration of 3:1 (v/w). The homogenate was centrifuged at 12000g for 15min at 4°C and the supernatant decanted into a clean tube. The protein content of the supernatant was

measured by the method of Bradford (ref here) and used for enzyme determinations.

Assay of Peroxidase and Chitinase Activities

Peroxidase activity of leaf disks extracts was determined using guaiacol as a substrate as described previously (Hammerschmidt et al. 1982). Chitinase activity was determined as follows. A 1% agarose in Potassium phosphate buffer 10 mM (pH 5.0) was prepared with a 1% (v/v) of glycol chitin which serves as chitinase substrate (Trudel and Asselin 1989). After polymerization, 3mm diameter plugs were carved using a small cork borer in the agarose. A 5 μ l aliquot of the total protein extract were placed in the wells (3 wells per sample), and incubated at 37 °C for 4 hours. After incubation, the reaction plate was rinsed and flooded for 10 min in 0.5 M solution of Tris-HCl (pH 8.9) with 0.01% (w/v) of calcoflour brightener 28 in order to stop the reaction and stain the plate. When viewed with UV transillumination, the areas of activity appeared as a well-defined dark area on a fluorescent background (Velasquez, Unpublished). Glucanase activity was determined using laminarin as substrate as previously reported (Biely et al. 1985).

PAGE and gel enzyme detection

Polyacrylamide minigels 1.5 mm thick, were prepared. For native gels, an anionic (pH 9.3) discontinuous system was used. An acrylamide concentration of 7% provided optimal separation of cucumber proteins.

Chitinase activity in PAGE was visualized as previously described (Trudel and Asselin 1989) with the following modification. The glycol chitin, substrate for chitinase, was added directly to the components of the gel before polymerization. Therefore, an overlay gel was not needed since the detection was done in the separation gel. Peroxidase activity was visualized in PAGE as previously reported (Smith and Hammerschmidt 1988). After electrophoresis, the gels were placed in a solution of 50 mg of 3-amino,9-ethylcarbazole, 10 ml of *N,N*, dimethylformamide, 200 μ l of 30% H_2O_2 and 190 ml of sodium acetate buffer (pH 5.0). Bands were visible within 10 min of incubation.

SOD and Catalase activity were visualized in gels by the method of Milosevic (Milosevic and Slusarenko 1996). The gels were scanned and analyzed by the software package Quantity-One (Bio-Rad Laboratories, Hercules, CA).

Detection of H_2O_2 in cucumber leaf disks.

To study H_2O_2 production by leaf disks, the Phenol red assay was used (Svalheim and Robertsen 1993). In this assay, the increase in absorbance at 610 nm resulting from the peroxidase-catalyzed oxidation of phenol red by H_2O_2 is measured. Ten leaf disks from SAR expressing plants were incubated in glass vials containing 1 ml 10 mM MES-KOH, pH 6.5, and 10 μ g of chloroamphenicol with or without fungal cell wall preparations from *C. orbiculare* that served as a source of elicitor. The cell wall preparation was added to the leaf discs in buffer.

Since the cell wall preparations were not soluble in water, the leaf disks containing the cell wall preparations (10 $\mu\text{g/ml}$) were placed in a shaker to maintain the cell wall preparation in solution. The segments were incubated at room temperature in the dark for 2 hours. Ten μL phenol red solution (10 g L^{-1} in distilled H_2O) was added and the mixtures were incubated for an additional two hours. The reaction was terminated by the addition of 20 μL of 1 M NaOH to each vial. The A_{620} of the incubation solution was read against a blank prepared in the same manner, but in the absence of leaf disks.

RESULTS

The effect of CHX in fungal growth and development:

Disease symptoms. The first anthracnose symptoms on untreated cucumber leaf disks were visible 48 hours after inoculation. The lesions in the area of inoculation appeared first as green-brown, water soaked areas that become necrotic and sometimes developed a chlorotic halo. Symptoms on induced cucumber leaf disks developed more slowly and less severely. SAR expressing leaf disks treated with CHX were infected by *C. orbiculare* to the same extent as the non-induced control plants. There were no noticeable differences in terms of lesion development between the control disks and the induced disks treated with CHX.

Cytological observations:

Twenty-four hours after inoculation, conidia had germinated and formed appresoria on leaf disks from all treatments. Penetration pegs were visible piercing the outer cell wall of the epidermal layer by forty-eight hours after inoculation. The rate of penetration was assessed for each of the treatments (Fig 3.2). Two hundred fully formed appresorium were observed in each of the treatments in up to ten different leaf disks. The results obtained show that the number of penetrations between the induced leaf disks treated with CHX was comparable to the number of penetrations observed in the non-induced leaf disks.

To determine CHX had an effect on the development of the fungus in induced tissue the fungal development was observed. Hyphal length was measured 72 hours after inoculation in all treatments. The results show that the growth rate of fungi in induced plants is greatly reduced (fig 3.3). In contrast, non-induced leaf disks allowed a considerable higher fungal growth within the tissue. The hyphal length at 72 hours after inoculation of induced CHX-treated leaf disks was similar than those in non-induced leaf disks. Therefore, leaf disks treated with CHX did not only allow more penetrations, but also tolerated a higher rate of fungal growth within the tissue (fig 3.4).

Papillae formation and lignin deposition in leaf disks treated with CHX.

Induced leaf disks floated in water developed normal papilla and lignin deposition associated with the site of penetration (fig 3.1A, 3.1C). The association of lignification at the site of penetration and resistance in cucumber plants has been reported (Hammerschmidt and Kuc 1982). Leaf disks of induced plants were treated with CHX and inoculated with a droplet of a spore suspension of conidia from *C. orbiculare*. In such leaf disks, papillae formation was drastically reduced and when present, it was unable to stop the pathogen from penetrating the plant tissue (fig 3.1B). Lignification in leaf disks treated with CHX was not primarily associated with areas of penetration. When present, the areas stained with the method of phloroglucinol-HCl were more generally associated with damage post penetration linked with cell damage. (Fig 3.1A)

Effect of challenge inoculation and CHX treatment on PR protein activity

In non-induced leaf disks, a sharp increase of chitinase activity was detected at 48 hours after inoculation with *C. orbiculare*. This is consistent with the observations that the defense mechanisms are further deployed after a pathogen attack. Non-induced, non-challenged leaf disks also had an increase of chitinase activity, however, the levels are far below the expressed by challenged leaf disks. Therefore, wounding and/or CHX treatment can also induce chitinase. Cycloheximide untreated and treated leaf disks and challenged with the fungus, also had a small increase of chitinase activity, but their levels were already higher due to SAR expression. Overall, chitinase activity is not hindered by CHX treatment. Glucanase activity, just as chitinase was not greatly reduced and its activity remain constant in induced leaf disks treated with CHX (data not shown).

The levels of peroxidase activity in induced plants compared to non-induced plants were higher at all times before and after inoculation as previously reported (Hammerschmidt et al. 1982). On the other hand, the treatment of leaf disks with CHX yielded a drastic reduction in POX activity. However the kinetics and in PAGE detection activity clearly show that peroxidase activity was greatly reduced in leaf disks treated with CHX (Fig 3.6A). However, CHX does not affect the activity of POX directly since the enzyme kinetics show that in the presence of CHX up to a concentration of 10 mM did not affect the activity of cucumber POX (Fig 3.6B).

In order to examine the levels of activity of POX in the presence of an elicitor, leaf disks of induced and non-induced plants were incubated in the presence of a fungal cell wall preparation (FCP) and its peroxidase activity determined at different times during the experiment. The results show that POX activity increases greatly in presence of the FCP. Changes on the activity of POX are detectable by 4 hours after exposure to the elicitor. The induced leaf disks exposed to the crude fungal cell wall preparations expressed the greatest peroxidase activity change (Fig 3.7)

The effect of CHX in the generation of Reactive Oxygen Species

The levels of production of the highly reactive oxygen species (ROS) and the activity of the primary enzymes that catalyze the production of these ROS were studied. At 96 hours after treatment with CHX, in PAGE detection of catalase and SOD was performed (Fig 3.8). The activity of both enzymes, catalase and superoxidase dismutase (SOD), were detected at the same levels before and after CHX treatment, therefore, establishing that CHX did not affect their activity. In this work, the effect of CHX clearly compromises the amount of H_2O_2 produced. In an experiment with leaf disks of induced plants, the detection of H_2O_2 was assessed in the presence of CHX. Data shows that the ability to elicit and increase the production levels of H_2O_2 in induced material is compromised by the protein synthesis inhibitor. (Fig 3.9).

The effect of treating levels of CHX in combination with a SAR Inducer.

As reported before the application of low concentrations of CHX in combination with SA produced a super induction of chitinase in tobacco (Horvath and Chua 1996). The effect of CHX in combination with the SA analog BTH also produced a systemic “super induction” of chitinase, β -1,3, glucanase and peroxidase in cucumber plants (Fig 3.10). After induction in leaf #1, leaf #2 was harvested and a detached bioassay for resistance was performed in addition to biochemical analysis of the induced plants. Ten micro-liter droplets of a spore suspension of *C. orbiculare* were placed on the leaves in a sterile Petri dish with a moist filter paper. The assessment of the disease progress was performed seven days after the inoculation. The number of lesions was greatly reduced in plants treated with BTH as well as with plants treated with the combination of BTH and CHX. Control plants allowed a larger number of lesions and a larger lesion size just as the plants treated with CHX alone (Fig. 3.11). However, there were no statistically significant differences between the lesion size or lesion number among the plants treated with BTH and plants treated with the combination BTH-CHX.

DISCUSSION

Hammerschmidt and Kuć have shown that the phenotype of SAR against *Colletotrichum orbiculare* is closely related to the ability of the epidermal cells to respond rapidly with deposition of lignified structures around the areas of possible penetration (Hammerschmidt and Kuc 1982). Lignification, in their study, was found to occur sooner and to be more intense beneath appressorium and around sites of penetration in induced compared to control leaves. This was also demonstrated in potato tissues where pre-treatment of the tissue with α -aminooxiacetic acid, a competitive inhibitor of PAL, induced a state of “susceptibility” associated with the lack of lignin deposition (Hammerschmidt 1984).

Penetration of *Colletotrichum orbiculare* into cucumber plants expressing SAR was found to be markedly reduced after challenge with conidia of the fungus. However, the treatment of induced leaf disks with CHX increased fungal penetration and did not affect fungal growth within the induced tissue. The SAR phenotype against *C. orbiculare* has been shown to be expressed in the epidermal cells (Xuei et al. 1988; Kovats et al. 1991; Stein et al. 1993). Thus the initial line of defense against pathogen attack is being compromised by the inhibition of protein synthesis. In studies elsewhere it has been found that the induction of phenylalanine ammonia lyase (PAL) activity was blocked by protein synthesis inhibitor, CHX or actinomycin and largely prevented by a protein kinase inhibitor (Mackintosh et al. 1994). PAL catalyses the formation of cinnamc acid

from phenylalanine, and the first step leading to the synthesis of phenylpropanoid phenolics such as lignin precursors. Thus, the inhibition of PAL expression or other enzymes leading to lignin biosynthesis is a possible explanation for the results described above. Hammerschmidt and Kuć have shown that the phenotype of SAR against *Colletotrichum orbiculare* is closely related to the ability of the epidermal cells to respond rapidly with deposition of lignified structures around the areas of possible penetration (Hammerschmidt and Kuc 1982). Lignification, in their study, was found to occur sooner and to be more intense beneath apresorium and around sites of penetration in induced compared to control leaves. This was also demonstrated in potato tissues where pre-treatment of the tissue with α -aminooxiacetic acid, a competitive inhibitor of PAL, induced a state of “susceptibility” associated with the lack of lignin deposition (Hammerschmidt 1984).

In addition to lignin deposition, papillae formation was also altered. For the most part papillae like structures were formed however, they were unable to restrict fungal penetration in induced leaf disks treated with CHX. In experiments with the defense-compromised *Arabidopsis nim1-1* and salicylate hydroxylase -expressing plants suggest that extrahaustorial callose production is enhanced by treatments that activate SAR, and that this induction may involve the NIM1/NPR1 pathway (Dempsey et al. 1999). In mutant plants *npr1* the callose deposition could not be rescued by applications of INA. In contrast, *nahg* plants, the ability to deposit callose was rescued by the application of INA. The authors noted that that callose deposition must occur for the phenotype of SAR

to be expressed but also noted the dependability on the NPR1 gene and SA. The observations made in this work suggest that protein synthesis might also drive papillae formation.

In this work, the induced plants treated with CHX were able to produce papillae, but these did not effectively stop pathogen penetration. SAR primes the plant to deploy callose deposition in a timely manner, but its ability to stop the fungi is protein synthesis dependent and the synchronized availability of the building blocks for its synthesis. It has not escaped our attention that a series of experiments have been done in order to assess the value of callose in stopping the pathogen penetration. Smart *et al* (Smart et al. 1986) concluded that callose deposition alone is not sufficient to stop pathogen attack. However, inhibition of callose synthesis using 2-deoxy-d glucose reduced papillae formation and led to enhanced penetration of powdery mildew *Erysiphe graminis* in barley suggesting that callose formation is important for resistance. (Bayles et al. 1990). Vance and Sherwood however, showed that the deployment of papillae is dependant of protein synthesis since CHX inhibited the production of papillae formation and allowed fungal penetration by spores of normally non-infective pathogens of reed canary grass (Vance and Sherwood 1976). From these experiments, it can be concluded that papillae formation is a key factor in the resistance mechanism, and the timely deposition of papillae and its proper lignification is a key factor of SAR. Since CHX also compromises PAL activity, it is also possible that the combination of callose deposition and lignification must be present in order for the phenotype of SAR in epidermal cells to occur.

H₂O₂ is believed to be important in lignin biosynthesis (Gonzalez et al. 1999), and is also antifungal (Joseph et al. 1998). The SAR-enhanced production of H₂O₂ was found to be sensitive to CHX treatment and was fully inhibited at 5 μ M concentration. This is consistent with an earlier report in which the same results were obtained in cucumber hypocotyls (Fauth et al. 1996). These authors concluded that CHX did not inhibit the H₂O₂ producing enzyme system. Our results show that indeed, catalase and SOD were not greatly affected by CHX treatment. This finding is important since it indicates that protein synthesis is required for the full production of H₂O₂ in SAR expressing cucumber plants. In tobacco, a catalase has been identified as a SA binding protein (Chen et al 1993). Since binding of SA to catalase inhibits its activity, increased amounts of SA could indirectly result in increased quantities of H₂O₂ in the tissue. Therefore the hypothesis that the mechanism of action of SA is to elevate the levels of H₂O₂, which then serves as an intermediate in the SA signaling pathway was born (Chen et al 1993). It was later found that BTH and INA acted similarly, they both block the activity of catalase (Yang et al. 1997). It is important to mention that H₂O₂ from the oxidative burst drives several plant defense responses including cross-linking of cell wall proteins (Levine et al. 1994; Brown et al. 1998). H₂O₂ production has been regarded as a key factor in the early defense response against pathogen attack in SAR expressing plants. Therefore when CHX compromises its production, it also compromises the full expression of SAR.

Induction of SAR by SA or its analogs (BTH, INA) is usually associated with the accumulation of Pathogenesis Related (PR) proteins. These are usually found in the intercellular fluids (IWF) of leaves of plants expressing SAR. Chitinases and β -glucanases and peroxidases are the most abundant in the apoplast. These are expressed at very low levels in non-induced plants, but their expression is highly increased when plants are expressing SAR. CHX did not affect the activity of the chitinases and glucanases. However, peroxidase activity is compromised by the presence of CHX.

The results of this work show that peroxidase activity is hindered by CHX treatment. The levels of H_2O_2 are also greatly reduced and the ability of induced plants to hinder fungal penetration and fungal growth is compromised. These findings in addition to previous evidence show that CHX also inhibits PAL activity (Mackintosh et al. 1994) thus limiting the availability of phenolic and lignin precursors have one thing in common. They are all involved directly in the early response stages against pathogen attack. Peroxidase activity has a function in the generation and utilization of H_2O_2 for cross-linking of protein and phenolics during the construction of papillae (Brown et al. 1998). Papillae are often lignified, and at the sites of potential penetration a higher concentration of H_2O_2 has also been found (Huckelhoven et al. 1999). Since peroxidase induction and H_2O_2 production are blocked by CHX these results indicate that *de novo* synthesis of protein is required for H_2O_2 generation, peroxidase activity, lignin synthesis and the construction of effective structures to stop fungal penetrations. The blockage

of induction of peroxidase by CHX has also been reported in cucumber hypocotyls (Svalheim and Robertsen 1990)

Chitinase and glucanase are thought to be a key component in the SAR mechanism. Chitinase mRNA increase slightly following cycloheximide application; however, its potent induction by salicylic acid was inhibited by cycloheximide treatment suggesting that protein synthesis is required for chitinase mRNA induction to take place. (Lawton et al. 1994). However, using a transgenic plant expressing a rice chitinase, it was found that CHX did not inhibit chitinase activity, (Kim et al. 1998). The fact that the growth of the fungal hyphae within the tissue of induced plants treated with CHX was comparable to non-induced plants questions the role of chitinase and glucanase in effectively restricting fungal growth. The higher activity of both enzymes was not a factor in the degree of fungal growth within the induced tissue.

When low levels of CHX are sprayed in combination with a SAR activator, the systemic “super induction” of the PR proteins glucanase and chitinase in addition to peroxidase takes place. This phenomenon although not totally understood, theoretically should prove useful to investigate the role of these PR proteins in the defense response of the plant. Although the levels of activity were statistically significant between the BTH induced plants and the CHX/BTH induced plants. The higher levels of PR expression of the CHX/BTH induced plants failed to decrease the development of the pathogen within the tissue.

Our experiments have show that the CHX treatment of SAR expressing cucumber leaf disks enhanced the susceptibility against the pathogen *C. orbiculare*. The lesions were comparable to the developed by leaf disks not expressing SAR. This suggests that protein synthesis is needed in order to keep the state of induction in cucumber plants expressing SAR. We are not suggesting that protein synthesis inhibition is the only factor responsible for breaking down of the resistance; CHX might have other unknown effects in the plant metabolism. Molecular approaches and mutation analysis might prove useful to disclose the specificities and the contributions of individual building blocks of the Systemic Acquired Resistance phenotype.

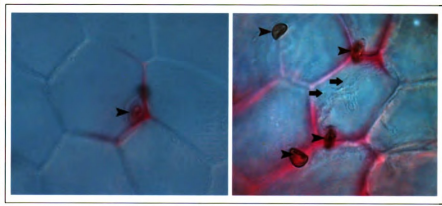


Figure 3.1A: Leaf discs from induced plants non treated with CHX (left panel) and CHX treated (right panel). Leaf discs were challenged with *C. orbiculare* and treated with phloroglucinol to detect lignin deposition 72 hours after inoculation. Induced plants non-treated with CHX were successful in stopping fungal penetration. Lignin deposition was observed directly under and around areas of possible penetration. In CHX treated leaf discs the areas of lignification were more spread, but the fungus was able to break through the lignified areas. Arrowheads indicate the appressorium, arrows indicate hyphae growing within the tissue. Pictures taken at magnification 100X

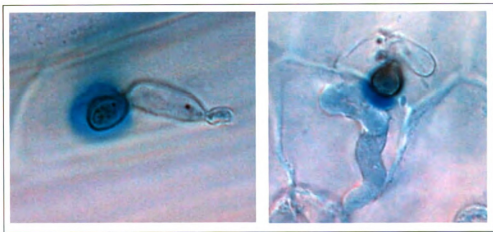


Figure 3.1B: Leaf Discs from induced plants non treated with CHX (left panel) and CHX treated (right panel). Leaf discs were challenged with *C. orbiculare* and stained with lacmoid blue to detect callus deposition at 72 hours after inoculation. Leaf disks from induced plants treated with CHX had callus deposition but were unable to stop penetration of the pathogen. Pictures taken at magnification 100X

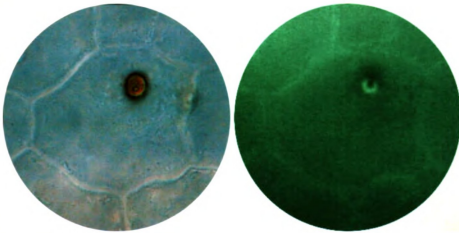


Figure 3.1C: Leaf disks from induced plants 48 hours after challenge with *C. orbiculare* stained with aniline blue to detect papillae formation. Left panel, picture taken under visible light, right panel taken with UV light. Note the small ring (papillae) directly under the appressorium. Pictures taken at magnification 100X

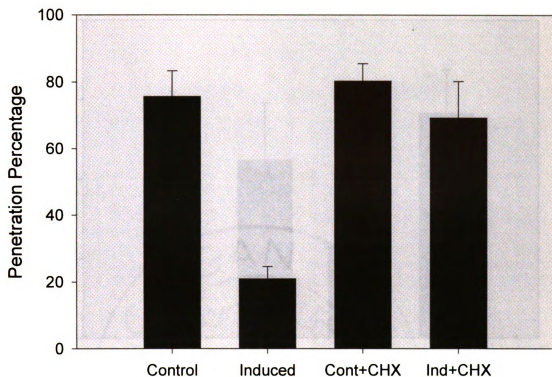


Figure 3.2. The effect of cycloheximide in the penetration rate of *C. orbiculare* 24 hours after inoculation with a 10 μ l droplet of a conidia suspension (10^6 spores ml^{-1}). Cleared leaf disks were observed under a Leica microscope and up to 200 fully formed appresoria were examined in 10 leaf disks for each treatment. A successful penetration was regarded as the visible presence of a penetration peg and a visible primary hyphae within the tissue.

Fungal Length 72 Hours After Inoculation

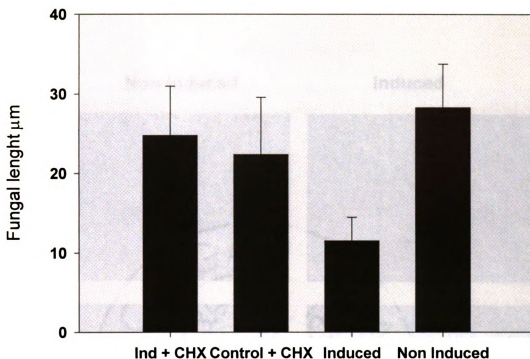


Figure 3.3: The effect of cycloheximide in the growth rate of the fungal hyphae within the tissue. Measurements were taken from the base of the appressorium to the hyphal tip of the invading pathogen. The measurements were taken 72 hours after inoculation with *C. orbiculare* and treatment with CHX. Statistical differences were only observed in the leaf disk from SAR expressing plants ($p < 0.01$).

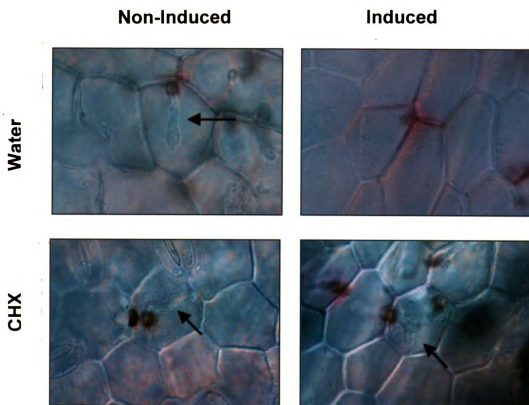


Figure 3.4: Micrographs of cucumber leaf disks under visible light taken with a Leica microscope. Arrows indicate the hypha within the tissue. Leaf discs were viewed 48 hours after inoculation, pictures taken at magnification 100X.

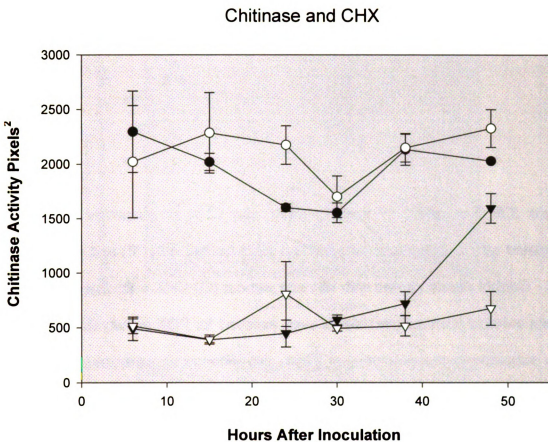
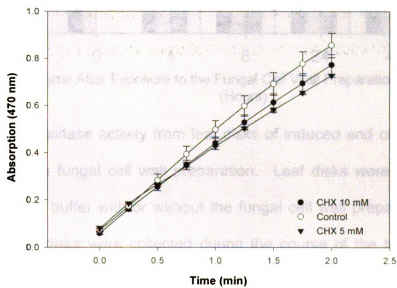
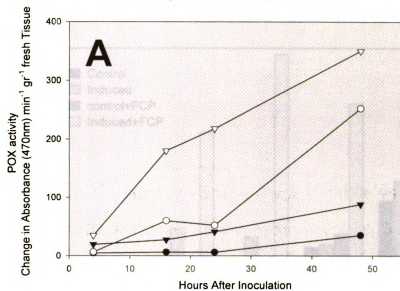


Figure 3.5. Detection of chitinase activity of leaf disks treated with cycloheximide during the process of infection by *C. orbiculare*. Leaf disks of induced plants treated with (○) or without CHX (●), and control plants treated with (▼) and without CHX (▽) were collected and their chitinase activity was assayed. It is apparent that chitinase activity was not affected by the presence of cycloheximide

Figure 3.6: The effect of the treatment with CHX to cucumber leaf disks inoculated with *C. orbiculare*. Induced leaf disks treated with (▼) and without (▽) CHX. Control plants treated with (●) and without (○) CHX. **B:** Cucumber total protein extracts from induced plants were assayed for POX activity using guaiacol as a substrate in the presence of CHX. No differences were detected in the initial enzyme kinetics up to the concentration used in the experiment (10 mM).

POX Activity in Leaf disks treated with CHX



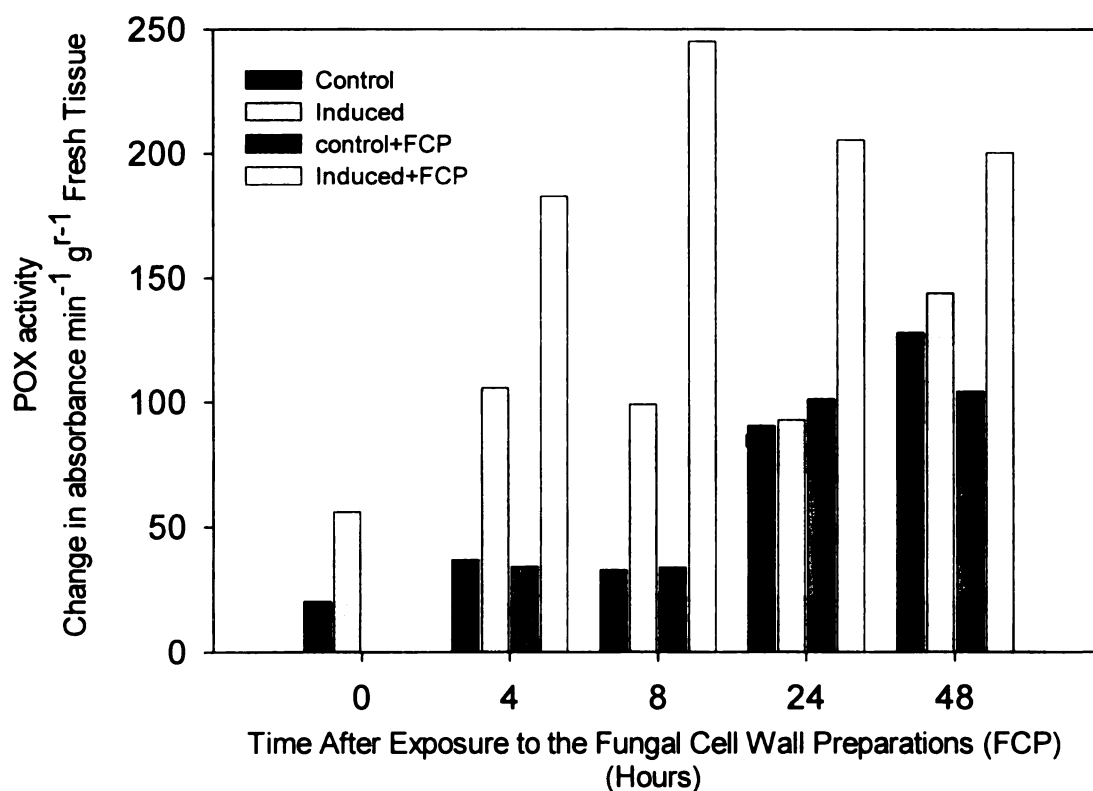
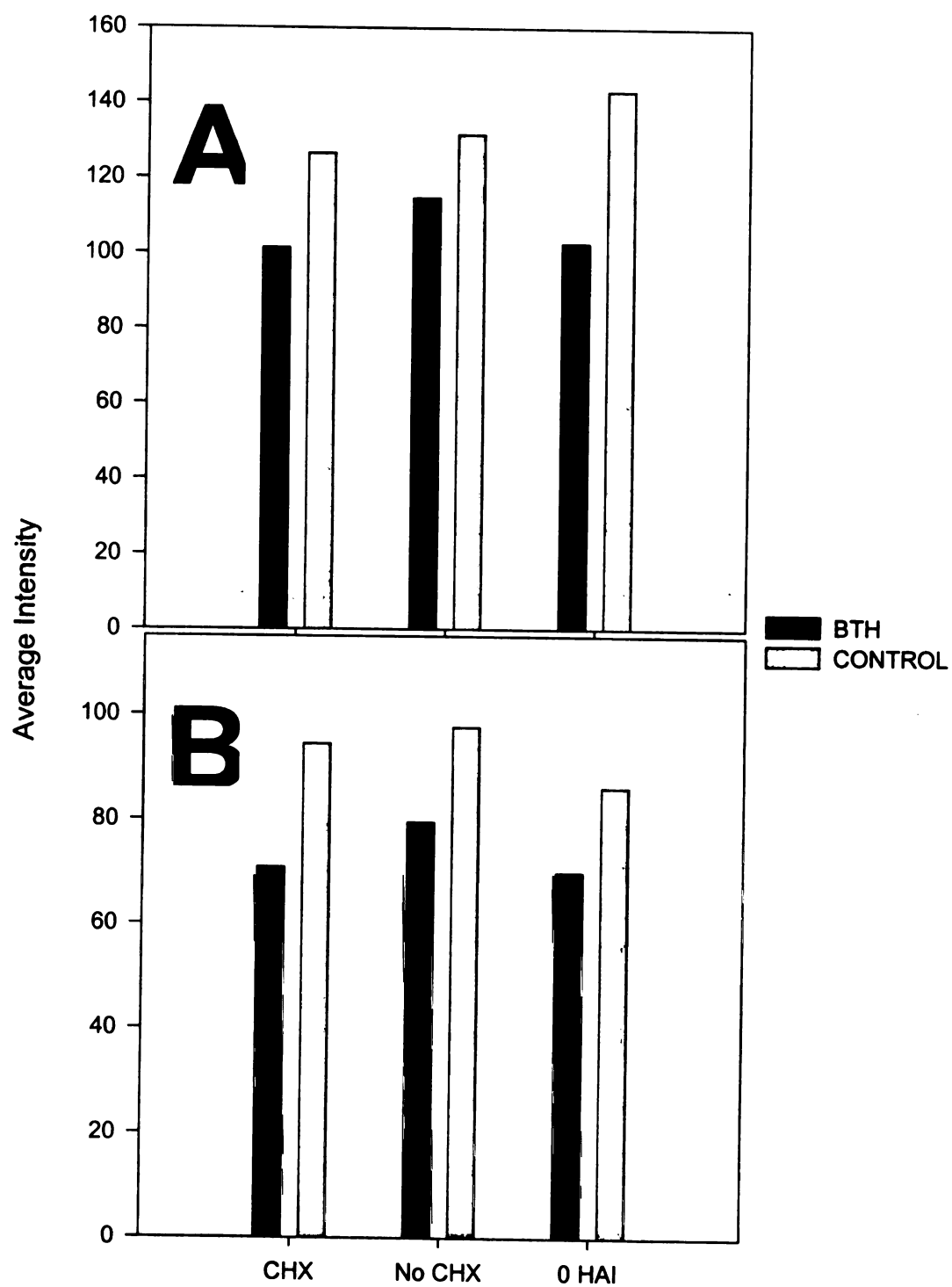


Figure 3.7: Peroxidase activity from leaf disks of induced and control plants in the presence of a fungal cell wall preparation. Leaf disks were collected and placed in Sodium buffer with or without the fungal cell wall preparation from *C. orbiculare*. Leaf disks were collected during the course of the treatment, their total protein extracted and the activity of POX measured using guaiacol as a substrate. Note the major differences of the cucumber leaf disks in the presence of FCP. This suggests that the accumulation and or activity of peroxidase increase beyond the induction phase of SAR.

Figure 3.8. Catalase Activity (A) and SOD activity (B) detected via PAGE activity staining. After staining, the gels were photographed and analyzed to detect the average intensity of the areas of enzymatic activity. Samples were collected 96 hours after inoculation, and 20 ug of total protein were loaded per well. In addition, samples from induced and non-induced plants at 0 hours of inoculation were also loaded (0 HAI).



Effect of CHX in the generation of AOS in leaf disks of cucumber

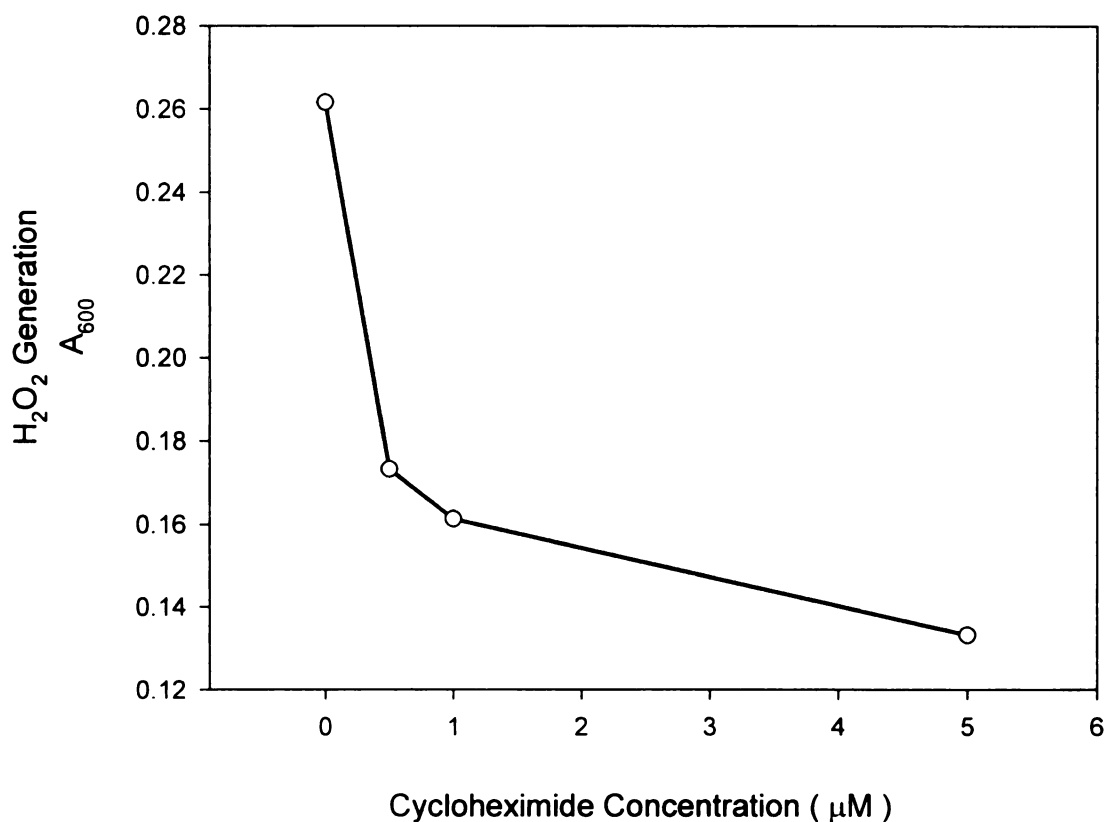


Figure 3.9: Inhibition by cycloheximide of the elicitation of H₂O₂ from leaf disks.

The induced leaf disks were conditioned with the indicated concentrations of cycloheximide followed by elicitation with 10 ug ml⁻¹ of crude fungal cell wall preparations for 30 min. Results are given from one representative experiment of three performed.

Figure 3.10: Gel electrophoresis detection acidic peroxidase (A) and chitinase (B) of plants sprayed with Water, BTH (20ppm) BTH+CHX and CHX (10 μ g/ml). 30 μ g of total protein was loaded in each well. Protein samples came from 2nd leaves of sprayed plants. (C) Glucanase activity detection. Detection of glucanase was done twice, graph above are the results of one of two different experiments.

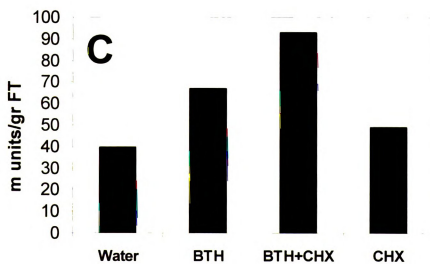
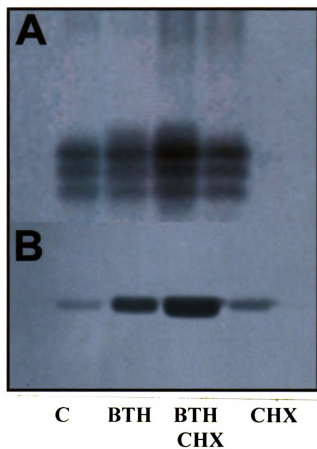
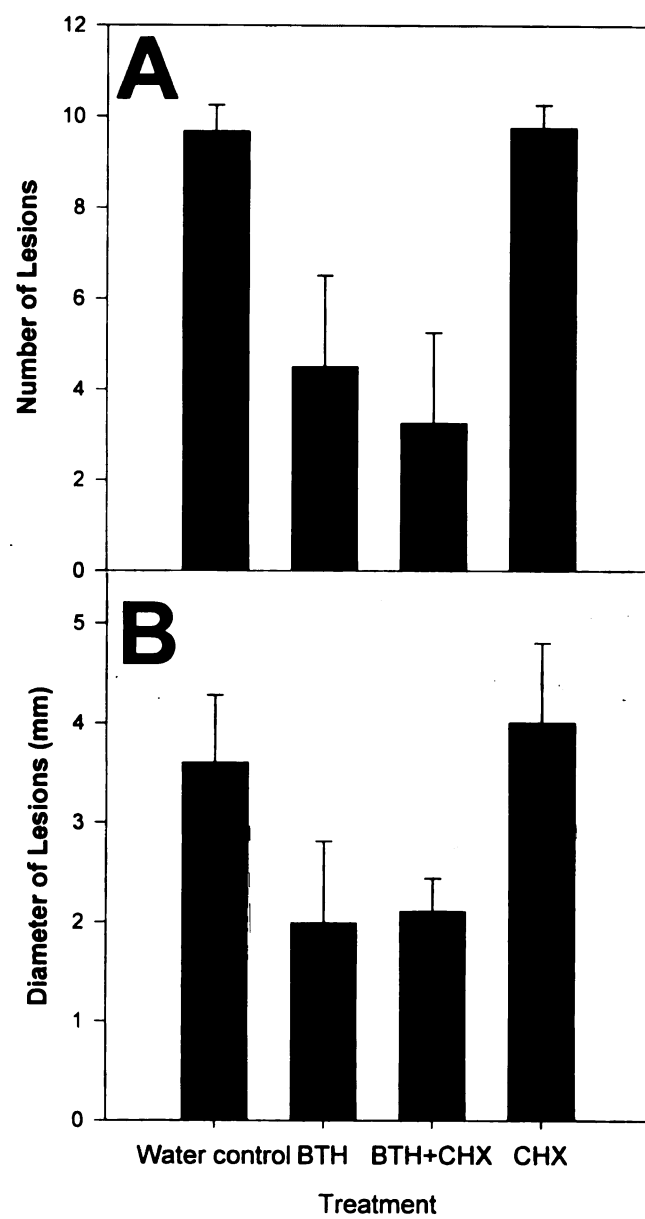


Figure 3.11. The systemic effect of induction of cucumber plants with BTH and BTH/CHX. CHX was sprayed in combination with an application of 20 PPM of BTH and the application of CHX was done at a rate of about 10 μ M solution. Second leaf was harvested and challenged with a spore suspension of *C. orbiculare* the disease assessment was done 7 days after inoculation and lesion size (A) and lesion number (B) were measured.



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CHAPTER IV

THE RESPONSE OF CUCUMBER CULTIVARS TO INDUCTION OF SAR IN
GREENHOUSE AND FIELD CONDITIONS AND THE SCREENING OF
RESISTANCE AGAINST *Colletotrichum orbiculare* AND *Dydymella bryoniae*.

ABSTRACT

Increase in activity of chitinase, a pathogenesis related (PR) protein, is associated with systemic acquired resistance induced in cucumber by plant activators and biological agents. Field and greenhouse experiments were conducted in order to correlate the amount of chitinase in different SAR expressing cucumber cultivars and the degree of resistance induced against *Colletotrichum lagenarium* and *Didymella bryoniae*. The results suggest that there is little correlation between the amount of chitinase induction and the degree of resistance acquired through SAR. However, the screening of these cultivars yielded a differential cultigen response to these two pathogens. Cucumber cultivars expressing SAR are generally resistant to *C. orbiculare* but are not always resistant to *D. bryoniae*. In addition, several cultivars expressing SAR developed more severe symptoms despite the high chitinase activity detected. Lesions number development by *C. lagenarium* varied significantly among cultivars, yet the size of the lesions did not. The variation in number of lesions generated by *D. bryoniae* was not as significantly different among cultivars whereas the size of the lesions varied greatly. This suggests different defense strategies against these two pathogens.

INTRODUCTION

Systemic acquired resistance (SAR) is a general defense response in plants that is characterized by the expression of pathogenesis-related (PR) genes (van Loon and van Kammen 1970). SAR can be induced after a necrotic response to an avirulent or virulent pathogen or by treatment with chemical SAR inducers such as the benzothiadiazole derivative, CGA-245704 (ASM), INA and salicylic acid (Lawton et al. 1996; Benhamou and Belanger 1998). The ground-breaking experiments of Kuc and coworkers have shown that plants expressing SAR are 'sensitized' to better withstand the infection of pathogens (Kuc 1982). Its action is nonspecific throughout the plant and reduces severity of diseases caused by all classes of pathogens. In cucumber plants, the expression of systemic acquired resistance against *Colletotrichum orbiculare* is characterized by the ability of the plant to block penetration by the formation of papillae, lignin, silicon deposition (Stein et al. 1993), and by an increase in peroxidase activity (Hammerschmidt and Kuc 1980).

Most of the research on cucumber SAR has studied the interaction with *C. orbiculare* (Kuc 1987). However, no two-pathogen species are completely similar in terms of their strategies for invading plants and causing disease. *Colletotrichum orbiculare*, relies on the formation of appressorium and mechanical penetration in order to infect plant material (Kovats et al. 1991; Stein et al. 1993). Alternatively, another pathogen of cucurbits, *Didymella bryoniae* has been reported to actively use pectolytic enzymes

during the colonization of plant tissue (Chilosi and Magro 1998), as well as penetration through the stomata (da Rocha, Personal communication). *Didymella bryoniae* (anamorph *Phoma cucurbitacearum*), the cause of gummy stem blight of cucurbits, and *Colletotrichum orbiculare*, which causes anthracnose of cucurbits, occur throughout the Eastern United States and are two of the most important diseases to attack cucurbits (Agrios 1997). The need to find control strategies that will be effective against pathogens attacking the cucurbit industry is very important and, SAR presents itself as a good potential control method against these diseases (Hammerschmidt and Dann 1997; Benhamou and Nicole 1999). Under field conditions, SAR has been reported to be effective in some cucurbits including cucumber (Caruso and Kuc 1977).

In cucumber, SAR has been mainly studied in a small number of cultigens (mostly Wisconsin SMR 58 and Straight 8). However, there are many others commercial varieties and breeding lines (Dijkhuizen et al. 1996; Staub et al. 1999). Therefore, it is easy to hypothesize that their resistance and SAR expression might vary significantly because of their genetic differences. To our knowledge there has not been an extensive study of a broad collection of cucumber cultigens for their SAR response. Consequently, a comprehensive genotype evaluation was performed.

The purpose of this work is to screen a collection of cucumber cultigens for their genetic differences in resistance against *C. orbiculare* and *D. bryoniae*. In addition, to evaluate their SAR expression levels under

greenhouse and field conditions. The correlation between the induction of resistance due to SAR and the amount of the PR protein expressed was also evaluated.

MATERIALS AND METHODS

Culture of host and pathogens.

In greenhouse, cucumber plants (*Cucumis sativus* L.) were planted in clay pots filled with Pro-Mix BX (Premier Peat Moss Corporation Marketing, New York, NY). The pots were grown under daylight supplemented with 14 h of fluorescent and incandescent light.

Field experiments were made during the growing seasons of 1998, 1999, and 2000 at the Plant Pathology research farm and at the Horticulture farm, both located in East Lansing, MI. Growing cultural practices were standardized throughout the experiments.

Cultures of *Colletotrichum orbiculare* were maintained on V-8 juice agar at room temperature in the dark. Conidia were harvested from 7-10 days cultures by flooding the dish with sterile water. Following filtration through cheesecloth, the spore concentration was adjusted to 10^6 spores mL⁻¹. *Dydymella bryoniae* was grown on ¼ strength Potato Dextrose Agar (PDA) at a temperature of 25° C in the dark. Inoculum preparations were done by flooding plates with a nutrient solution of Casamino acids (0.05 g, Difco Co.) and sucrose (1.0g, EM Science Co.) in distilled water (100 ml). The surface of the agar was scraped with a rubber spatula, and the mix was filtered through two layers of cheesecloth. The spore suspension was standardized to a concentration of 10^6 spores mL⁻¹. *Rhizoctonia solani* was maintained in PDA. Inoculum was prepared from 3-4 day old cultures, agar plugs (5 mm

diameter) from the edge of the growing mycelia were taken and placed on small wounds done to the cucumber fruit.

Induction of SAR

In Greenhouse experiments, the cucumber plants were induced to express SAR when the first true leaf (leaf 1) was fully expanded and the leaf above (leaf 2) one-third to one-half expanded. The induction was achieved by spraying the plants with the benzothiadiazole derivative, CGA-245704 (ASM) (Friedrich et al. 1996) at a rate of 20 PPM of active ingredient in water. Induction of SAR in field experiments was done using a solution of ASM with water (20 ppm) and sprayed using a compressed air sprayer. The induction was done when the majority of the plants had two fully expanded true leaves.

Detached leaf resistance bioassay

For both, greenhouse and field experiments, leaves were collected and brought into the lab. They were cut along the midrib and inoculated with 10 droplets of 10 µl of a spore suspension of either *C. orbiculare* (10^6 /ml) in water or *D. bryoniae* (10^6 spore/ml) in a casamino acids/glucose solution. The half leaves were placed in sterile Petri dishes underlined with moist filter paper. Seven days after challenge, the number of lesions allowed per half leaf was counted and the lesion size diameter (mm) assessed.

Cucumber fruit bioassay

Cucumber fruits were harvested when fruit 1¾ to 2 inches in diameter were first observed. The fruit was harvested into clean, plastic containers, washed in chlorinated water and placed in trays underlined with moist paper towels. A small wound was done in each cucumber fruit using a razor blade and a 5 mm agar plug of *Rhizoctonia solani* was placed on top of the wound. The cucumber fruits were kept under high humidity conditions. About 8-10 days after inoculation, the fruit was cut in half directly over the wound and the damage was measured in two dimensions, lesion diameter and lesion depth.

Assay of Peroxidase and Chitinase Activities

Peroxidase activity of leaf extracts was determined using guaiacol as a substrate as described previously (Hammerschmidt et al. 1982). Chitinase activity was determined as follows. A 1% agarose in potassium phosphate buffer 10 mM (pH 5.0) was prepared with a 1% (v/v) of glycol chitin which serves as chitinase substrate (Trudel and Asselin 1989) and poured in a 15 cm diameter Petri dish. After solidification, 3mm diameter plugs were carved using a small cork borer in the agarose. Five µl of the total protein extract were placed in the wells (3 wells per sample), and incubated at 37 °C for 4 hours. Following incubation, the reaction plate was rinsed and flooded for 10 min in 0.5 M solution of Tris-HCl (pH 8.9) with 0.01% (w/v) of calcoflour brightener 28 in order to stop the reaction and stain the plate. When viewed with UV translumination, the areas of activity appeared as a well-defined dark

areas on a fluorescent background. The diameter of the circular area of activity was measured and used for data analysis.

Experimental design.

Greenhouse experiments were done based on a complete randomized block design. In comparing induced plants and control plants, 3 blocks per treatment of at least 5 plants per block were analyzed.

In 1998 and 1999, two field experiments were done to study genetic resistance differences of a cucumber collection at the Horticulture research farm. In addition an experiment to study the effect of ASM was done in the Botany and Plant pathology research farm. These experiments were based on a randomized complete block design. In 2000, one main experiment was performed to study ASM effect on cucumber plants. The experiment was designed as a split plot with cultigens as main plots and treatments (control and ASM) as subplots.

RESULTS

Greenhouse screening of cucumber cultigens revealed different levels of resistance after treatment with the synthetic plant activator acybenzolar-S-methyl ("ASM", "Actigard"). Initial observation using five cultigens revealed that the levels of expression of SAR significantly enhanced the ability of the treated plants to hinder fungal invasion as well as fungal spread within the tissue (fig 4.1). In both, lesion size and lesion number, statistical analysis revealed that comparison within varieties (control vs. treatment) were statistical significant at all levels.

When the basal levels of resistance in control plants was analyzed, the levels expressed in control plants did not present differences among the varieties tested in terms of lesion number. In terms of lesion size and the ability of the plant to hinder fungal growth within the tissue, two groups were observed. Pioneer and National Pickling had a slightly higher resistance than SMR 58, Straight 8 and MM 80 (fig 4.1).

The induced levels of resistance were found to behave similarly in both measurements, lesion size and lesion number reduction. Pioneer being the more resistant and Straight 8 being the most susceptible.

In addition, all five cultigens had expressed an increase in chitinase as well as peroxidase activity after ASM treatment (fig 4.2). Additionally, it was observed that the basal levels of chitinase activity were non statistically different. However, when the plants were induced with ASM, the levels of chitinase were increased and three statistical significant groups were

observed. (fig 4.2). However, when the number of cucumber cultigens was increased, the results became more complex. Although the majority of the cultigens treated with ASM had fewer lesions and reduced lesion size, the reductions were not always at significant levels. The majority of the cultigens were also more successful stopping the initial infection than actually reducing fungal growth within the tissue as shown by the number of lesions observed (table 4.1). The bulk of the cultigens had a significant reduction of lesion number even though some of the same cultigens had no differences between the control and treated plants in terms of lesion size. Nonetheless, PI 390240 and PI 390259 were more successful reducing fungal growth within the tissue by limiting the lesion size than stopping the pathogen from initial infection.

There is a differential response of plants expressing SAR against pathogens with different mode of invasion.

In order to study the response of SAR against two different pathogens with different modes of infection, plants were induced with ASM and challenged with two different pathogens. *Colletotrichum orbiculare*, uses the formation of appressorium and mechanical penetration to infect the plant material (Kovats et al. 1991; Stein et al. 1993). Alternatively, *Didymella bryoniae* has been reported to penetrate through stomata (da Rocha, Personal communication) and actively use pectolytic enzymes during the colonization of plant tissue (Chilosi and Magro 1998).

Seven days after induction with ASM the second true leaf was harvested and brought into the lab. The leaf was cut along the center vein and one half was challenged with *C. orbiculare* and the other half was challenged with *D. bryoniae*. The treated plants expressed an increase in resistance against *C. orbiculare*.

Conversely, the plants treated with ASM and challenged with *D. bryoniae* gave results that were unexpected. In almost half of the cultigens tested against this pathogen, the treated plants allowed higher incidences of disease caused by this organism when compared to the control plants. Higher amount of disease in ASM treated plants occurred even in lines that expressed good induced resistance to *C. orbiculare* (fig 4.3). The treatment of cucumber plants with ASM appears to predispose some the cultigens to become more susceptible to *D. bryoniae*. However, there were some cultigens that did express a higher degree of resistance to *D. bryoniae* after ASM treatment. A second experiment was done to corroborate the findings described above, the results shown (fig 4.4) clearly demonstrates that there is a differential expression of SAR induced by ASM against *C. orbiculare* and *D. bryoniae*. Chitinase activity in this experiment shows that the plants treated with ASM have higher expressions of the enzyme. The differences in expression of resistance are statistically significant.

There is a genotypic difference between cultigens to express natural resistance against Colletotrichum orbiculare and Dydimella bryoniae.

Experiments performed at the Horticulture farm at Michigan State University during the 1998 and 1999 growing seasons were structured to observe the genotype genetic response to *C orbiculare* and *D. bryoniae*.

When the leaves were infected with *C. orbiculare*, all of the cultigens tested, supported the same number of lesions (1998 trial). However, the cultigens were different in their ability to restrict lesion development after initial infection (fig 4.6, tables 4.2, 4.3) In this trial, the lesion numbers between cultigens vary somewhat. However, the trend is still the same. Most cultigens were not successful in stopping the initial penetration.

When plants were challenged with *D bryoniae*, the cultigens also varied in their response (table 4.4). There was a significant variation in lesion number, but no size among the cultigens. Thus, the response of the plants against *D. bryoniae* seems to be more effective in stopping the initial penetration rather than the spread of the fungus within the tissue (table 4.4).

While most of the cultigens were more successful reducing the size of the lesions against *C. orbiculare*, the opposite occurs when challenging the plant with *D. bryoniae* (fig 4.7). On the other hand, the cultigens were more successful in reducing the lesion number against *D. bryoniae*, as opposed to *C. orbiculare*. This variation is easily seen in figure 4.8.)

Cucumber Cultigens present little genetic difference in resistance against *Rhizoctonia solani*

During the 1998 and 1999 growing seasons, cucumber fruits were collected from field experiments performed at the experimental farms of horticulture and plant Pathology farms at Michigan State University. The results obtained indicate that there was no genotypic variation between the genotypes (Data no shown). In fact, the results imply that there is no significant genetic resistance variation among all of the cultigens.

ASM application does not enhance the resistance of cucumber fruits against *R. solani*.

Cucumber fruits were obtained from an experiment in which the plants were induced to express SAR with ASM. Results indicate SAR, is not effective against *R solani* in the fruit. Since, the levels of resistance between ASM treated plants, and control plants were not statistically different, data from both samples were combined for analysis of variance comparing the cucumber genotypes. No getonotypic differences were obtained; all of the cultigens were infected at the same rate. A random sampling of cucumber fruits were analyzed and their chitinase activity measured, although there was not enough data to perform a full statistically analysis, the observations seem to suggest that the amount of chitinase induction between cucumber fruits obtained from control and treated plants was different. There was an

increase of chitinase activity from cucumber fruits from plants treated with ASM (data no shown).

Levels of Resistance against a challenge inoculation of *C. orbiculare* in cucumber treated with ASM.

In 1999, a pilot experiment was performed with three varieties of cucumber, SMR-58, MM86 and Straight 8 in order to investigate the effect of treating plants with ASM. At seven days of the treatment, the leaves were collected and challenged with *C. orbiculare*. The data obtained in this preliminary experiment shows that a treatment with ASM does not provide the level of protection observed under greenhouse conditions. In fact, there were no measurable differences between the cultigens as well as between control plants and induced plants. Two leaf harvests were analyzed seven days after induction (fig 4.9).

A more comprehensive experiment was performed in 2000. Data obtained this year suggests that the levels of protection obtained from ASM applications in the field do provide some resistance to the fungus. Interestingly, the levels of acquired resistance is more pronounced in the ability of the plant to stop pathogen spreading rather than the ability to stop initial penetration. Of the sixteen varieties tested, ten had significant differences between the ASM treated plants and their controls (table 4.4). However data obtained from three cultigens, (County fair, H19 and M21),

suggests that their control plants had higher levels of resistance than the ASM treated plants under field conditions.

In terms of lesion number, as observed in the genotypic screenings, the differences between the control and induced plants did not yield well-defined differences. However, in this particular experiment four out of sixteen varieties had lower number of lesions after the induction. Interestingly, one of those varieties, H-19 was one of the varieties that allowed a larger lesion size after induction (table 4.5).

As another assessment of the level of SAR induced in the plants, chitinase activity was measured (table 4.6). These results suggest that the levels of chitinase activity is significant different between the control and the ASM treated plants. However, it is important to note that 3 of the 16 varieties (Anka, Sumpter and PI 231054), had levels of chitinase higher in control plants than in the treated plants. M-21 did not express an increase of chitinase and the other 12 did express higher levels of chitinase after ASM treatment.

Correlation between the chitinase expressed and the resistance acquired.

Analysis of levels of induced chitinase and resistance show that they are not significantly correlated. Figure 4.10 shows the distribution of one of those correlations for field grown plants. In greenhouse conditions the correlations are also non-significant.

DISCUSSION

Systemic Acquired Resistance has been researched over the last years as a potential control method for a broad spectrum of diseases in many species. A compound [azibenzolar-S-methyl (ASM)] that activates the plant's natural defense has been released commercially. This activator has been registered for a variety of cultigens. The manufacturer's web site (<http://www.syngenta.com>) cites bananas, tomatoes and tobacco as main crops, but cucurbits and other species are also included. Most of the experiments and the development of the SAR technology have been done under greenhouse conditions and a few field experiments have been published, in some cases negative results have been reported. In one study, the authors state that the treatment with acibenzolar-S-methyl alone was effective in reduction of the disease severity in many but not all situations (Huang et al. 2000). This statement suggests that SAR is not effective against all diseases or under all conditions, which is also demonstrated in this work. The fact that the levels of SAR induction are mostly effective against *Colletotrichum orbiculare* but not, in many cases, to *Didymella bryoniae* supports Huang et al.

Several conclusions can be drawn from my results. First, the response of induced cucumber plants against *Colletotrichum orbiculare* in greenhouse conditions seems to be consistent across the collection of cultigens tested. According to the literature, the phenotype of SAR in cucumber is expressed in the epidermal layer by decreasing the number of successful penetrations

(Kovats et al. 1991). In the epidermis, enhanced formation of papillae beneath appressoria, and increase lignification (Hammerschmidt and Kuc 1982) are correlated with reduced development of infection hyphae. The data in this work supports this observation, since most cultigens treated with ASM were more effective in reducing the number of successful penetrations than reducing the spread of the fungus within the tissue. However, five out of 40 varieties tested did not have a significant reduction of lesion number and two out of those five were more successful in reducing the fungal growth within the tissue. When it comes reducing the growth of the fungus within the tissue, the majority of the cultigens were successful in reducing the fungal growth. The fact that the majority of the cultigens behaved in a similar way in terms for their response to SAR induction against *Colletotrichum orbiculare* penetration suggests that the differences in terms of the SAR phenotype expression is generalized across the specie. On the other hand, in reducing the lesion size, thus reducing the fungal growth within the tissue, cultigens presented some differences. Not all cultigens were able to decrease the levels of disease in ASM treated plants.

The increased resistance associated with SAR has been thought to be linked to the action of PR proteins. The function of some PR proteins is unknown, while others have been found to be β -glucanases and chitinases (Van Loon 1997). Thus, given that β -glucans and chitin are major constituents of the fungal cell wall, such enzymes have an obvious anti-microbial role.

Therefore, since not all of the varieties were able to decrease the fungal growth within the tissue questions the role of these enzymes in resistance.

When the plants were challenged with *Dydymella bryoniae*, a fungus with a different infection strategy, a variation in response among cultigens was observed. The ability of the ASM treatment to enhance susceptibility to some of the cultigens to infection by *Didymella bryoniae* seems to suggest that there is a negative physiological effect caused by ASM in the plants. However, in some varieties the opposite occurs: treatment with ASM actually reduced the infection of *D. bryoniae*. Since some cucumber varieties were more resistant after ASM induction seems to suggest that there is a genetic difference in such plants that allows them to increase their defenses against *D. bryoniae*. On the other hand, a published experiment reports that SAR is effective against *D. bryoniae* (Mucharromah and Kuc 1991) in cucumbers. However, the inducers in this particular experiment were *C. orbiculare* and aqueous solutions (20 or 50 mM) of oxalate, potassium phosphate dibasic (K_2HPO_4) or tribasic (K_3PO_4). This brings up a question, does the type of induction play a factor in the resistance against *Dydymella bryoniae*?

It is also possible that there is a morphological difference among the cultigens that can explain the difference (e.g. stomata or hydathodes). ASM may cause these natural openings to behave differently by opening longer time periods, or increasing their opening size (da Rocha personal communication).

Additionally, the strategy of infection of the fungus and the effect of the induction in the plant might play a role in this differential display of resistance against *D. bryoniae*. For instance, *C. orbiculare* relies on the formation of an appressorium to penetrate, and the phenotype of SAR has been clearly demonstrated to be expressed in the epidermal layer (Kovats et al. 1991; Stein et al. 1992). On the other hand, *D. bryoniae* has the ability to penetrate through natural openings such as stomata (da Rocha personal communication) or by using pectolytic enzymes to help in their initial penetration (Chilosi and Magro 1998). It also happens that *Dydymella bryoniae* produces a polygalacturonase (PG) when infecting cucumber, in fact, it is the first enzyme produced followed by other cell-wall degrading enzymes (Chilosi and Magro 1998; Zhang et al. 1999). Because many plant species produce polygalacturonase-inhibiting proteins (pgip) (De Lorenzo et al. 2001), the possible role of these proteins in cucumber is also worth investigating. Interestingly, transcripts of pgip's accumulate in *P. vulgaris* hypocotyls in response to wounding or treatment with salicylic acid (Bergmann et al. 1994).

SA and JA dependant defense pathways interact, for example the SA analog INA and ASM can suppress the JA dependant defense gene expression. (Penacortes et al. 1993; van Wees et al. 2000), possibly through the inhibition of JA biosynthesis and action (Penacortes et al. 1993). Thus, over expressing the SA dependant pathway by ASM application could have detrimental effects on the Jasmonic acid/Ethylene pathway that confers

resistance against insects and certain group of pathogens. It is possible that because of the *D. bryoniae* mode of infection (pectolictic enzyme production) the JA dependant pathway might be a better fit for expression of resistance against this pathogen.

Screening of a cucumber collection under field conditions for resistance to *D. bryoniae* and *C. orbiculare* has provided interesting results. In the 1998 trial, the levels of basal resistance were clearly expressed in the ability of the plants to stop fungal growth within the tissue, however there were no differences between the cultigens in reducing the number of lesions. In the 1999 trial, the results were consistent with the findings of 1998. The cucumber cultigens show little variation in reducing the lesion number, but the ability of the plants to stop fungal growth within the tissue and reduce lesion size is evident. In contrast, resistance against *Dydimella bryoniae* was expressed in reducing the initial penetration of the fungus. This dissimilarity is very interesting and suggests that the levels of basal resistance against both pathogens come from different defense responses in the plants. As mentioned before, the initial penetration by *C. orbiculare* is via an appressorium. Therefore, The fact that there is little variation in the number of lesion across the cultigens clearly establishes the argument that SAR activates the defense response at the epidermal layer, since in greenhouse conditions the number of lesions is reduced when plants are expressing SAR. In the case of *D. bryoniae*, the lesion number varies significantly across the

cultigens, suggesting that there is a genetic control of this part of the host resistance.

SAR induction in the field with a biotic inducer such as *Colletotrichum orbiculare* in cucumber or by plant growth-promoting rhizobacteria (pgpr) has been reported in a variety of species including cucumber (Wei et al. 1996); (Caruso and Kuc 1977; Caruso and Kuc 1977). ASM has also been reported to increase levels of resistance against a variety of pathogens in a variety of species (Cole 1999; Pappu et al. 2000; Csinos et al. 2001; Romero et al. 2001) including the post harvest protection of cucurbits against some fungi (Huang et al. 2000). During the 1999 trials, an experiment was design to observe the levels of resistance acquired after ASM induction in field conditions. Cucumber fruits were no protected against *R. solani* by ASM application. There has been evidence that the there is an antagonism of the and ISR pathways (Pieterse et al. 2001). An application of ASM is enough to induce in the plant the higher levels of SAR, and suppress the JA pathway and ISR. It could be possible that ISR is actually effective against *R. solani* as it is against other pathogens of cucurbit fruits as recently shown (Huang et al. 2000).

The results of the 1998 trial in which the basal resistance of the cucumber cultigens was tested against *R. solani* supports the data obtained in 1999. Both years, there were no differences among cultigens and as in the case of the 1999 trial, no differences between control and induced plants were observed. It has not escaped our attention that the resistance might be

expressed in the epidermal layer of the fruit. By causing the small wound in the epidermis in order to cause infection the fungus might be surpassing the actual site of resistance.

The data obtained in the field in 1999 suggests that under field conditions, SAR is not as effective as under greenhouse conditions. In 2000, the experiment performed spraying the plants with ASM, shows that the levels of resistance against *C. orbiculare* seems to be more effective in reducing the lesion size rather than reducing the number of lesions. As stated before, SAR phenotype is expressed in the epidermal layers, which reduce the number of successful penetrations. In this particular experiment and in the 1998 trial, no dramatic differences were found in reduction of lesion number. Interestingly using chitinase activity as marker for SAR suggests that SAR activation was successful. The majority of the cultigens had a higher chitinase activity than plants from the control plots. However, this marker is not generalized in all cultivars since some did not express an enhanced resistance, yet their chitinase activity was higher in ASM treated plants.

The levels of chitinase induction in all experiments, field and greenhouse did not correlate with the reduction of lesion size at any statistical level. This finding is rather controversial since the induction of SAR has been correlated with disease suppression. However, the correlation is more of a qualitative marker, rather than a quantitative marker. As shown in this work, the levels of chitinase in induced plants are usually higher than in control plants yet, the ability of the plant to defend itself seems to be unrelated to the

chitinase activity level. So far there is no real evidence that chitinase induction has a direct involvement in the suppression of disease other, than serving as a marker for induction of SAR. The basal levels of PR proteins have been suggested to be used as a marker for resistance (Vleeshouwers et al. 2000). However, before any recommendations are to be made, a comprehensive study needs to be done in order to validate these claims.

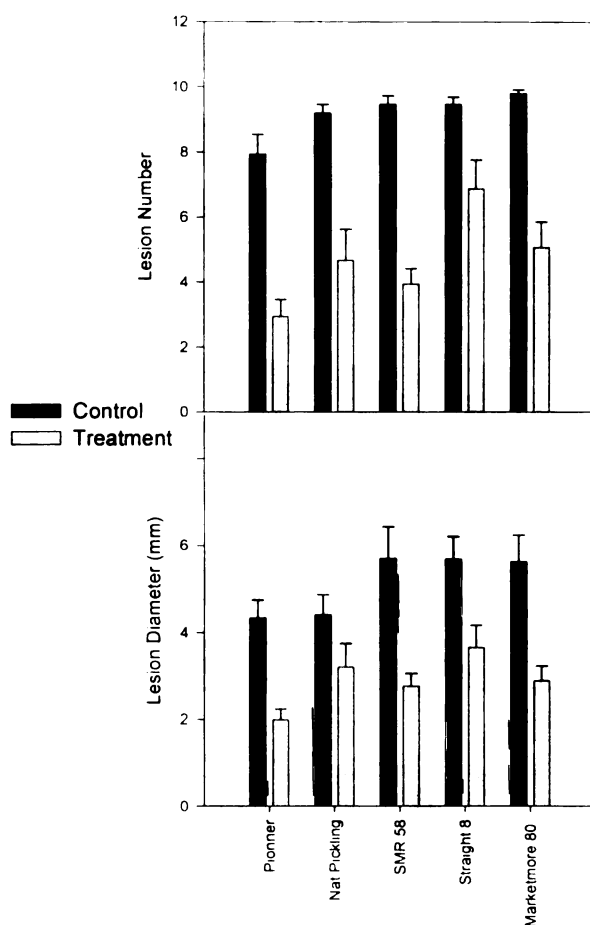
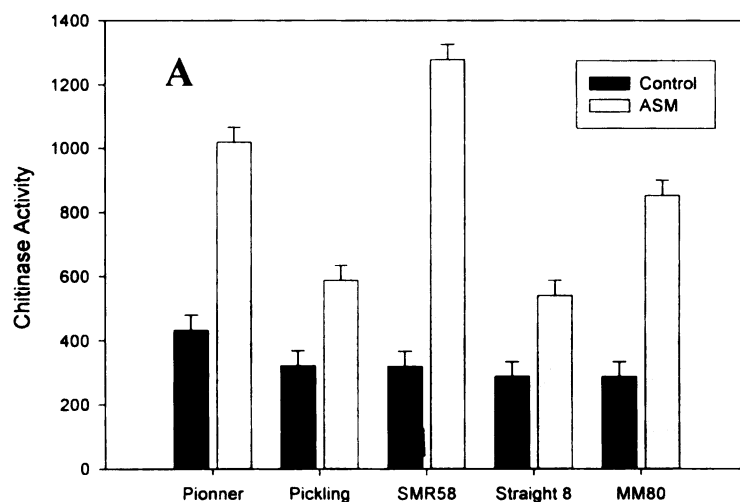


Figure 4.1: Lesion size and lesion number analysis of greenhouse experiment of cucumber plants treated with ASM and challenged with droplets of 10 μ l of a spore suspension of *Colletotrichum orbiculare* (10^6 spores/ml). Measurement disease severity was made seven days after challenge. Basal levels of resistance (black bars) no statistical differences were observed in lesion number between cultivars. Lesion diameter, however, pioneer and Nat Pickling were a statistical different group from SMR58, Straight 8, and MM80.



B

	SMR58	Pioneer	MM80	Pickling	Straight 8
Control	A	A	A	A	A
ASM	A	B	B	C	C

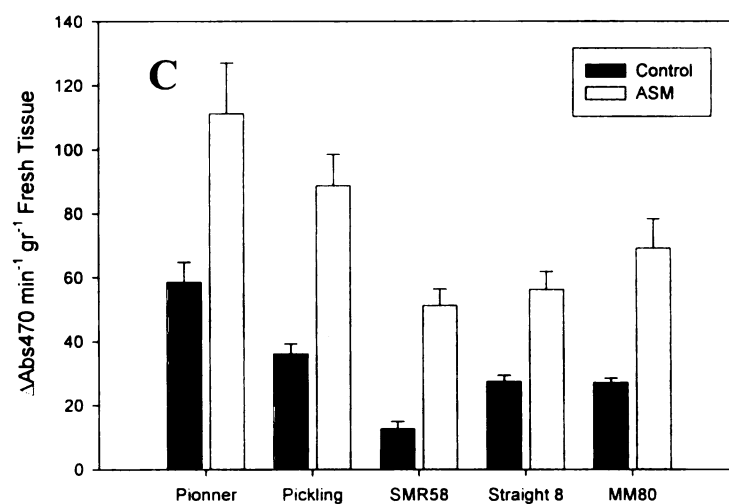
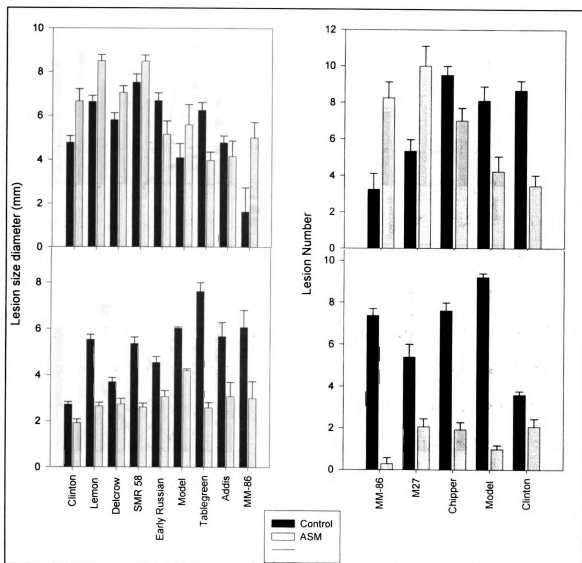


Figure 4.2: Chitinase activity (A) and peroxidase activity (C) analysis of 5 commercial cultivars of cucumber grown in greenhouse conditions. B is the statistical analysis of chitinase activity comparing the basal levels and induced levels of the enzyme.

Table 4.1: Greenhouse screening of cucumber cultigens expressing SAR induced with ASM and challenged with the fungi *Colletotrichum orbiculare*. The experiment was performed in three different phases; SMR58 was used as a reference in each of the experiments. Notice the SMR58 results, Despite the fact that the numbers vary the statistical analysis outcome was the same. The symbol * represents the presence of a significant difference between the control and the plants treated with ASM. A larger number of cultigens expressed higher resistance by suppressing the number of lesions than reducing the actually spread of the pathogen within the tissue.

		Lesion size			Lesion Number		
	Cultigen	Control	Treatment	S*	Control	Treatment	S*
1	1160 Dwarf	2.896	2.179		8.600	4.000	*
2	Ames	4.233	1.965	*	9.000	0.500	*
3	C. hardwickii	3.598	2.714		8.200	1.000	*
4	Calypso	1.918	3.885		4.250	0.400	*
5	Carolina	2.824	2.246		6.000	3.200	*
6	Castle	3.11	1.779	*	5.800	4.500	*
7	Collet	3.419	1.074	*	7.400	1.333	*
8	Coolgreen	4.177	1.134	*	9.500	0.600	*
9	County Fair	3.984	1.56		6.250	0.200	*
10	Early Pick	5.348	7.393	*	7.200	1.750	*
11	Freemont	3.549	2.33	*	8.250	0.400	*
12	GY14	3.757	3.534		8.750	5.500	*
13	H19	3.082	-7.77E-15	*	9.333	0.000	*
14	M21	3.047	2.505		7.000	2.500	*
15	Marbel	3.359	2.803		9.250	3.600	*
16	MM 76	4.356	1.816	*	10.000	1.500	*
17	Model	6.026	4.194	*	9.200	4.000	*
18	Poinssette	3.296	-1.52E-14	*	10.000	0.000	*
19	Regal	2.834	1.564		6.000	1.750	*
20	SMR 58	5.471	4.234	*	7.750	5.400	*
21	SMR 58	3.53	2.414	*	9.500	2.667	*
22	SMR58	7.118	2.505	*	10.000	2.500	*
23	Straight 8	3.909	3.27	*	9.750	5.750	*
24	Sumpter	3.543	2.753	*	8.750	3.800	*
25	TG 52 M	5.687	3.78	*	9.667	3.500	*
26	W 1983	3.247	2.153		7.500	3.667	*
27	W 5096	2.847	2.853		4.600	1.400	*
28	Wautoma	5.933	2.133	*	10.000	2.000	*
29	P76	2.791	1.44		7.750	0.750	*
30	PI 288238	4.832	4.961		5.444	6.400	
31	PI 234517	1.689	1.993		7.250	2.333	*
32	PI 267942	6.141	3.081	*			
33	PI 279468	5.077	4.923		2.000	2.800	
34	PI 330628	3.596	2.699		8.000	4.000	*
35	PI 390240	6.956	5.709	*	8.000	6.333	
36	PI 390244	5.714	6.63		8.500	6.000	
37	PI 390259	6.939	5.329	*	6.000	5.200	
38	PI 391570	3.531	3.152	*	5.600	3.250	*
39	PI 426170	5.194	3.166		7.000	3.200	
40	PI 432865	5.95	3.513	*	7.250	2.000	*
41	PI 432865	4.627	3.326		9.750	1.333	*
42	PI 432867	3.982	0.99	*	8.200	0.250	*
43	PI 432890	3.754	0.546	*	7.800	0.500	*
43	Pioneer	3.381	1.67	*	7.200	1.000	*
44	PI 466922	4.848	2.772	*	8.600	2.200	*

Figure 4.3: Differential response of plants expressing SAR and challenged with two different pathogens. Plants were grown under greenhouse conditions and induced with ASM. Seven days after induction, the second leaves were collected and brought into the lab. The leaves were cut along the midrib and half of each leaf was challenged with one pathogen and the other half with the second pathogen. Upper panels represent the leaves challenged with *D. bryoniae*. Lower panels are leaves challenged with *C. orbiculare*. Black bars represent control leaves and gray bars represent the leaves treated with ASM. Only plants with statistical significance are depicted in the graph.



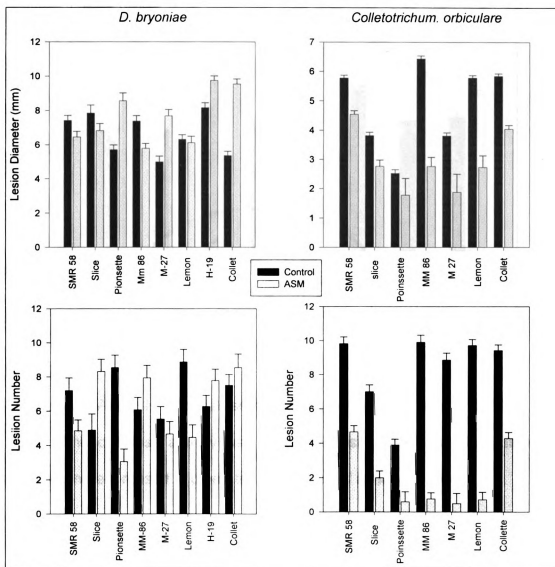


Figure 4.4: Analysis of lesion size and lesion number of cucumber cultivars grown under greenhouse conditions and induced to express SAR by ASM. Leaves were collected and challenged against *C. orbiculare* and *D. bryoniae*.

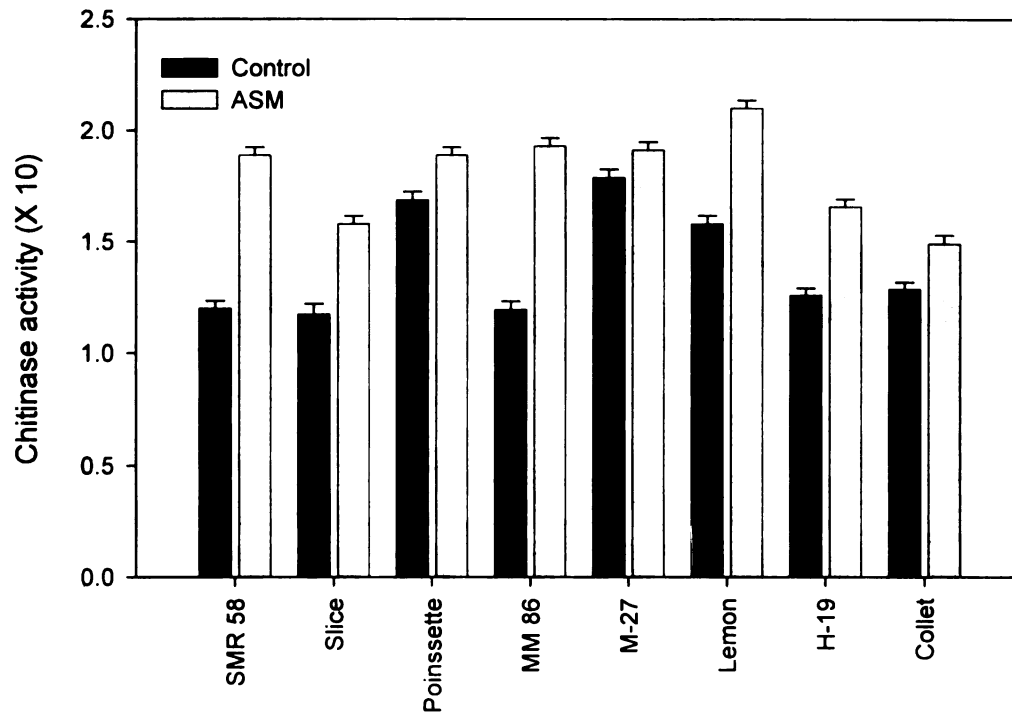


Figure 4.5: Analysis of chitinase activity at seven days after induction with ASM. The chitinase activity was measured using the agar plate diffusion method and expressed in diameter of the area of activity in mm.

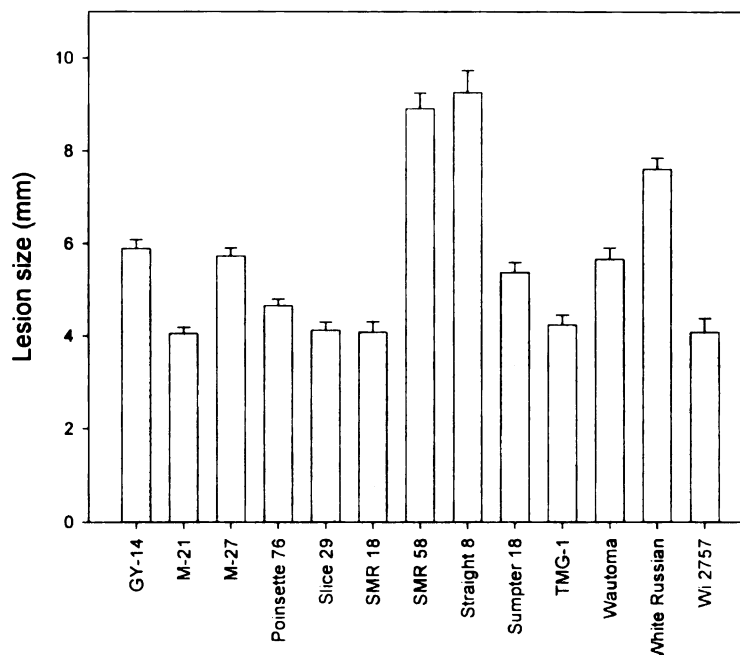


Figure 4.6. Data analysis of the lesion size of the field trial conducted in 1998.

Variety	Mean	Stdev	
M-21	4.057	1.151	A
SMR 18	4.082	1.021	AB
Wi 2757	4.086	2.353	AB
Slice 29	4.128	1.622	AB
TMG-1	4.245	1.726	AB
Poinsette 76	4.659	1.552	AB
Sumpter 18	5.378	2.004	B
Wautoma	5.66	1.612	B
M-27	5.737	1.835	B
GY-14	5.897	1.777	B
White Russian	7.61	2.632	C
SMR 58	8.919	2.483	D
Straight 8	9.262	2.087	D

Table 4.2: Statistical analysis of the cucumber leaf lesion size data obtained from the field test in 1998. The leaves were challenged with *C. orbiculare* and measured seven days after inoculation.

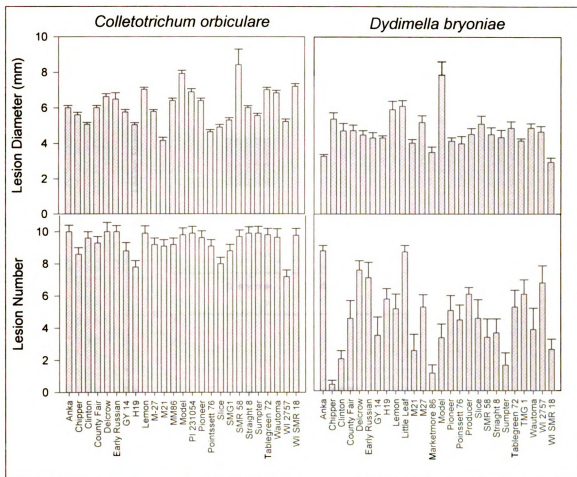


Figure 4.7: Statistical analysis of the data from the detached bioassay for *Colletotrichum orbiculare* and *Dydimella bryoniae* (1999 field trial). The youngest fully expanded leaf were brought into the lab and challenged. The measurement of the size of the lesions was done 7 days after inoculation

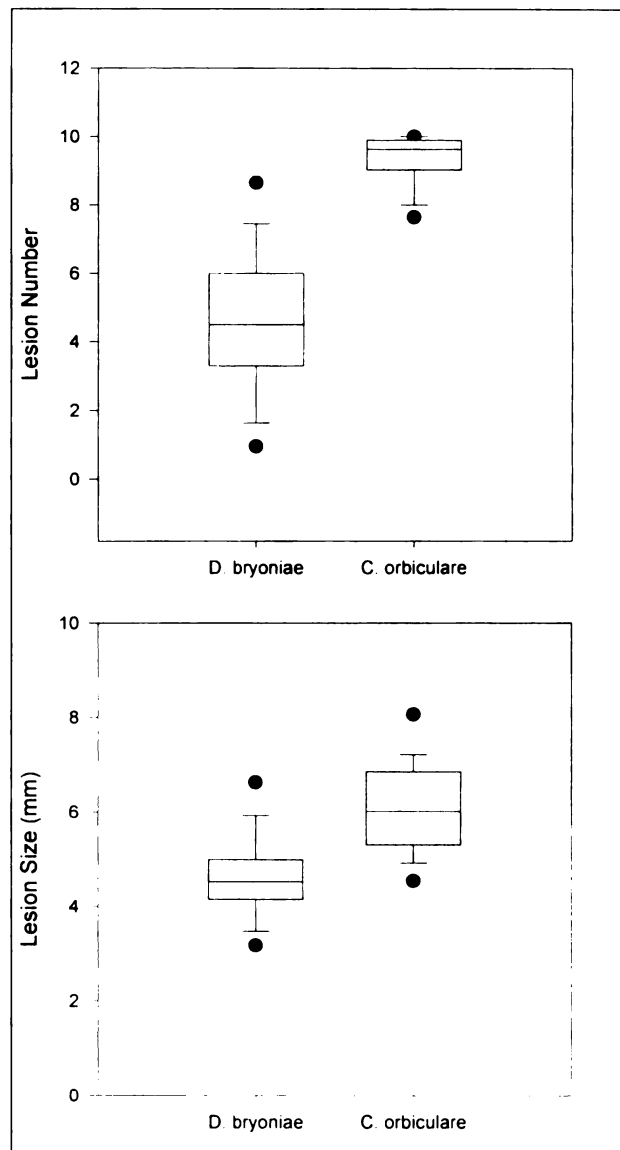


Figure 4.8: Data analysis of the average of the means of the cultivars tested in a field experiment in 1999. Lesion size and lesion numbers means of the varieties tested were used to perform this analysis. Note the differences of variation between lesion size and lesion number for each challenge pathogen.

Field Data 1999
Challenge pathogen *Colletotrichum orbiculare*

Cultigen	Lesion Number Average	Significance P=<0.001	Cultigen	Lesion size mean (mm)	Significance P=<0.001
H 19	7.423	B	Lemon	7.11	A
M 21	7.526	AB	M 21	3.86	G
Slice	7.538	AB	Slice	4.175	G
WI 2757	7.76	AB	Pointssett 76	4.2	G
Pointssett 76	7.808	AB	WI 2757	4.665	FG
TMG 1	7.808	AB	H 19	4.7	FG
Chipper	8.28	AB	TMG 1	4.97	F
Clinton	8.5	AB	M-27	5.065	F
Sumpter	8.6	A	Clinton	5.18	F
M-27	8.846	AB	Anka	5.315	EF
GY 14	9.077	A	Sumpter	5.36	EF
Marketmore 86	9.208	A	GY 14	5.53	EF
Anka	9.231	A	Chipper	5.56	DEF
County Fair	9.24	A	Marketmore 86	5.76	CDEF
Straight 8	9.462	A	Pioneer	5.79	CDEF
SMR 58	9.538	A	Producer	5.795	CDEF
Tablegreen 72	9.615	A	Early Russian	5.82	CDE
Pioneer	9.667	A	Little leaf	5.9	CDE
WI SMR 18	9.708	A	County Fair	5.91	CDE
Lemon	9.75	A	Straight 8	6.325	BCD
Little leaf	9.765	A	Wautoma	6.435	ABCD
Early Russian	9.852	A	Delcrow	6.52	ABCD
Wautoma	9.857	A	SMR 58	6.52	ABCD
Delcrow	9.9	A	WI SMR 18	6.68	ABCD
Model	9.929	A	Model	6.72	ABC
			Tablegreen 72	6.885	AB

Table 4.3. Analysis of cucumber cultivars grown in field conditions. A detached leaf bioassay was performed by challenging the leaves with a spore suspension of *C. orbiculare*.

Field Data 1999
Challenge pathogen *D. bryoniae*

Cultigen	Lesion number average	Significance	Cultigen	mean lesion size	Significance
		P=<0.001			P=<0.001
Anka	8.7	A	WI SMR 18	2.937	E
Little Leaf	8.633	AB	Anka	3.279	E
Delcrow	7.5	ABC	Model	3.498	E
Early Russian	7.025	ABC	Producer	3.99	DE
WI 2757	6.7	ABC	M21	4.025	DE
TMG 1	6	ABCD	Pointssett 76	4.134	DE
County Fair	4.5	ABCDE	TMG 1	4.151	DE
H19	5.7	ABCDE	H19	4.313	D
Lemon	5.1	ABCDE	GY14	4.318	D
M27	5.2	ABCDE	Sumpter	4.342	D
Pioneer	5	ABCDE	Early Russian	4.485	D
Poinsett 76	4.4	ABCDE	Straight 8	4.509	D
Producer	6	ABCDE	Slice	4.521	D
Slice	4.5	ABCDE	WI 2757	4.657	D
Tablegreen 72	5.2	ABCDE	Clinton	4.715	D
GY 14	3.45	CDE	County Fair	4.73	CD
Model	3.3	CDE	Tablegreen 72	4.865	CD
SMR 58	3.325	CDE	Wautoma	4.876	CD
Straight 8	3.6	CDE	SMR 58	5.103	BCD
Wautoma	3.8	CDE	M27	5.189	BCD
WI SMR 18	2.575	CDE	Chipper	5.379	BCD
Clinton	2	DE	Lemon	5.905	BC
M21	2.5	DE	Little leaf	6.094	AB
Sumpter	1.6	DE	Pioneer	7.862	A
Chipper	0.4	E			
Marketmore 86	1.1	E			

Table 4.4. Analysis of cucumber cultivars grown in field conditions. A detached leaf bioassay was performed by challenging the leaves with a spore suspension of *D. bryoniae*.

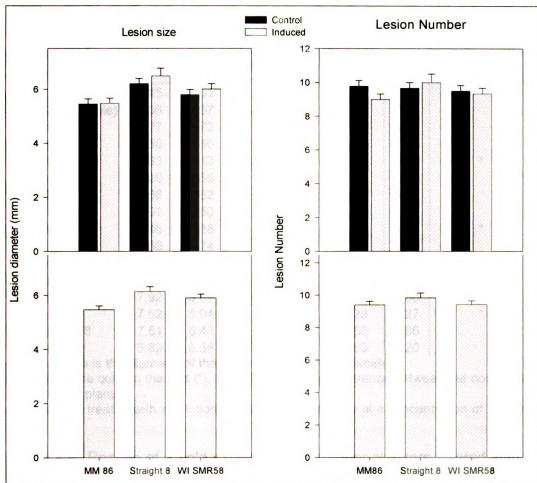


Figure 4.9: Field experiment of three cultivars of cucumber treated with ASM (20 ppm). Leaves were harvested and challenged with a spore suspension of *Colletotrichum orbiculare* (10^6 spores/ml). Lesions were assessed seven days after challenge. Upper panels indicate the analysis the control and the induced plants. Since no statistical differences were found between the controls and treated plants, the data was combined and analyzed for each cultivar. Lower panels are the results of the analysis. No statistical differences were found between the three cultivars tested.

	Lesion Size ^a			Lesion Number		
	Control	ASM ^c	Significance ^a <i>P</i> <0.05	Control	ASM ^c	Significance ^b <i>P</i> <0.05
Anka	6.45	6.91		7.33	7.00	
Clinton (male)	7.06	5.57	*	7.42	7.63	
County fair	4.77	5.72	*	7.55	6.96	
GY14	5.50	5.56		5.87	7.83	
H19	4.63	5.43	*	8.36	8.31	*
Lemon	6.20	6.55		9.35	8.69	
M21	5.68	6.62	*	8.23	6.34	
M-27	6.01	6.50				
MM 86	8.46	7.38	*	9.73	8.37	*
PI 231054	5.30	5.74		7.38	8.25	
Pioneer	6.68	5.86	*	9.70	8.12	
Poinssette	5.38	4.70	*	8.60	7.78	*
Slice	7.92	5.07	*	8.39	7.86	
SMR58	7.52	5.94	*	9.23	9.27	*
Straight 8	7.51	6.48	*	9.68	9.86	
Sumpter	6.89	6.38		8.83	8.20	

a: Lesion size is the diameter of the lesion measured in millimeters.

b: Significance column marked (*), there is a statistical difference between the control and the ASM treated plants.

c: Plots were treated with a solution of ASM diluted in water at a concentration of 20 ppm.

Table 4.5: Results of a field experiment cucumber cultivars treated with ASM and control plants. Plants were treated with ASM. Seven days after treatment, leaves were brought into the lab for analysis. They were challenged with a spore suspension of *C. orbiculare* and the lesion assessment was done seven days after the challenge

	Chitinase Activity ^a		Significance ^b P<0.05
	Control	ASM	
Anka	1.370	1.205	*
Clinton (male)	1.277	1.394	*
County Fair	1.085	1.216	*
GY14	1.165	1.481	*
H19	1.258	1.622	*
Lemon	1.087	1.252	*
M21	1.310	1.343	
M27	1.390	1.467	*
MM86	1.090	1.295	*
PI 231054	1.426	1.213	*
Pioneer	1.083	1.218	*
Poinsette	1.201	1.428	*
Slice	1.200	1.381	*
SMR58	1.010	1.168	*
Straight 8	1.097	1.246	*
Sumpter	1.396	1.345	*

a: chitinase activity measured by the agar diffusion plate expressed in diameter of the area of activity.

b: statistical analysis was made combining the two harvests and the two replicates per harvest. Each sample was measure in triplicates

Table 4.6: Chitinase levels of cucumber plants from a field experiment at the Horticultural farm, Michigan State University. Plants were induced to express SAR with a treatment of ASM and leaves were collected seven days after induction. Leaves were frozen and ground in liquid nitrogen. The chitinase activity levels were determined using the agar plate diffusion test.

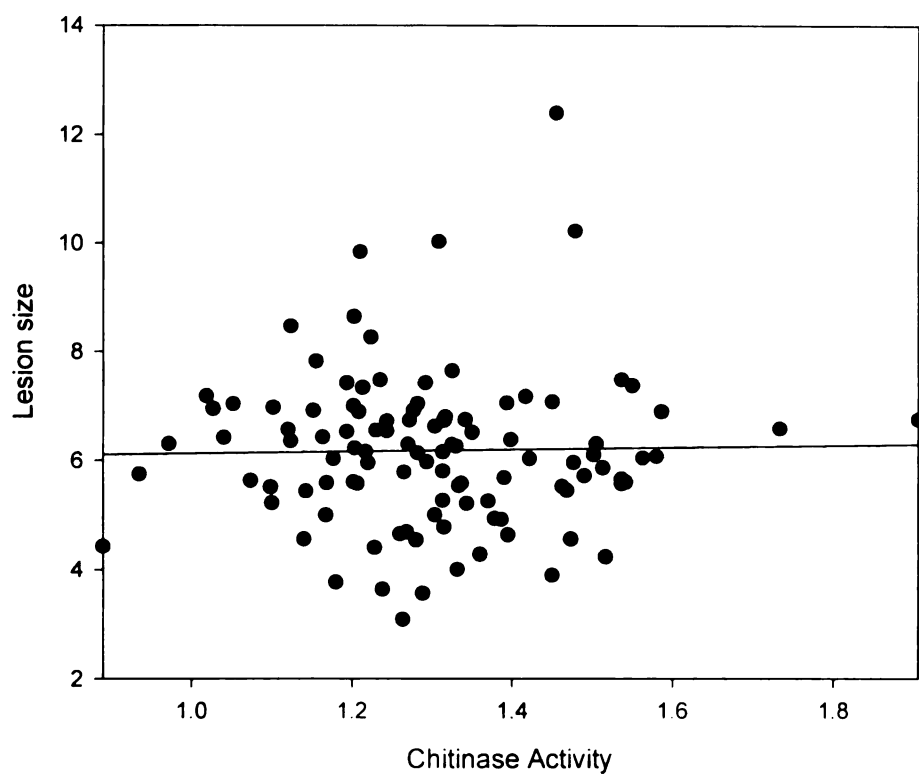


Figure 4.10 Correlation between the Lesion size and the chitinase activity of induced plants in the field. No correlation was found.

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APPENDIX I

THE TRANSFORMATION OF CUCUMBER EXPRESSING SENSE AND
ANTISENSE cDNA SEQUENCE OF ACIDIC CHITINASE TYPE III.

ABSTRACT

The cDNA chitinase sequence of acidic chitinase type III from cucumber was cloned in the *Agrobacterium* vector *pGA643* in the sense and antisense directions. Transgenic cucumber plants (*Cucumis sativus* L.) were produced by inoculating cotyledon explants with *Agrobacterium tumefaciens* bearing a Ti plasmid with NPT II gene for kanamycin resistance. Kanamycin resistant plantlets, (resulting from self-pollination of transgenic plants) were evaluated for their tolerance to *C. orbiculare* under greenhouse conditions. ASM treated transformed plants did not demonstrate significant differences in their ability to withstand pathogen attack. Non-ASM treated plants bearing the antisense construct were more susceptible to pathogen spread within the tissue after initial penetration. Thus it suggests that the levels of acidic chitinase are important for the basal levels of resistance prior to induction. The amount of acidic chitinase activity was suppressed in plants bearing the sense and antisense constructs possibly due to gene silencing.

INTRODUCTION

Induced resistance has been recognized as an inducible defense response mechanism to pathogens since the turn of the century (Chester 1933). Recently, studies have been undertaken to quantify the intensity of the defense response in the induced plant (Ryals et al. 1996). The discovery that proteins, known as Pathogenesis Related (PR) proteins, were associated with the induction of resistance (Gianinazzi et al. 1970; van Loon and van Kammen 1970) suggested that these could be used as markers for SAR. In cucumber, the acidic type III chitinase has been associated with the induction of SAR (Lawton et al. 1994; Narusaka et al. 1999) and has been used extensively as a SAR marker. In addition to serving as a biochemical marker, chitinase has also been hypothesized to have an active role in the defense of plants expressing SAR. Among the reasons behind this line of thought is the fact that acidic chitinase has been found to have antifungal activity *in vitro* (Ji and Kuc 1996). It accumulates in the apoplast (Boller and Metraux 1988), thus placing it in the path of any invading fungi. The time of induction of acidic chitinase also correlates with the onset of resistance in plants expressing SAR (Kastner et al. 1998). Using molecular techniques, the acidic chitinase type III cDNA sequence has been elucidated (Metraux et al. 1989), and its regulation and gene structure has also been reported (Lawton et al. 1994).

The discovery of DNA delivery and transfer by *Agrobacterium tumefaciens* into plants has resulted in the production of several transgenic lines bearing chitinase of various types. For instance, cucumber plants have been transformed to express a rice chitinase and reported to have enhanced resistance to gray mold (*Botritis cinerea*) (Tabei et al. 1998). Raharjo *et al* (Raharjo et al. 1996), has also transformed cucumber with chitinase encoding genes from petunia, tobacco and bean. They report that, in both growth chamber studies using whole plants and *in vitro* inoculations conducted with detached leaves, no differences in disease development (rate and final levels) were detected between transgenic and non-transgenic plants (Punja and Raharjo 1996). These transgenic plants were made to enhance resistance based on the circumstantial evidence that chitinase might play an active role in the defense mechanism of the plant. These findings have provided little evidence concerning the role of chitinase in cucumber plants. Therefore, the circumstantial evidence in which chitinase is an active player in the defense mechanism is still questionable.

In this work using molecular techniques, constructs bearing the antisense and sense strands of the chitinase type III from cucumber regulated by the cauliflower mosaic virus S35 promoter were used to transform cucumbers. The transgenic lines were used to assess the role of chitinase in the defense response against pathogens.

MATERIALS AND METHODS

Plant Material

Cucumber plants cultivar Wisconsin SMR58 were used. The seeds were washed in distilled water and soaked for 30 min. Seed coats were carefully removed, following the removal of the coats; the seeds were surfaced sterilized as described elsewhere (Raharjo et al. 1996). The uncoated seeds were treated with a 10% solution of commercial bleach in distilled water for 10 minutes and rinsed 3 times with distilled water. Following the bleach treatment, the seeds were treated with a solution of 70% ethanol for 30 seconds and washed with distilled water a minimum of three times. The seeds were placed on germination media consisting of 4.4 g MS basal media (Murashige and Skoog 1962) amended with Sucrose 30 g/L, BAP 2 mg/L, ABA 1mg/L and Gelrite 2.5 g/L for 24 hours in the dark. All chemicals used were tissue culture grade obtained from Sigma Inc. (St. Louis MO) or otherwise specified. Explants for co-cultivation were pieces of 1 day old cotyledons of about 1-2 mm².

Vector construction

The complete cDNA chitinase type III sequence was isolated via PCR from cDNA previously prepared by RT-PCR. The primers used to amplify the genomic DNA were synthesized to contain one of two restriction enzyme sites *Xba I* and *Cla I*. Two primer sets were synthesized and after a PCR reaction

the product contained the restriction sites at 3' and at the 5' of the sequence. In order to make the sense construct the primers used were 5'-TAC ATC GAT AAG AAA GCT CTT TAA GCA ATG G-3' and 5'-TAT CTA GAT T GGA GAA GAT GAA GTC TCA CTT T-3'. To make the antisense construct, the primers used were 5'-TAT CTA GAA AGA AAG CTC TTT AAG CAA TGG-3' and 5'-TAC ATC GAT TGG AGA AGA TGA AGT CTC ACT TT-3' (fig 5.1). The boxed partial sequence denotes the restriction sites introduced to the PCR product to allow directional cloning. The primers amplified from position 2 to position 986 of the cDNA sequence (Metraux et al. 1989). Directional cloning of the sense and antisense sequences of the cDNA was done into the *Agrobacterium tumefaciens* plasmid *pGA643* (kindly provided Grumet's group). The antisense and sense sequences were cloned in the plasmid to be constitutively expressed by the S35 promoter of the cauliflower mosaic virus. *Agrobacterium tumefaciens* strain LBA4404 was transformed with the construct and used for explant transformation.

Transformation procedure

The *Agrobacterium* was grown in LB media with 100 mg L⁻¹ of the antibiotic kanamycin. A single bacterial colony was inoculated into 15 ml of liquid medium with 100 mg L⁻¹ of kanamycin and incubated at 29 °C on shaker at 250 rpm for 24 hours. The *Agrobacterium* culture was diluted 100 fold. A small amount of the bacterial suspension was placed in a Petri dish and the partially germinated seedlings were added. The cotyledon pairs were

separate and sliced in the *Agrobacterium* suspension. The edges were cut and discarded and the remaining piece of cotyledon was sliced into 4-6 pieces and co-cultivated in the *Agrobacterium* for 10 min. Following incubation, the explants were blotted onto sterile filter paper and placed on shoot induction media consisting of 4.4 g MS basal media (Murashige and Skoog 1962) amended with Sucrose 30 g/L, BAP 2 mg/L, ABA 1mg/L and Gelrite 2.5 g/L and 0.4 g L⁻¹ of Timetin and cultured in the dark for 3 days. After the proper incubation, the explants were retrieved and rinsed with distilled water and placed in selection media, shoot induction media amended with kanamycin 25 mg L⁻¹. The explants were cultured for seven days at room temperature with amended fluorescent lighting.

When shoots regenerated in the kanamycin amended media, they were excised and transferred to MS media with kanamycin but without growth regulators. Roots developed after 2-3 transfers onto the same medium. The plantlets were transferred to sterile soil and maintained in a growth chamber at 26 °C and 16-h/day photoperiod.

Confirmation of transformation

Total nucleic acids were extracted from putatively transgenic plants and from untransformed plants (negative) controls. A small piece of tissue (100 mg) was frozen in liquid nitrogen and ground using a mortar and pestle in 1 ml eppendorf tubes. The resulting powdered tissue was suspended in a mixture of 200 µl TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and 200 µl

saturated phenol solution and vortexed vigorously for 1 min. Following centrifugation, (14,000 g, 5 min room temperature), the isolated DNA was purified by ethanol precipitation. Precipitated DNA was dissolved in 50 µl of sterile distilled water, and 1 µl was used for PCR reaction.

Two specific primer sequences for the *NPTII* (kanamycin resistance gene) coding region were synthesized, forward primer 5'-TCG GGA GCG GCG ATA CCG-3' and reverse primer 5'-GGT TCT CCG GCC GCT TGG-3'. The isolated DNA in combination with the primers were used to set up a PCR reaction (25 µl) consisting of 1x Taq buffer (MgCl₂-free), 1 mM MgCl₂, 200 uM of dNTP's 0.5 uM of each oligo-nucleotide primer, 1 µl of the isolated genomic DNA and 2 units of Taq polymerase (Invitrogen Inc. Carlsbad CA). The volume was brought up to 25 µl with distilled water. PCR reactions was carried out in a thermocycler (Perkin Elmer, Boston MA) under the following conditions: the reaction was incubated at 94 °C for 6 minutes followed by 25 cycles of 94 °C for 45 sec, 62 °C for 1 min and 72 °C for 2 min. The last cycle was the same with the exception that the DNA extension at 72 °C was carried out for 10 min. The PCR products were analyzed by electrophoresis on a 1.0% agarose gels and detected using ethidium bromide staining.

Detection of resistance against Colletotrichum orbiculare in transgenic cucumber plants.

Leaves from transformed and control seedlings (untransformed plants) were collected and brought into the lab. They were cut along the midrib and

inoculated with 10 droplets of 10 μ l of a spore suspension of *C. orbiculare* (10^6 spores/ml) in water. The half leaves were placed in sterile Petri dishes underlined with moist filter paper. Seven days after challenge, the number of lesions allowed per half leaf was counted and the lesion size diameter (mm) measured.

Chitinase detection

Chitinase activity was determined as follows. A 1% solution of agarose in potassium phosphate buffer 10 mM (pH 5.0) was prepared with a 1% (v/v) of glycol chitin, the chitinase substrate (Trudel and Asselin 1989). Thirty ml of agarose solution were poured in a 15 cm Petri dish. After solidification, 3 mm diameter plugs were carved using a small cork borer in the agarose. Five μ l of the total protein extract were placed in the wells (3 wells per sample) and incubated at 37 °C for 4 hours. Following incubation, the reaction plate was rinsed and flooded for 10 min in 0.5 M solution of Tris-HCl (pH 8.9) with 0.01% (w/v) of calcoflour brightener 28 in order to stop the reaction and stain the plate. When viewed with UV transillumination, the areas of activity appeared as a well-defined dark area on a fluorescent background. The diameter of the circular area of activity was measured and used for data analysis. The acidic chitinase from intra cellular fluids was detected by using a modified method previously reported (Trudel and Asselin 1989). The substrate, glycol chitin was added to a non-denatured polyacrilamide gel electrophoresis instead of using an overlay gel as the authors suggested.

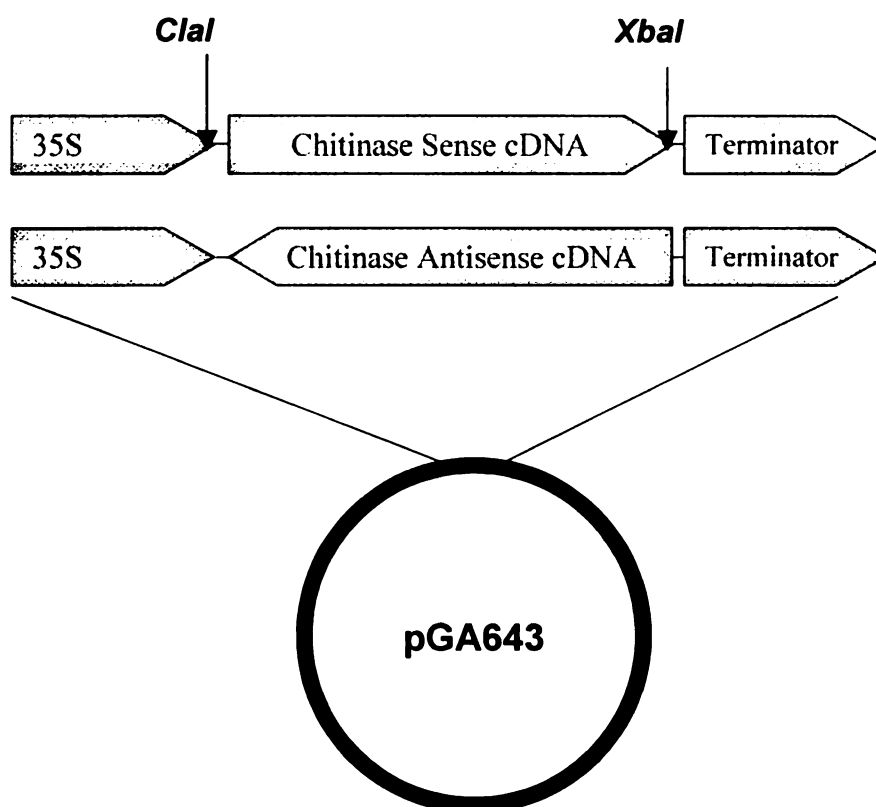


Figure 5.1: Vector construction for transformation of cucumber plants. The chitinase cDNA sequence was amplified using two sets of primers that introduced the *Clal* and *Xba I* restriction sites. The plasmid *pGA643* was cut with the same enzymes that allowed directional cloning to produce the sense and the antisense constructs.

RESULTS

Development of kanamycin resistant plantlets.

Multiple shoots were induced from both explants infected by *Agrobacterium* and control explants (uninfected with *Agrobacterium*) in basal MS media without kanamycin. When the shoots were exposed to kanamycin (25 $\mu\text{g ml}^{-1}$) amended media and Timentin (400 $\mu\text{g ml}^{-1}$), untransformed shoots exhibited slow growth and eventually were bleached. Shoots that survived this selection were subcultured to a fresh medium of the same composition where they further developed (Fig 5.2). These kanamycin-resistant shoots were assumed to have neomycin phosphotransferase (NPT II). The integration of the NPT gene was confirmed via PCR (fig 5.3). All kanamycin positive plantlets from PCR were acclimatized in growth chamber and transferred to soil and placed in the greenhouse to be selfed. After acclimatization and growth under greenhouse conditions, there was no difference between transgenic and non-transgenic cucumber plants from viewpoints of growth and morphological characters. The plants were selfed and seeds obtained and used for resistance experiments.

Resistance to Colletotrichum orbiculare.

Sense and antisense seedlings (T1 generation) were screened for the NPTII gene, which confirmed the insertion of the transformation construct. Transformed seedlings were transplanted into sterile soil and grown under

greenhouse conditions. The cucumber plants were induced to express SAR when the plants' first true leaf (leaf 1) was fully expanded and the leaf above (leaf 2) one-third to one-half expanded. The induction was achieved by spraying the plants with the benzothiadiazole derivative, CGA-245704 (ASM) (Friedrich et al. 1996) at a rate of 20 ppm of active ingredient in water. Leaves were harvested and challenged with a spore suspension of *C. orbiculare* seven days after induction. Statistical analysis of the data collected revealed that there were no significant changes in terms of lesion size or number between the ASM treated sense, antisense and control plants. On the other hand, when non ASM treated plants (controls, sense and antisense) were compared, the data obtained shows significant differences (fig 5.4). Statistical analysis shows that the number of lesions did not vary between the transgenic and control plants. However, the development of the fungi, determined by the lesion size, varied between constructs. Transgenic plants expressing the antisense construct allowed a larger lesion size when compared with the sense and the control plants. Interestingly, the control plants and the plants expressing the sense construct were not significantly different in terms of lesion development.

The levels of chitinase activity in transgenic plants.

The levels of the acidic type III chitinase activity in transgenic plants were monitored by gel electrophoresis with protein samples extracted from intracellular fluids. The levels of acidic chitinase activity were similar in sense

and antisense plants, but the levels were higher in control plants (Fig 5.5). Additionally, the levels of total chitinase activity measured by the agarose gel diffusion activity assay presented no differences at any level between the transgenic plants and the control plants.

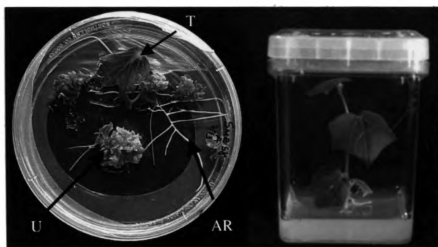


Figure 5.2: Transgenic shoots and transgenic plants regenerated from cotyledon explants. Left picture, Petri dish containing regenerated shoots in kanamycin amended media. T: kanamycin resistant shoot, AR: adventitious root, U: untransformed, non kanamycin resistant shoot. Right picture, kanamycin resistant shoots in elongation media.

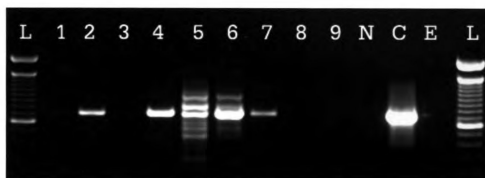


Figure 5.3: PCR analysis of regenerated shoots after *Agrobacterium* co-cultivation. Lanes from 1-9 DNA samples from different adventitious shoots, L: DNA ladder, N: non transformed plant, C: vector containing the kanamycin resistant gene, E is negative control, no template. Plants 2, 4 and 7 were kept and used further.

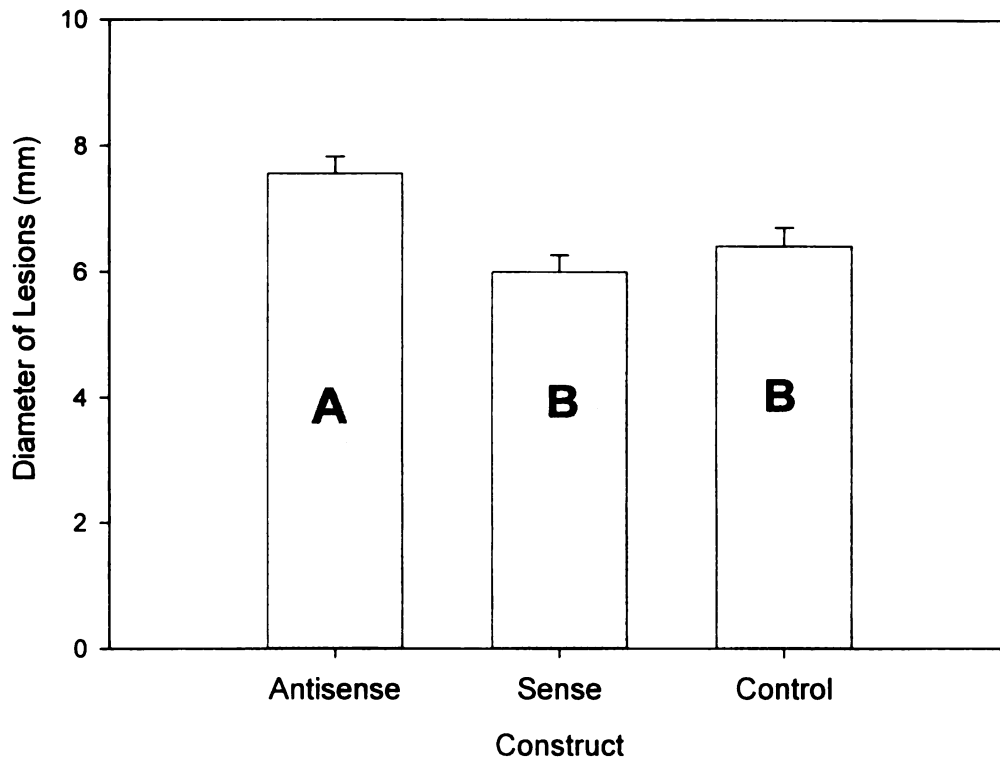


Figure 5.4: Resistance to *Colletotrichum orbiculare* of non ASM treated transgenic plants bearing the sense and the antisense construct of chitinase type III under the control of the S35 promoter. Three groups of at least 15 plants for each treatment were used data analysis. Control plants are non-transformed plants. The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P = <0.001$).

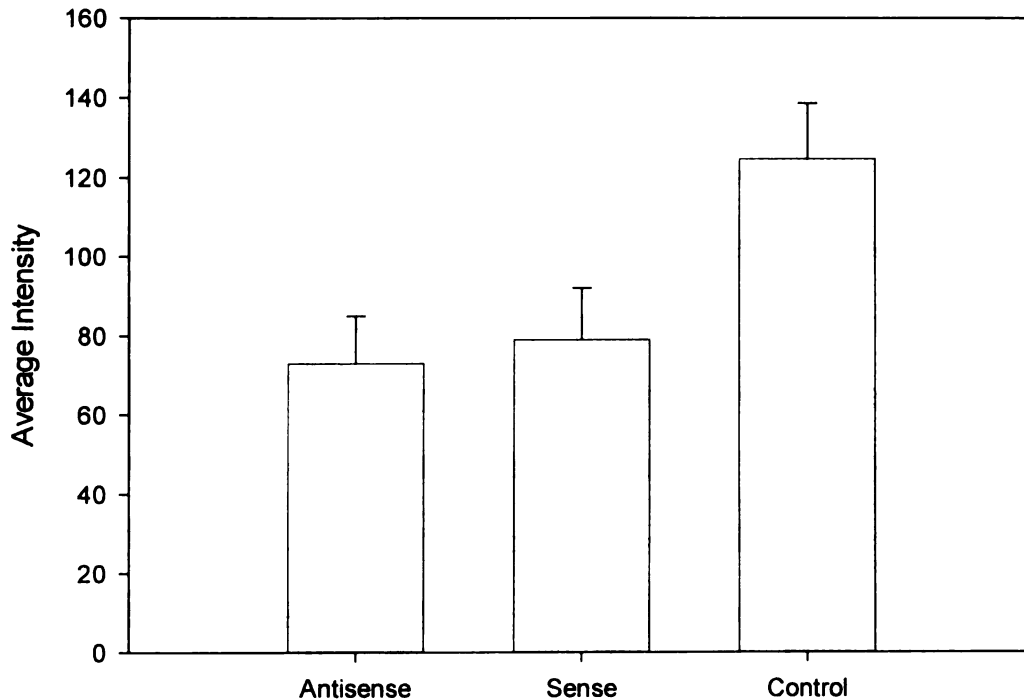


Figure 5.5: Acidic chitinase of transgenic and control plants. Three different extractions were made from intracellular fluids of transgenic seedlings. Each extraction was loaded the combination of at least 5 transgenic seedlings. 30 μ g of total protein was loaded in an acrylamide gel electrophoresis in non-denatured conditions. The chitinase was detected using the method of Trudel and Asselin. The bands generated were calculated using a software package (Quantity one, Bio Rad Inc). The average intensity of the bands was calculated. No significant changes were observed between the transgenic plants, but they did had less activity as compared to the control plants.

DISCUSSION

This work demonstrates the introduction of the cucumber chitinase type III cDNA sequence was introduced in the sense and antisense directions in cucumber. Our transformation efficiency was not higher than 5% other reports of transformation of cucumber plants have also been successful, with a low transformation efficiency (Broglie and Broglie 1993; Raharjo et al. 1996; Tabei et al. 1998). In the regeneration system employed in this experiment, one-day-old cotyledons used as explants proved novel and efficient as opposed to current cucumber transformation methods. This method, recently developed (Tabei et al. 1998), and adapted by the Grumet (personal communication) will contribute to the more efficient production of transgenic cucumbers.

When treating the transgenic plants with ASM, the levels of expression of resistance in plants (sense, antisense and control plants) were to the same degree. In other words, there were no statistically measurable differences in terms of lesion development or number of lesions allowed. In the systemic acquired resistance phenotype, the levels of chitinase activity rises with the process of induction and with increased levels of resistance. The observations shown here demonstrate that the levels of lesion development and lesion size are not affected by the construct present in the plants. This is rather interesting since it has been suggested that the levels of resistance due to SAR are tightly correlated to the increase of the PR proteins in particular, chitinase and β -glucanase. The fact that the level of resistance

was unchanged in the transgenic plants suggests that chitinase suppression or overexpression does not affect the normal levels of resistance in control plants after SAR induction. This fact, contests the argument that chitinase is an active player in the defense of plants due to SAR.

An interesting finding is the basal levels of resistance in transgenic and control plants. The non-induced plants expressing the antisense construct allowed for a larger lesion size when compared to controls and plants expressing the sense construct (fig 5.4). No differences in resistance were found between the control plants and the plants expressing the sense construct. Interestingly, no effect in disease development caused by *Colletotrichum orbiculare* was found in transgenic cucumbers expressing petunia and tobacco chitinases (Punja and Raharjo 1996). However the chitinase expressed in this experiment was of basic nature. There is only one report of transgenic plants expressing an acidic chitinase from *Lycopersicum chilense* (Tabaeizadeh et al. 1999). The data obtained from transformed plants (tomato) in this report suggested that the levels of resistance in transgenic plants demonstrated improved resistance to *Verticillium dahliae* race 2. However, these experiments only suggest that acidic chitinase could potentially be able to increase the level of resistance in tomato, in cucumber however it could be different. Additionally antisense experiments with β -glucanase, another PR protein, did not exhibit increased susceptibility to *C. nicotianae* infection. These results suggest that expression of the beta-1,3-glucanase isoform blocked by antisense transformation is not necessary for

'housekeeping' functions of *N. sylvestris* nor defense against the fungal pathogen tested. Chitinase, and glucanase, have been topics of heated debate over their role in the plant. Even with the antisense insertion of glucanase, it failed to become "less resistant" than control plants. The plants did not exhibit increased susceptibility to *Cercospora nicotianae* infection (Beffa and Meins 1996). These results suggest that expression of the beta-1,3-glucanase isoform blocked by antisense transformation is not necessary for 'housekeeping' functions of *N. sylvestris* nor defense against the fungal pathogen tested.

The fact that only in non-induced plants, the antisense construct was able to make a significant reduction in the levels of disease by allowing the fungi to spread further into the tissue suggest that constitutive levels of chitinase could potentially be of importance for housekeeping of the resistance functions in cucumber. This suggests that the constitutive levels of chitinase are important for the basal resistance in plants, and the over expression of the gene does not enhance the ability of the plants to withstand pathogen attack.

The difference in the level of total chitinase activity among the transgenic and control plants was not significant. However, the acidic type III chitinase expression varied between constructs. Interestingly, the levels of acidic chitinase expressed in the transgenic plants were lower than in control plants. Experiments have shown that transgenes introduced into plants can interact with homologous host genes resulting in the inactivation of both genes

(Jorgensen 1991; Matzke and Matzke 1995). A phenomenon called co-suppression or plant gene silencing. As reported, some plants transformed with chitinase genes failed to overexpress (Tabaeizadeh et al. 1999), and in some cases it reduces the total chitinase expression (Neuhaus et al. 1991). In this study, the lack of over expression of chitinase activity in plants expressing the sense construct might explain the fact that no detectable differences were found in the resistance against *C. orbiculare*. On the other hand, despite the fact that no significant differences were obtained in acidic chitinase expression between the sense and the antisense transgenes, the levels of non-transgenic plants were considerably higher. The transgenic cucumber plants expressing the antisense construct, were successful at reducing the translation of the chitinase transcripts. This fact, could be responsible for the increase of susceptibility to the spread of the fungus within the tissue.

The most widely used approach to enhance resistance in plants against pathogens, has been the overexpression of hydrolytic enzymes, mainly glucanases and chitinases which belong to the group of the PR proteins (Neuhaus 1999; Punja 2001). Following expression of several chitinases in a range of transgenic plant species, has proven that the levels of chitinase induction does not necessarily is translated to an enhanced resistance. This might indicate that differences exist in sensitivity of fungi to chitinase. But most importantly, the levels of induction in most experiments do not correlate with the levels of resistance acquired. These results

questions the effort and the potential to express hydrolytic enzymes in plants to enhance resistance. Additionally, it has been also suggested that pathogen-inducible PR proteins do not play a major role in the resistance (Van Loon 1997). Thus the idea of generating transgenic plants with PR proteins to enhance the resistance against pathogens could be challenged.

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APPENDIX II
ISOLATION OF A cDNA FRAGMENT OF THE NPR1 GENE FROM
CUCUMBER PLANTS

ABSTRACT

Systemic Acquired Resistance and Induced Systemic Resistance are regulated by NPR1 gene. The present work reports the isolation and partial sequence of a cDNA fragment of the NPR1 of cucumber plants. cDNA was prepared via reverse transcription. Degenerate primers were designed from the alignment of NPR1 cDNA sequences from rice, *Arabidopsis* and tobacco. The PCR product obtained was cloned and sequenced, results show that the NPR1 cDNA partial sequence from cucumber is highly homologous to the reported *Arabidopsis* sequence. Its translated sequence also shows ankyrin repeats a hallmark of the NPR1 genes reported in the literature.

INTRODUCTION

Plants are in constant contact with pathogens. Thus, they have evolved complex mechanisms of defense. These include, among others, a mechanism that is activated by exposure to pathogens. Systemic Acquired Resistance (SAR) is a state of enhanced resistance to pathogens, and it is correlated to the deployment of a battery of several Pathogenesis Related (PR) proteins (Van Loon 1997; Metraux 2001). From the whole battery of genes deployed during the SAR response, there has not been one conclusive report of showing a single gene to be essential for SAR. This suggests that the coordination of deployment of these genes might be more important than a single gene deployed.

Regulation of the Systemic Acquired Resistance has been shown to be dependant on the NPR1 gene. The *Arabidopsis* NPR1 gene controls the onset of systemic acquired resistance (Cao et al. 1994). Using mutagenesis, a mutant, *npr1* (nonexpressor of PR genes), was discovered to be non responsive the SA application and other SAR-inducing treatments (Cao et al. 1994). Further characterization showed that the mutant displays little expression of pathogenesis-related (PR) genes and exhibiting increased susceptibility to infection (van Loon et al. 1998). Characterization of the NPR1 gene has shown that it has ankyrin repeats (Cao et al. 1997), suggesting that these repeats are important for NPR1 function. Other key experiments with the NPR1 gene include the transformation of the cloned wild-type NPR1 gene into *npr1* mutants. The transgenic gene

complementation not only restored the mutation but also rendered the transgenic plants more resistant to infection by *P. syringae* in the absence of SAR induction (Cao et al. 1997; Cao et al. 1998). Another complementation experiment also showed similar results in addition to reporting that overexpression of NPR1 gene prones the plants to be more responsive to SA or a SA-dependent signal (Friedrich et al. 2001).

The NPR1 has also been shown to regulate Induced Systemic Resistance (ISR). This resistance pathway relies on the activation of the Jasmonic acid pathway (van Loon et al. 1998). Because rhizobacteria-mediated ISR was found to be independent of SA and not associated with PRs (Pieterse et al. 1998), it was expected that ISR would still be expressed in this mutant. However, the *npr1* mutant of *Arabidopsis* did not express ISR mediated by the rhizobacterium WCS417 (Pieterse et al. 1998). This implies that NPR1 functions beyond the expression of PR-genes and is required for both pathogen and rhizobacteria-mediated systemic induced resistance. This can possibly explain why SAR and ISR are phenotypically similar. In both, the defensive capacity of the plant against a broad spectrum of pathogens is enhanced after an initial induction. Yet, PRs are induced concomitant with SAR expression, whereas activation of PR genes is not part of the pathway leading to ISR in *Arabidopsis*. Apparently, NPR1 differentially regulates defense responses mediated by different signaling pathways. Despite the source of induction, the *NPR1* gene seems to potentiate the defense mechanisms typical of SAR and ISR (van Loon et al. 1998).

Cucumber plants have been used extensively for the study of SAR, and their importance as model has been increasingly noted. The SAR mechanism was initially characterized using cucumber plants, and to our knowledge no NPR1 sequence has been isolated from cucumber. This work describes the isolation of the NPR1 cDNA sequence from cucumber.

MATERIALS AND METHODS

Cucumber cDNA:

Cucumber plants var. Wisconsin SMR 58 were grown in a growth chamber. The cucumber plants were induced to express SAR with the benzothiadiazole derivative, acibenzolar-S-methyl (CGA-245704, ASM) (Friedrich et al. 1996) at a rate of 20 ppm of active ingredient in water. Total RNA was isolated from leaves as previously described. mRNA was isolated using mRNA isolation kit using paramagnetic beads (Promega Inc). cDNA was made from mRNA using reverse transcriptase kit (Roche Inc). The cDNA obtained was used for downstream applications.

Primer selection:

Alignment of the published amino acid sequences in the database of NPR1 was performed (fig 6.1). Degenerate primers were designed from the conserved regions of the amino acid alignment of the sequences of *Arabidopsis*, rice and tobacco NPR1. The primer combination AAR GCI YTN GAY WSN GAY GAY (forward), and NGC NAR NBC NAC NCK RTT YTC (reverse) was the one primer pair that amplified a distinct band from cucumber cDNA. The primer combination corresponds to the amino acid sequences KALDSDD and ENRVALA which are present in the three NPR1 amino acid sequences from the species above mentioned.

PCR optimization:

The optimization of the PCR reaction was performed in a Perkin Elmer Thermocycler machine. The successful amplification of the NPR1 fragment of cucumber cDNA was done using the following parameters. The PCR reaction (25 μ l) consisting of 1x Taq buffer (MgCl_2 -free), 1 mM MgCl_2 , 200 μ M of dNTP's 0.5 μ M of each oligo-nucleotide primer, 1 μ l of a 1:100 dilution of the cDNA made and 2 units of High fidelity Taq polymerase (Invitrogen Inc. Carlsbad CA). The volume was brought up to 25 μ l with distilled water. PCR reactions were carried out in a thermocycler (Perkin Elmer Inc.) under the following conditions: The reaction was incubated at 94 °C for 6 minutes followed by 30 cycles of 94 °C for 45 sec, 55 °C for 1 min and 72 °C for 2 min. After each cycle the annealing temperature was lowered by 0.5 °C. The last cycle was the same with the exception that the DNA extension at 72 °C was carried out for 10 min. The PCR products were analyzed by electrophoresis on a 1.0% agarose gels and detected using ethidium bromide staining.

Cloning of the PCR product:

After optimization of the PCR reaction, a product was obtained (fig 6.2). The bands were extracted from the gel using a PCR cleaning kit (Promega Inc), the end product was cloned into a plasmid (Topo II) which allows the cloning of PCR products with an adenine overhang (Invitrogen Inc). The ligation reaction was done according to the manufacturers recommendations. The resulting ligation product was used for the

transformation of *E. coli* competent cells as previously described (Sambrook et al. 1998). The cells were plated on a kanamycin amended media (50 µg/ml) and incubated at 37 °C overnight. The transformed cells formed white colonies due to the disruption of the *lacZ* gene by the insert. Plasmid DNA was obtained quantified and sequenced.

Arabidopsis	1	DTTIDGFADSYETSTSFVATDNTSSIVYLAAEQVLTGPDVSAIQLNSFESMDS
Tobacco	1	NSRTA.S..ND..SS.SICCIGGGMTEFFSPET---SFAEITS.KR..ETL..I..AS
Rice	1	EPPTSHVT--NAFSDSDSASVEEGG-----D---ADA..E..RR..DNLAAA.R..
Arabidopsis	61	D---DFYSAAKLVLSD---G-REVSF...VLAFFSEFKSALAAAKKEK--DSNNITAVK
Tobacco	58	LPEF.YFA....V.G...PCK.IPV...I.....P...NLFC-----..CKKEKNSS.
Rice	49	E.DFA.LA...RIAVPCGGG.GGDLLV.....P.LRGVE.RRAAAAAGGGGEGGER
Arabidopsis	112	LAKITAK---DY...GFDSVVT...AVVSSRRVRPP...GVSEAAENEC...ACRFANDM
Tobacco	107	V...VM....EH...SY.A.MS...L..GK...S...D.CV.V.ND.S.....A.L
Rice	108	...R.LLGGGGEEV...YEALRL..D.L..G..GDLAACL.V...DA..G.H...A..
Arabidopsis	168	LE...YLAFFIFRIP...VTLY...PHELVV...VVIETLVI...KLANICGFACMLIDRKEII
Tobacco	163	V...TS.T.Q.S...DNF.....IL...TAAD.VMMV.SV.....ER..SS.I...
Rice	168	AC...EA..ST.QVA...TN.F...R...L...EVDNL.L...SV...L.N.S...E...LDMV
Arabidopsis	228	KK...V...MVSLEPSESELVFEIIRK...EVKVK---...VNSNMHKA...DDDDIEVVK
Tobacco	223	...IIT.D.A...HDI...Q.T.S.A...QG.ESNGFPD...KRI.R.....V..LQ
Rice	228	R...L..IT.....PQV...Q...ALS...IS..EN.GFPN...RRI.R.....V...R
Arabidopsis	284	LAKEDHINIFACRHFAYAYNMVATDTLKDIAVNRHPRPGYTVLHVAMPKFNQ
Tobacco	283	M...R.G..T...Y...Y...DA...TAED.D.A...I..Q.S.....K
Rice	288	M...T.GC...F...Y...FH.DS...IT.E...D.A.....I..R.R..K
Arabidopsis	344	LILNLEFGASASEALLEGRTALMAFQA...MAVECNIP...QCHSIFGRDVEILEDK
Tobacco	343	IVV...T...RP.DLSD...K..Q...RLRL.DFSKS...EG.SASND...I.....AER
Rice	348	I.V...T...READVAFD...K.VQ.S.RL.KQGDYFGVT...EG.P.P.D...I.....AER
Arabidopsis	404	EEIPROVFP...FAVDADEKMTL...DLENHVA...P...P...A...E...M...G...C...E...F...I...M...T...
Tobacco	403	D.LLGEASV.L.M.G.D.R.K...Y.....G...KL...M...KV...D...QVD...S...PLA...
Rice	408	D.LLGEASV.L.M.GES...RGR...Y.....RIM...M...RV...D...QVD...L...NLG...
Arabidopsis	464	LEPDLTGTR...SFGVKIAP...RILEPHOSFLK...K...K...TVELGHRFFP...CSAUL...EQ...M...N...C...E...
Tobacco	463	IG-KKMANAQ...IVDLNE...K.K...LNM...R...R.....E...NK...DAD...
Rice	468	GA-NPPPERQ...IVDLNES...IMK...LA...MT.....N...K...D...
Arabidopsis	524	LTQLACGED...AEKRLQ...KQYMEI...E...K...AFSEENL...ELGNSSLTD...T...ST...K...T...G...K...R...
Tobacco	522	.SEI.YMGN...E.QL...L...I...T...T...KE...YDKTNN-I...S...CS...T...K...V...D...K...
Rice	525	E.DPVSIGR...SAE---RK.FHDL...DV...Q...H...KE...NDR.G--L...S...S...T...I...AI...
Arabidopsis	584	SNRKFSHRRR 593
Tobacco	581	P.KLPFRK-- 588
Rice	580	PR----- 582

Figure 6.1: Alignment of Amino acid sequences of the NPR1 gene. Arrows indicate the position of the degenerate primers used in this work. The sequences used were accession numbers AAM65726, AAM62410, BAB12719.

RESULTS

After PCR optimization a faint, yet well-defined band was observed in the PCR reaction (fig 6.2). This band was gel purified and cloned into the *Topo 2* plasmid (Invitrogen Inc). A series of clones that contained the right size fragment were sent for sequencing. The obtained sequences were blasted for homology to the published database. A group of clones was observed to have very strong homology to the *Arabidopsis* NPR1 amino acid sequence (fig 6.3) and when comparing the nucleic acid sequence the homology was even higher (fig 6.4)

A BLAST search of gene bank showed that the NPR1 gene cDNA sequence from cucumber also encodes a sequence which highly homologous to ankryn (fig 6.5) The ankryn repeat concesus has been identified in diverse group or proteins involved in cell structure transcription and regulation, cell differentiation and enzymatic and toxic activities (Cao et al. 1997) In short, the amino acid sequence deduced from the partial cDNA sequence from cucumber shares a very high similarity with the NPR1 cDNA sequence from *Arabidopsis*.

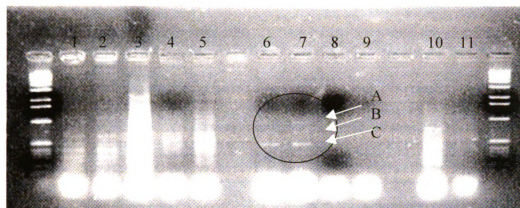


Figure 6.2. Agarose gel electrophoresis of a PCR product using degenerate primers to amplify the NPR1 gene of cucumber. Three distinct bands (A, B and C) were purified from the gel and cloned. Band A encoded the NPR1 partial sequence of cucumber. Lanes (1-11) represent the PCR product of several PCR reactions under different parameters. Bands 6 and 7 showed distinct bands that could be isolated and cloned.

Arabidopsis	MDTTIDGFADSYEISSTSFVATDNTDSSIVYLAAEQVLTGPDVSALQLLSNSFESVFDSP	60
Cucumber	-----	1
Arabidopsis	DDFYSDAKLVLSDGREVSFHRCVLSARSSFFKSALAAKKEKDSNNTAAVKLELKEIAKD	120
Cucumber	-----	1
Arabidopsis	YEVGFDSVVTVLAYVYSSRVPPPKGVSECADENCCHVACRPAVDFMLEVLYLAFIFRIP	180
Cucumber	-----	1
Arabidopsis	ELVTLYQRHLLDVVDKVVIEDTLVILKLANICGKACMKLLDRCKEIIVKSNDVMVSLEKS	240
Cucumber	-----	1
Arabidopsis	LPEELVKEIIDRRKELGLEVPKVKKHVSNVH	300
Cucumber	-----	29
Arabidopsis	-----	360
Cucumber	-----	89
Arabidopsis	-----	420
Cucumber	-----	149
Arabidopsis	-----	480
Cucumber	-----	193
Arabidopsis	-----	538
Cucumber	-----	252
Arabidopsis	-----	593
Cucumber	-----	308

Figure 6.3: Clustal alignment of the Cucumber NPR1 translated sequence and the *Arabidopsis* NPR1 sequence (Accession # AAM65726)


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NPR1Cucumber: 154 NHRNPRGYTVLHVAAMRKEPQLILSLLEKGASASEATLVGRTALMIAKQ 300
      N +  GYT LH AA +  +I LL+ AS +E T+ G TAL IA++
ANK3:          740 NAKTKNGYTALHQAQQGHGTHIINVLLQNNASPNELTVNGNTALAIARR 788

NPR1Cucumber: 16  DDIELVKLLK-----EDHTNLDDACALHFAVAYCNXXXXXXXXXXXXXXXXXNHRNPRGYT 180
      D +  V+LLL+      +D TN D ALH A A+C      N +  G+T
ANK3:          328 DHLNCVQLLLQHNVPVDDVTN-DYLTALHVA-AHCGHYKVAKVLLDKKASPNAKALNGFT 385

NPR1Cucumber: 181 VLHVAAMRKEPQLILSLLEKGASASEATLVGRTALMIA 294
      LH+A + +++ LL+ GAS T G T + +A
ANK3:          386 PLHIACKKNRIRVMELLLKHGASIQAVTESGLTPIHVA 423

NPR1Cucumber: 172 GYTVLHVAAMRKEPQLILSLLEFGASASEATLVGRTALMIAKQ 300
      GYT LH+AA + + + SLLE GA A+ T G ++ +A Q
ANK3:          614 GYTPLHIAAKKNQMDIATSLLEYGADANAVTRQGIASVHLAAQ 656

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Figure 6.5: Sequence similarities of NPR1 amino acid sequence with mouse ankyrin 3 (ANK3). Three regions produced the highest scoring pairs. The sequence similarities are 42% 32% and 48%. The Identical and similar (+) amino acids are highlighted in bold letters.

DISCUSSION

In the search for novel methods of disease control, Systemic Acquired Resistance (SAR) is a potential candidate. However, the search of genes that would serve as markers and as targets for disease resistance improvement has been difficult. The NPR1 is important because it regulates the deployment of the battery of SAR and ISR genes. In *Arabidopsis*, the NPR1 has been characterized and has served to find other genes in relationship to SAR. For example, The *npr1 Arabidopsis* mutant could be rescued by another mutation, *sn1* (Li et al. 1999). The *npr1-sni1* double mutant was then able to induce PR expression when treated with INA as inducer (Li et al. 1999). It has been proposed that the NPR1 gene is a positive regulator of SAR, which is repressed in wild type plants by SNI1 gene. It has been suggested that the role of NPR1 is to remove the SNI1 repression (Li et al. 1999). If this is correct and the fact that the NPR1 basal levels remain the same in induced and control plants, these levels might be an clue for the level of SAR protection among cultivars. However, the fact that the *npr1-sni1* mutant is able to respond to exogenous applications of SA and INA, it also opens the possibility of a pathway independent of NPR1 (Li et al. 1999). Nevertheless, the study of the NPR1 basal levels and its correlation with SAR expression ability is worthwhile aiming to provide a marker for SAR potentiation since SAR levels positively correlate with pathogen resistance. This hypothesis is supported by the overexpression of the NIM1 (NPR1) gene in *Arabidopsis*. Transgenic lines expressing the NPR1 gene exhibited

heightened responsiveness to SAR-inducing compounds. Activation of SAR in transgenic plants was detected when treated with SA concentrations that would not activate SAR in wild plants (Friedrich et al. 2001) . In addition, NPR1 has also been found to differentially interact with members of the TGA/OBF family which is implicated in the activation of SA-responsive genes, including PR-1 (Zhou et al. 2000). Increased levels of PR-1 activation were found in *Arabidopsis* plants overexpressing the NPR1 gene (Friedrich et al. 2001). In addition, *npr1* mutants presented an abnormal callose response and hypersusceptibility to *Peronospora parasitica* thus compromising resistance (Donofrio and Delaney 2001). The phenotype was rescued by exogenous applications of SA, but it seems dependant on the NPR1 pathway. It seems then, that the NPR1 gene does play an important role in the expression and strength of the SAR response.

PR protein basal expression was found to be correlated to non-specific resistance ins *Solanum* species to *Phytophthora infestans* and it has been suggested that these levels might be used as biochemical markers for germplasm selection for resistance (Vleeshouwers et al. 2000). This finding not only provides partial evidence of the importance of measuring basal levels of enzyme activity, but also encourages further studies at the level of mRNA via RT-PCR and northern analysis.

In short, the cucumber NPR1 partial sequence isolated in this work is almost identical to the cDNA NPR1 sequence of *Arabidopsis*. Because NPR1 overexpression can result in reduced pathogen growth on the plants with the

use of less fungicide (Friedrich et al. 2001), not only will reduce increase pathogen resistance to pesticides by exposing the pathogen to less of the active ingredient. Thus the screening for plants with higher NPR1 expression might be a strategy to look for the “magic” biochemical marker for SAR.

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FUTURE DIRECTIONS

It is well established that a large number of genes are expressed in plants upon induction of Systemic Acquired Resistance (SAR). Some of those genes encode proteins for which a function has been hypothesized based on correlational and *in vitro* studies. The enhanced expression of acidic chitinase in cucumber plants expressing SAR is one of these genes. However, mounting evidence has challenged the hypothesis of a direct role of chitinase against the invading pathogen.

The overall results of the present study suggests that the role of the pathogenesis related (PR) protein does not directly affect the outcome of the resistance conferred by SAR in cucumber plants. Direct evidence shows that the presence of high levels of acidic chitinase and other PR proteins in the tissue do not hamper the growth of the invading pathogen. Additionally, no correlation was found between the levels of resistance by SAR and levels of expression of acidic chitinase in the SAR expressing plants. Many comparisons were made between the levels of chitinase between SAR-expressing plants and control plants. Although, the data were statistically significant, their biological significance is questionable. On the other hand, chitin oligomers have been shown to elicit lignification and other defense responses in plants, (Roby et al. 1987; Barber et al. 1989; Barber and Ride 1994). Hence, that does not exclude the possibility that acidic chitinase in SAR expressing plant might release elicitors from the invading pathogen that trigger defenses (Hammerschmidt 1999). In order to more fully understand the role of chitinase or other PR proteins, the study of

genetically modified plants engineered to silence or under express chitinase or other PR proteins may help to understand their role in resistance. In this work, preliminary experiments have been performed, and transgenic lines under expressing chitinase have been generated. The further study of these transgenic plants is needed in order to enhance the understanding of the role of acidic chitinase in SAR expressing plants.

Evidence is provided to support the idea that the state of resistance by SAR is protein synthesis dependant. Blocking protein synthesis in SAR expressing cucumber plants leads to the breakdown of the resistance accompanied by a higher rate of fungal growth within the tissue. Additionally, peroxidase (POX) activity and H₂O₂ production are hindered. The link between the decrease of POX activity, the low H₂O₂ production and the breakdown of resistance is unclear thus the investigation of such relationship is important.

In greenhouse and field experiments, a differential expression of SAR against pathogens with different mode of entry was found. Some cucumber cultigens expressing SAR are resistant to *Colletotrichum orbiculare* but not to *Dydimella bryoniae*. On the other hand, several other cultigens expressing SAR are resistant to both pathogens. The morphological, biochemical or genetic differences that make this differential response to SAR induction could potentially lead to the discovery of new resistant genes or defense mechanisms/interactions. For instance, because many plant species produce polygaractuonase-inhibiting proteins (pgip) (De Lorenzo et al. 2001), the possible role of these proteins in cucumber plant defense against *Dydimella bryoniae* is

worth investigating. Interestingly, transcripts of *pgip*'s accumulate in *P. vulgaris* hypocotyls in response to wounding or treatment with salicylic acid (Bergmann et al. 1994). It is possible to hypothesize that some cultigens might express the *pgip*'s in response to SAR induction, making the cultigens resistant to *Dydimella bryoniae* in addition to *C. orbiculare*.

The access to collections of cucumber cultigens and crossing lines, in addition to this differential expression of resistance is a goldmine yet to be explored. Crosses between cultigens that express this differential resistance and the generation of molecular markers to track and pinpoint the source of this response can provide more information of how plants expressing SAR restrict differentially pathogen development.

The search for genes that would serve as markers and as targets for disease resistance improvement has been difficult. The NPR1 gene is important because it regulates the deployment of the battery of SAR and ISR genes. In this work a fragment of the NPR1 gene has been isolated, which is almost identical to the cDNA NPR1 sequence of Arabidopsis. NPR1 over expression results in reduced pathogen growth (Cao et al. 1994; Friedrich et al. 2001). Screening of cucumber cultigens levels of expression of NPR1 and their correlation with resistance induced by SAR could yield a potential marker for SAR which is worth investigating.

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