This is to certify that the

dissertation entitled

Investigation of the Role of Plant Cell Wall Degrading Enzymes in Host-Pathogen Interactions

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Plant Pathology

Major professor

Date Feb. 10, 1997

INVESTIGATION OF THE ROLE OF PLANT CELL WALL DEGRADING ENZYMES IN HOST-PATHOGEN INTERACTIONS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1997

ABSTRACT

INVESTIGATION OF THE ROLE OF PLANT CELL WALL DEGRADING ENZYMES IN HOST-PATHOGEN INTERACTIONS

By

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This research focused on determining whether cell wall degrading ezymes secreted by the fungal pathogen, *Cochliobolus carbonum*, had an essential role in the colonization of maize tissue. Three secreted proteases (Alp1a, Alp1b, Alp2) were purified and characterized.and the corresponding genes (*ALP1*, *ALP2*) were cloned and sequenced. Biochemical features of Alp1a and Alp1b and analysis of a mutant deficient in the production of *ALP1* indicated that Alp1a and Alp1b were differentially glycosylated forms of the same gene product. The ability of *alp1*, *alp2*, and *alp1/alp2* mutants to cause disease on maize is discussed.

Similarly, three 1,3-1,4-β-glucanases (Mlg1a, Mlg1b, Mlg2) were purified and characterized, the genes encoding these proteins (*MLG1*, *MLG2*) were cloned and sequenced, and *mlg1* mutants were generated. Again, the biochemical data and the analysis of the *mlg1* mutants indicated that Mlg1a is the glycosylated form of Mlg1b. The capacity of a strain deficient in the synthesis of *MLG1* gene products to colonize maize tissue is discussed.

Redundancy is the root of all evil.

ACKNOWLEDGMENTS

No one works on a project for four years without the help of others. Therefore, I would like to thank all the people in the lab who have pitched in to help me accomplish my goals. I would especially like to thank John Pitkin for literally standing by me for most of the last four years even though I was typically off by ten-fold. I learned most of my biting sarcasm from John, while the remainder came from Robin Buell. It goes without saying that everything I ever learned about the history of the PRL and my predecessors was taught to me by John Scott-Craig. If the PRL should be unfortunate enough to lose John to another university, they should make him sign a secrecy agreement. I thank Ambro van Hoof for being a very important part of my life. I also thank Fabienne Hamburger for doing all those dreaded PABA's with a smile on her face.

A great deal of thanks goes to my family for supporting me through the last nine years of school. I promise some day I will get a "real job".

I thank my committee members, Dr. Jonathan Walton, Dr. Kenneth Keegstra, Dr. John Ohlrogge, and Dr. Gregory Zeikus for their guidance. A special thanks goes to the faculty and staff of the PRL for giving me every opportunity to succeed in science. I feel that the scientific standard set by the PRL is one of the highest and I am proud to say that I received my Ph.D. while being a member of the PRL. I thank the NIH-Biotechnology

Training Program for supporting me for most of my tenure at MSU. Thanks to John Ryals, I was given the chance to fulfill my Industrial Internship at CIBA-GEIGY. During my time there, I gained a new perspective on science and met Jörn, who as Jonathan said, took care of me.

Finally, I would again like to thank Dr. Jonathan Walton for his patience with me even though I had a tendency to redecorate his office and "foolishly" send letters for which I deserved a sailboat ride to Alcatraz. I am now convinced that all of his great ideas come from the New York Times crossword puzzles and solitaire.

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INTRODUCTION

The Pathogen

Cochliobolus carbonum R. R. Nelson (anamorph, *Bipolaris zeicola* (G. L. Stout) Shoemaker = *Helminthosporium carbonum* Ullstrup) is the causal agent of northern leafspot of maize (*Zea mays* L.). This species of fungus can be found as several different races, which defines host specificity. Race 1, first described by Ullstrup (1941), produces a host-selective phytotoxin called HC-toxin (Pringle and Scheffer 1967) which is required for pathogenicity of this fungus on maize (Scheffer and Livingston 1984). This toxin is a cyclic tetrapeptide (Figure 1) which can be isolated as several forms, each with varying activities (Rasmussen and Scheffer 1988b, Liesch et al. 1982, Pope et al. 1983, Walton et

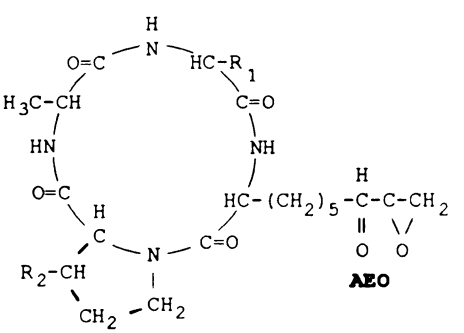


Figure 1. Structure of HC-toxin and derivatives (Leisch et al. 1982, Pope et al. 1983, Rasmussen and Scheffer 1988b, Walton et al. 1982).

al. 1982). However, toxicity absolutely requires the presence of the epoxide group and the carbonyl group of Aeo (2-amino-9,10-epoxy-8-oxodecanoic acid) (Walton and Earle 1983, Ciufetti et al. 1983, Kim et al. 1987). Toxin production is conditioned by a single genetic locus, *TOX2* (Nelson and Ullstrup 1961, Scheffer et al. 1967). Biosynthesis of HC-toxin involves a cyclic peptide synthetase, HTS (Walton 1987, Walton and Holden 1988). The 15.7 kb open reading frame of the gene, *HTS1*, encoding this enzyme is found in two copies in most race 1 isolates of *C. carbonum* (Ahn and Walton 1996). Simultaneous disruption of both copies of *HTS1* results in a loss of HC-toxin production and a concommitant loss in pathogenicity on maize (Panaccione et al. 1992).

In addition to the cyclic peptide synthetase, several other genes (*TOXA, TOXC, TOXD*), unique to toxin producing isolates of *C. carbonum,* have been found which may be involved in HC-toxin biosynthesis. The protein encoded by *TOXA* exhibits a high degree of similarity to small molecule efflux pumps and may be involved in secreting HC-toxin and/or protecting *C. carbonum* from the toxic effects of HC-toxin (Pitkin et al. 1996). Supporting the argument that the *TOXA* gene product is essential for protection of the fungus from HC-toxin, Pitkin et al. (1996) were not able to recover mutants with disruptions in the two copies of *TOXA*. *TOXC*, which appears to encode a fatty acid synthetase, might be involved in the synthesis of Aeo (Figure 1) (Ahn and Walton, 1997). Gene disruption of all three copies of *TOXC* result in a loss of toxin production and pathogenicity. *TOXD*, found in three copies in *C. carbonum* race 1, has no known role in biosynthesis of HC-toxin (unpublished data). Gene disruptions of the *TOXD* loci have no effect on toxin production or pathogenicity. In addition to the genes already identified,

there may be genes encoding proteins which participate in cyclization of the toxin and regulation of toxin production.

Resistance to race 1 is governed by a single dominant gene, *Hm* (Nelson and Ullstrup 1964). Meeley and Walton (1991) found that a cell-free extract from a resistance maize genotype (*Hm/hm*) was able to inactivate HC-toxin by the reduction of the carbonyl group on Aeo (Figure 1). Later, Meeley et al. (1992) showed that this HC-toxin reductase activity was found only in extracts from maize genotypes resistant to *C. carbonum* race 1 (*Hm/hm*, *Hm/Hm*) and not in those that were susceptible (*hm/hm*). These studies demonstrated that the biochemical activity of the enzyme encoded by the *Hm* locus was an HC-toxin reductase. Johal and Briggs (1992) subsequently cloned the *Hm* gene and showed that it had sequence similiarity to other known reductases.

HC-toxin has a variety of effects on susceptable tissues and cells. It can inhibit the biosynthesis of chlorophyll (Rasmussen and Scheffer 1988a), inhibit mammalian cell division (Walton et al. 1985), and increase the uptake of certain amino acids, ions, and nitrate (Yoder and Scheffer 1973a, 1973b). It seems that HC-toxin is not really a toxin but rather a cytostatic agent which may in some way alter or block the ability of the host to mount a defense response, thus allowing the fungus to ramify in the host tissue. Towards defining the mode of action of HC-toxin, Brosch et al. (1995) have shown that three forms of maize histone deacetylase were significantly inhibited by HC-toxin. This may indicate that HC-toxin affects gene regulation by altering histone binding to DNA.

A second race of *C. carbonum*, race 2, does not produce HC-toxin. *HTS1*, *TOXA*, *TOXC*, and *TOXD* are missing in race 2 isolates of *C. carbonum* (Ahn and Walton 1996).

The morphology of race 2 lesions (small, necrotic flecks) on both susceptible (*hm/hm*) and resistant (*Hm/hm*) genotypes of maize is similar to that of race 1 on *Hm*/- maize (Nelson and Ullstrup 1961). If HC-toxin is present in a race 2 inoculation, the fungus is able to colonize susceptible maize leaves in a manner similar to race 1 (Comstock and Scheffer 1973). Collectively, the introduction of *C. carbonum*-resistant maize varieties and the possible fitness disadvantage of race 1, due to the burden of toxin production (Leonard 1987), has made race 2 the predominant isolate found in maize fields (Leonard 1978).

Though *C. carbonum* is not an economic threat to maize growers today, it is an excellent system to study plant-pathogen interactions. It grows well under most conditions, it is genetically stable, there are two mating types which allow for Mendelian genetic analyses, transformation-mediated gene disruptions can be performed routinely, and most importantly the disease determinant (HC-toxin) has been defined and shown to be the key factor for a successful disease phenotype on susceptible maize.

The Host-Pathogen Interaction

Pathogens have devised a diverse array of mechanisms to colonize host tissues. Bacterial pathogens can invade plant tissue by taking advantage of natural openings due to injury, hydathodes, stomata, and spaces where lateral roots have emerged. In addition, cell wall degrading enzymes are required by several of the soft-rotting bacteria to macerate tissue and cause disease (Liao et al. 1988, Boccara et al. 1988). Viruses can only be introduced by vectors such as fungi and insects or mechanically. Phytopathogenic fungi, on the other hand, use not only natural openings of plants but also generate structures

specialized for host penetration. Some rust fungi search out stomatal openings by orienting germ tubes (primary hyphae originating from the spore) at right angles to the cell wall junctions until a stomatal apparatus is located (Staples and Macko 1980, Allen et al. 1991). Upon sensing a stomate, germ tube growth ceases, an appressorium (a specialized structure which facilitates adhesion and penetration) is formed over the opening, and the penetration peg pushes between the guard cells down into the substomatal cavity.

Howard and Ferrari (1989) have demonstrated that mechanical pressure is necessary for *Magnaporthe grisea* to penetrate a surface as resistant as a cell wall. High internal hydrostatic pressure is generated in the appressorium by an increase in solutes and water influx. This pressure can be maintained because of a layer of melanin in the appressorium which is impermeable to solutes (Rast et al. 1981). The pressure is then focused at the appressorium pore, located at the point of attachment to the plant surface, and a penetration peg is forced through the plant cell wall. This does not occur in melanin-deficient *M. grisea* mutants, which are consequently nonpathogenic (Chumley and Valent 1990). At least two other fungal species, *Pyricularia* and *Colletotrichum*, also require melanized appressoria for penetration of the host plant (Kubo et al. 1991, Woloshuk et al. 1980, Rasmussen and Hanau 1989).

An alternative to mechanical pressure is cell wall dissolution. For many years, plant pathologists have tried to use the knowledge gained from plant cell wall structural analysis to determine what components of the plant cell wall are most important for cell wall structural integrity and protection from the environment. As it is yet unkown what cell wall macromolecules in any plant species maintain wall integrity, plant pathologists have

had to make educated guesses about which pathogen-secreted cell wall degrading enzymes should be investigated first. Studies concerning the interactions between *Leptosphaeria maculans* and oilseed rape (Easton and Rossall 1985), *Colletotrichum lindemuthianum* and bean (Wijesundera et al. 1989), *Verticillium albo-atrum* and tomato (Cooper and Wood 1980), and *Gaeumannomyces graminis* var. *tritici* and wheat (Dori et al. 1995) have demonstrated a correlation between the levels of various cell wall degrading enzyme activities and the formation of disease. Le Cam et al. (1994) have even shown that the difference in timing and magnitude of secretion of cell wall degrading enzymes by four different isolates of *Mycocentrospora acerina* may determine the degree of aggressiveness on carrot. Using monoclonal antibodies against major plant wall constituents, Xu and Mendgen (1997) demonstrated that the density of carbohydrate epitopes decreased upon infection with *Uromyces vignae*. The authors propose that the density decrease was due to the action of degradative enzymes secreted by the fungus. Many more studies on the identification of individual enzymes and their potential role in virulence of fungi have been reviewed (Cooper 1984, Walton 1994).

Of these numerous studies, only two cell wall degrading enzymes, in a defined number of host-pathogen interactions, have been shown to have a potential role in virulence. The work of Dickman et al. (1989) demonstrated that the introduction of a cutinase gene from *Nectria haematococca* into the obligate wound pathogen *Mycosphaerella* enabled this fungus to breach an intact cuticle and be pathogenic on papaya. Moreover, cutinases may be important for attachment to the plant surface as the addition of cutinase to *Uromyces viciae-fabae* spores increased the adhesion to bean leaves 2-fold (Deising et al. 1992). A

UV-induced, non-pathogenic mutant of *Pyrenopeziza brassicae* was characterized as also being deficient in protease production (Ball et al. 1991). The non-pathogenic phenotype co-segregated with protease deficiency and both could be complemented with a 40-kb genomic cosmid clone. To date, the gene encoding this protease has not been cloned and disrupted to demonstrate that pathogenicity is unequivocally due to a single protease.

In the case of *C. carbonum*, 90% of fungal penetrations are at cell junctions and 10% are through stomatal openings (Jennings and Ullstrup 1957, Murray and Maxwell 1975). Therefore, host invasion is primarily by mechanical pressure and/or enzymatic degradation. Melanin-deficient mutants of both *C. miyabeanus*, a pathogen of rice, and *C. carbonum* are fully pathogenic (Kubo et al. 1989, J. Pitkin unpublished data). Therefore, if mechanical pressure is the means by which these fungi penetrate the host tissue, then there must be some other component, yet to be found, that allows for the increase in hydrostatic pressure in the appressorium.

Supporting the hypothesis that cell wall degrading enzymes play a role in maize colonization by *C. carbonum*, Murray and Maxwell (1975) concluded from an ultrastructural analysis that the initial penetration by *C. carbonum* was probably due to enzymatic degradation rather than mechanical means. They observed that the host cuticle was not pushed inwards by the penetration peg, but instead appeared to be initially dissolved and then ruptured by mechanical pressure. Furthermore, they suggested that the swollen endoplasmic reticulum above the penetration site may be involved in the transport of cell wall degrading enzymes and material for the growth of the penetration peg. In a related fungus, *C. sativus*, penetration and growth of the hyphae between the host cells is

believed to involve both cell wall depolymerization and mechanical mechanisms (Huang and Tinline 1976).

The cell wall degrading enzymes that have been identified from *Cochliobolus* fall into many different classes. Deshpande and Deshpande (1968) and Nelson and Sherwood (1968) first demonstrated that *Helminothosporium atypicum* and *C. carbonum*, respectively, secreted pectin degrading enzymes. Later, cellulase, cutinase, and xylanase activities were characterized in *C. sativum*, *C. carbonum*, and *C. heterostrophus* (Muse et al. 1972, Bateman et al. 1973, Baker and Bateman 1978). More than a decade later, Walton and Cervone (1990) purified and characterized an endopolygalacturonase (*PGN1*) from *C. carbonum*. The gene corresponding to this protein was next cloned and a null mutant was generated to evaluate the role of pectin degradation on pathogenicity of maize (Scott-Craig et al. 1990). Subsequently, twelve more secreted cell wall degrading enzymes were purified and characterized from *C. carbonum* (Table 1). The genes corresponding to eleven of these proteins have been cloned and five additional genes for other cell wall degrading enzymes have been cloned by homology with sequences from known genes (Table 1). Transformation-mediated gene disruptions have been performed for most of these genes and virulence of the resulting null mutants evaluated (Table 1). To date, no single cell wall degrading enzyme secreted by *C. carbonum* is essential for the successful colonization of this fungus on maize. Even a strain deficient in multiple xylanases has no effect on virulence; however, residual xylanase activity was detected *in* vitro (Apel-Birkhold and Walton 1996).

TABLE 1. Cell Wall Degrading Enzymes of *C. carbonum*.

Enzyme Activity	*Purified*	*Gene Name*	*Mutant Phenotype*
α-Arabinosidase	X[a]		n.d.
Serine Protease (Trypsin-Like)	X[b]	*ALP1*[b]	#[b]
Serine Protease (Subtilisin-Like)	X[b]	*ALP2*[c]	#[c]
Endo-β-1,4-Glucanase		*CEL1*[d]	#[d]
Cellobiohydrolase		*CEL2*[e]	n.d.
Cutinase		*CUT1*[f,g]	#[f,h]
Exo-β-1,3-Glucanase 1	X[i]	*EXG1*[j]	$[k]
Exo-β-1,3-Glucanase 2	X[f]	*EXG2*[f]	n.d.
Endo-β-1,3-1,4-Glucanase/1,3-Glucanase 1	X[l]	*MLG1*[l]	#[l]
Endo-β-1,3-1,4-Glucanase/1,4-Glucanase 2	X[l]	*MLG2*[f]	n.d.
Endopolygalacturonase	X[m]	*PGN1*[n]	#[n]
Exopolygalacturonase	X[o]	*PGX1*[o]	#[o]
Pectin Methylesterase	X[g]	*PME1*[g]	n.d.
Endo-β-1,4-Xylanase 1	X[p]	*XYL1*[q]	#[q]
Endo-β-1,4-Xylanase 2		*XYL2*[s]	#[s]
Endo-β-1,4-Xylanase 3		*XYL3*[s]	#[s]
Endo-β-1,4-Xylanase 4	X[t]	*XYL4*[g]	n.d.
β-Xylosidase	X[a]	*XYP1*[u]	n.d.

- $, indicates an altered phenotype was observed *in vitro* due to mutation.
- #, indicates no difference in phenotype *in vivo* or *in vitro* due to mutation.
- n.d. = not determined.
- a, Ransom and Walton 1996; b, Murphy and Walton 1996; c, Görlach et al. 1997b; d, Sposato et al. 1995; e, J.-H Ahn unpublished data; f, J.M. Görlach unpublished data; g, J.S. Scott-Craig unpublished data; h, J.W. Pitkin unpublished data; i, van Hoof et al. 1991; j, Nikolskaya unpublished data; k, Schaeffer et al. 1994; l, Görlach et al. 1997a; m, Walton and Cervone 1990; n, Scott-Craig et al. 1990; o, Scott-Craig et al. 1996; p, Holden and Walton, 1992; q, Apel et al. 1993; s, Apel-Birkhold and Walton 1996; t, P.C. Apel-Birkhold unpublished data; u, R. Ransom unpublished data.

In light of these frustrating results, several interesting observations have been made. First, xylanase activities and gene expression patterns indicated that of the three xylanase family G class xylanases, only *XYL1* and *XYL3* are expressed *in planta*. In contrast, only *XYL1* and *XYL2* are expressed *in vitro,* with enzyme activity only being detected for Xyl1(Apel-Birkhold and Walton 1996). Second, a mutant of *EXG1* is still pathogenic but is impaired in its ability to grow on 1,3-β-glucan as the sole carbon source (Schaeffer et al. 1994). Third, a double disruption in *PGN1* and *PGX1* diminishes nearly 99% of all polygalacturonase activity, yet there is no measurable differences in the growth rate or dry weight of this mutant compared to wild type when grown on pectin as the sole carbon source (J.S. Scott-Craig, unpublished data). Support of growth may be due to detectable pectin methylesterase activity. Multiple gene disruptions in all the presently identified xylanases (*XYL1* through *XYL4*) and pectin-degrading enzymes (*PGN1, PGX1, PME1*) are currently in progress.

Whether or not cell wall degrading enzymes have a direct impact on the penetration of maize by *C. carbonum* is still unclear. One must realize, however, that cell wall degrading enzymes may not only have a direct role in penetration, they may also be important in fungal growth and differentiation, as the already mentioned role for cutinase in adhesion (Deising et al. 1992). Independently, two groups of researchers demonstrated that *S. cerevisiae* mutants deficient in a secreted exo-1,3-β-glucanase (SPR1 or SSG1) had reduced spore thermoresistance and delayed mature ascus formation (San Segundo et al. 1993, Muthukumar et al. 1993). They speculated that the exo-1,3-β-glucanase may soften the pre-existing wall or, as seen in *Candida albicans*, function as a transferase to

elongate 1,3-β-glucans being synthesized by glucan synthetase. Moreover, cell wall degrading enzymes could have a direct or indirect role in warning the plant that the pathogen is present. For instance, an endo-β-1,4-xylanase protein was identified as being responsible for ethylene biosynthesis and necrosis (Sharon et al. 1993, Bailey et al. 1990). Cell wall degrading enzymes might also release oligosaccharides from their own wall or from that of the plant wall which then act as potent elicitors of the hypersensitive resistance response (Côté and Hahn 1994). Finally, wall depolymerases undoubtedly function as a means of saprophytic nutrient acquisition and this may be their only role in some phytopathogenic organisms.

This study was undertaken to evaluate the role that several different plant cell wall degrading enzymes play in the colonization of maize by *C. carbonum*. Three proteases were purified and characterized, the corresponding genes were cloned, and gene disruption experiments were performed to investigate the involvement of proteases in the growth and virulence of *C. carbonum*. The purification and biochemical charactization of three enzymes able to degrade 1,3-1,4-β-glucans was also accomplished. The genes encoding these enzymes were obtained and a gene disruption in one was performed to evaluate its role in the growth and virulence of *C. carbonum*. Furthermore, N-terminal amino acid sequence of an exo-β-1,3-glucanase aided in the cloning of its corresponding gene.

CHAPTER 1

Jenifer M. Murphy and Jonathan D. Walton. 1996. Three extracellular proteases from *Cochliobolus carbonum:* cloning and targeted disruption of *ALP1*. Mol. Plant-Microbe Interact. **9:** 1091-1098.

Chapter 1

THREE EXTRACELLULAR PROTEASES FROM *COCHLIOBOLUS CARBONUM:* CLONING AND TARGETED DISRUPTION OF *ALP1*

Abstract

Three extracellular serine proteases (Alp1a, Alp1b, Alp2) from *Cochliobolus carbonum* were purified and characterized. Of eight carbon/protein substrates tested, total protease activity was highest when the fungus was grown on medium containing collagen. Alp1a and Alp1b are members of the trypsin family (EC 3.4.21.4), and Alp2 is a member of the subtilisin family (EC 3.4.21.62). Alp1a, Alp1b, and Alp2 have monomer molecular masses of 25-kD, 30-kD, and 38-kD, respectively. Alp1b is glycosylated whereas Alp1a is not. The gene encoding Alp1a, *ALP1*, was isolated using PCR primers based on two amino acid sequences: one obtained directly from the N-terminus of Alp1a and another that is highly conserved in other trypsins. The transcriptional start site was determined using RACE and the intron structure and polyadenylation site were determined from a cDNA clone. An internal fragment of *ALP1* was used to create Alp1a null-mutants by transformation-mediated gene disruption. Total protease activity in the mutants was reduced by 35 to 45%. By chromatographic analysis, the mutants had lost two peaks of UV absorption and the two protease activities corresponding to Alp1a and Alp1b, which, together with the biochemical data, indicates that Alp1a and Alp1b are products of the

same gene. The *in vitro* growth and disease phenotypes of the *ALP1* mutants were indistinguishable from the wild type strain; therefore, ALP1 is not by itself required for pathogenicity.

Introduction

The first barrier a phytopathogenic microbe encounters on its host is the plant cell wall, and cellular pathogens secrete a variety of wall-depolymerizing enzymes. The role, if any, of these enzymes in the process of pathogenesis has been the subject of intensive research (Bateman and Basham 1976; Cooper 1983; Walton 1994).

Although the plant cell wall is mainly composed of polysaccharides, at least five classes of structural proteins and numerous classes of enzymes are present (McNeil et al. 1984; Showalter 1993). During pathogenesis the expression of many structural proteins and enzymes are up-regulated and secreted into the plant cell wall as part of a general defense response (Showalter 1993; Alexander et al. 1994). Insofar as these wall proteins are important to plant defense, effective pathogens may require extracellular proteases to degrade them. The proteases secreted by a pathogen might also be important as activators of any of its cell wall degrading enzymes that are secreted as zymogens (Drapeau 1978; Rypniewski et al. 1993; Moormann et al. 1993), or as processors of toxins (Howard and Buckley 1985) and elicitors (van den Ackerveken et al. 1993). Proteases might also have a role in pathogenesis by increasing the permeability of the plant plasma membrane (Tseng and Mount 1974). Many plants produce protease inhibitors, which suggests that plants have evolved mechanisms to counter pathogen proteases (Ryan 1990).

Extracellular proteases are produced by many phytopathogenic bacteria and fungi, e.g., *Xanthomonas alfalfae* (Reddy et al. 1971), *Monilinia fructigena* (Hislop 1982), *Colletotrichum lindemuthianum* (Ries and Albersheim 1973), and others (Porter 1966). An aspartic protease gene has been isolated from *Cryphonectria parasitica* (Choi et al. 1993). A protease mutant of *Cladosporium cucumerinum* had wild type symptom development on cucumber seedlings, but residual protease activity was present (Robertsen 1984). No reduction in virulence was observed in a metalloprotease mutant of *Erwinia chrysanthemi* EC16 when inoculated on either potato tubers or chrysanthemum stems (Dahler et al. 1990). A protease-deficient mutant of *Xanthomonas campestris* pv. *campestris* had reduced virulence when introduced into the cut vein endings of turnip leaves (Dow et al. 1990). A UV-induced mutant of the fungus *Pyrenopeziza brassicae* that had lost both protease activity and pathogenicity could be complemented for both traits by a 40-kb genomic cosmid clone (Ball et al. 1991).

One definitive test of the role of any particular gene in pathogenesis is the construction of a null mutant using targeted gene disruption (e.g., Scott-Craig et al. 1990). Towards the goal of testing the role of proteases in plant pathogenicity, we report here the characterization of three proteases that *C. carbonum* secretes when grown on collagen, and the sequence and disruption of *ALP1*, which encodes two of these proteases.

Results and Discussion

Characterization of Alp1a, Alp1b, and Alp2.

To facilitate the study of proteases, we investigated which growth conditions maximized total protease activity. Collagen was tried as a substrate because it is a hydroxyproline-rich glycoprotein like the extensins of the plant cell wall. Stimulation of the de novo synthesis as well as cross-linking of structural wall proteins such as extensin in response to pathogen attack is thought to be an important plant defense response (Showalter 1993; Lawton and Lamb 1987). Therefore, fungal proteases that are induced in planta to degrade wall structural proteins might also be induced in culture by collagen. Preliminary experiments indicated that of the potential protease substrates, *C. carbonum* produced most total protease on medium supplemented with collagen. Other protein sources (casein, gelatin, and bovine serum albumin) yielded less total protease activity than collagen (Figure 2). Low levels of protease activity were observed on media supplemented with pectin, corn bran, or 2.0% sucrose (Figure 2). In the presence of collagen, 0.2% sucrose stimulates protease production (Figure 2), an effect also seen with polygalacturonase (Walton and Cervone 1990) and xylanaes (Holden and Walton 1992). High protease production on protein substrates and low production on other substrates indicate that total protease activity in *C. carbonum* is substrate-induced and partially catabolite-repressed.

Culture filtrates were concentrated by rotary evaporation, dialyzed, and then passed over a low-pressure anion-exchange column to remove acidic proteins and pigments. Alp1a, Alp1b, and Alp2 were then separated by cation-exchange HPLC. Alp1a was

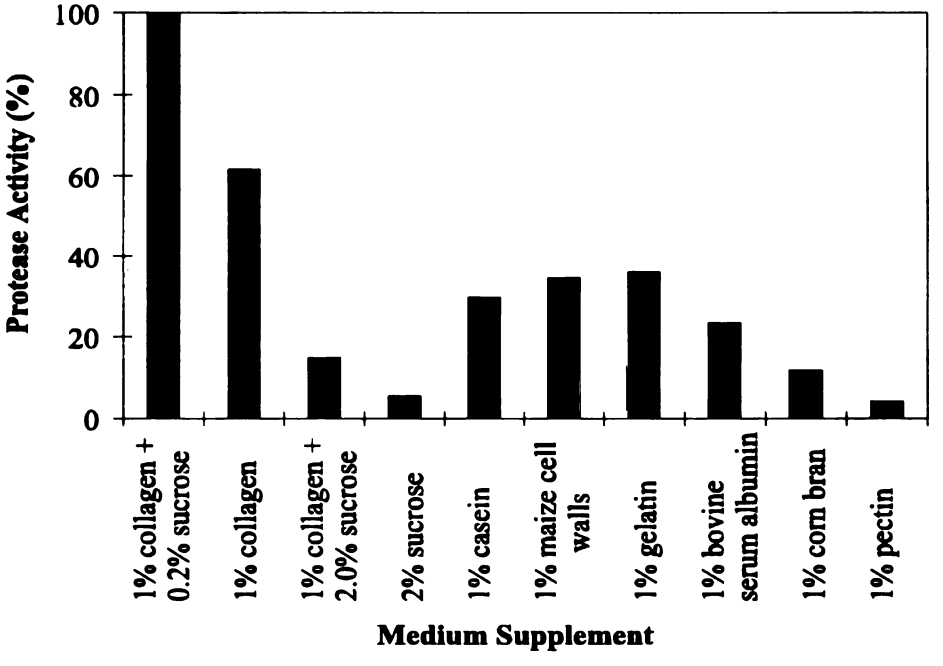


Figure 2. Effect of different media supplements on total protease activity in culture filtrates of *C. carbonum*. Protease activities are shown as a percentage of the highest activity measured. Experiment was repeated three times with similar results. Data from one experiment is presented in this figure.

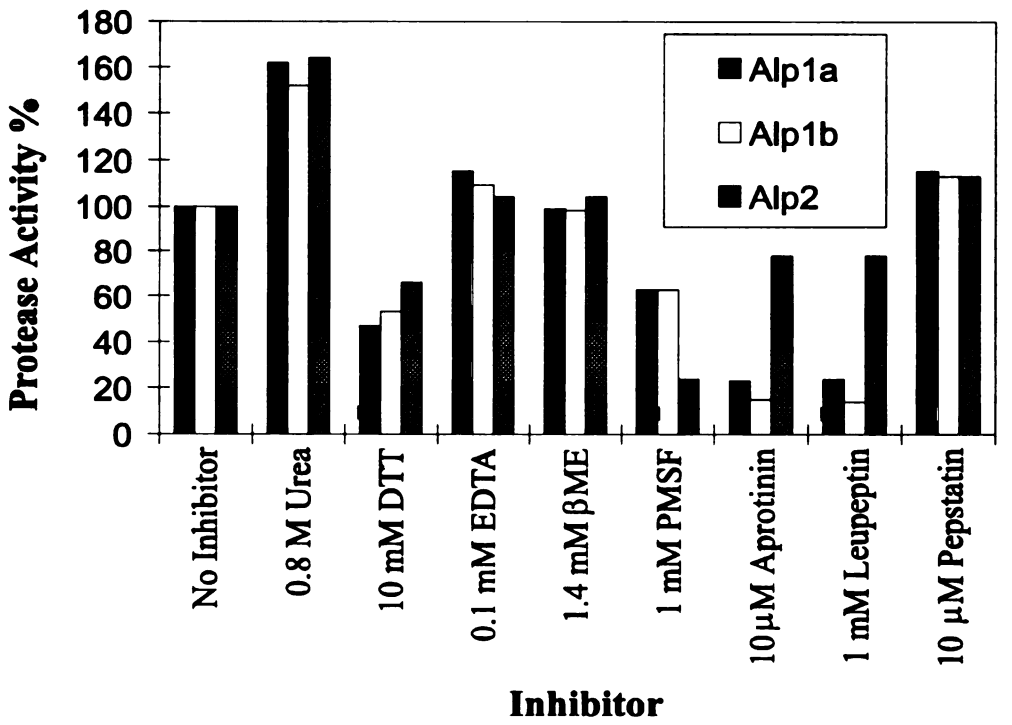


Figure 3. Effect of protease inhibitors on Alp1a, Alp1b, and Alp2. Each enzyme was incubated with azocasein in the presence of the inhibitor being tested for 30 min at 45°C. Protease activities are expressed as a percentage of activity obtained in the control. Data are the average of two experiments. DTT, dithiothreitol; EDTA, ethyelenediamine-tetraacetic acid; βME, β-mercaptoethanol; PMSF, phenylmethylsulfonylfluoride.

further purified by hydrophobic-interaction HPLC and sequenced at the N-terminus. By SDS-PAGE, Alp1a, Alp1b, and Alp2 had M_r's of 25-kD, 30-kD, and 38-kD, respectively, similar to serine proteases (North 1982). Alp1a and Alp1b activities were inhibited strongly by aprotinin (77% and 85%, respectively) and leupeptin (76% and 96%, respectively) and weakly by PMSF (37% and 37%, respectively) (Figure 3), suggesting that these proteases are related to trypsin (Gebhard et al. 1986; Powers and Harper 1986). Alp2 was more sensitive to PMSF (81% inhibition) than to aprotinin or leupeptin (40% and 47% inhibition, respectively) (Figure 3), suggesting that it is related to the subtilisin family of proteases (Ottensen and Svendsen 1970). Alp1a, Alp1b, and Alp2 were less sensitive to other major classes of protease inhibitors (Figure 3).

Periodic acid/Schiff staining indicated that Alp1b is a glycoprotein whereas Alp1a lacks glycosylation (data not shown). All three enzymes were most active between pH 7 and pH 8, but each showed some activity over the pH range of 5 to 11. Temperature optima were similar for all three enzymes. Each enzyme was as active at 45°C as at 55°C but lost activity at 65°C and above. Alp1a and Alp1b seemed to be less stable than Alp2 based on the observation that after being stored at -20°C and then separated by SDS-PAGE, Alp1a and Alp1b were degraded whereas Alp2 was not.

To test the possibility that plant cell wall structural proteins might be substrates for these proteases, salt-extractable extensin was purified from maize stylar tissue (Murphy and Hood 1993). Neither crude culture filtrates nor the proteases individually or in combination could degrade extensin, whereas in parallel experiments casein was degraded to small peptide fragments (data not shown). Therefore, these proteases probably do not

have a role in degradation of this class of maize cell wall structural proteins during pathogenesis.

Although no evidence for additional proteases was found, we cannot exclude the possibility that *C. carbonum* makes other proteases, which might have been overlooked because (1) they are unstable, (2) they are not produced on the substrates tested, (3) they are acidic and therefore retained by the DEAE-cellulose pre-treatment, or (4) they are active only below pH 5, under which conditions the substrate, azocasein, precipitates.

Isolation and characterization of ALP1.

The N-terminal amino acid sequence of Alp1a was determined to be IVGGTTAAAGEYPFIVS (indicated by double-underlining in Figure 4). A search of the non-redundant databases using BLASTP (Gish and States 1993) identified a 76% identity with the N-terminus of a 22-kD trypsin-like protease from *Fusarium oxysporum*. A 48-fold degenerate oligonucleotide based on the amino acid sequence EYPFIV was used in conjunction with a 256-fold anti-sense degenerate oligonucleotide coding for the amino acid sequence VAGWGA (also indicated by double-underlining in Figure 4), which is a highly conserved internal amino acid sequence of many trypsins. Using these two primers and DNA isolated from a *C. carbonum* cDNA library as template in PCR, a 330-bp product was generated. BLASTX analysis of the PCR product showed a high degree of similarity with trypsin-like proteases. The PCR product was used as a probe to screen a *C. carbonum* cDNA library. A 1.0-kb cDNA clone (pC8-6.1) was isolated and sequenced, and also used to screen a library of *C. carbonum* genomic DNA.

Oligonucleotide primers that had been used to sequence the cDNA copy of *ALP1* were used to sequence, on both strands, approximately 1.5-kb of genomic DNA covering *ALP1*.

Figure 4 shows the sequence and structure of *ALP1* and its deduced amino acid sequence. The start of the *ALP1* message was determined by sequencing three independent RACE products (Frohman et al. 1988). The context of the first ATG, 91-bp downstream of the transcription start site (CACCATGCGT) (Figure 4), is in good agreement with the 5' end of the consensus sequence for *Neurospora* translation initiation (CAMMATGGCT where M = C or A) (Edelmann and Staben 1994). Sequences typical of promoters of lower eukaryotes, TATAA and CAAC (Gurr et al. 1987), are located 34- and 72-bp, respectively, upstream of the transcription start site (Figure 4). A single 74-bp intron deduced by comparing the cDNA and genomic sequences of *ALP1* is indicated by lower-case letters (Figure 4). The 5' (G^GTAAGTTCACTCA; consensus G^GTAAGTNNYCNYY, where Y = T or C) and 3' (AACAG; consensus WACAG, where W = A or T) donor sites as well as the splice branch site (AACTAACA; consensus WRCTRACM, where R = A or G) and intron length are consistent with other introns of *C. carbonum* and other fungi (Apel et al. 1993; Scott-Craig et al. 1990; Sposato et al. 1995; Edelmann and Staben 1994). No AATAAA polyadenylation signal sequence (Gurr et al. 1987) could be identified before the polyadenylation site 229-bp downstream of the stop codon (Figure 4).

ALP1 is predicted to encode a mature protein of 261 amino acids and a mass of 24.5-

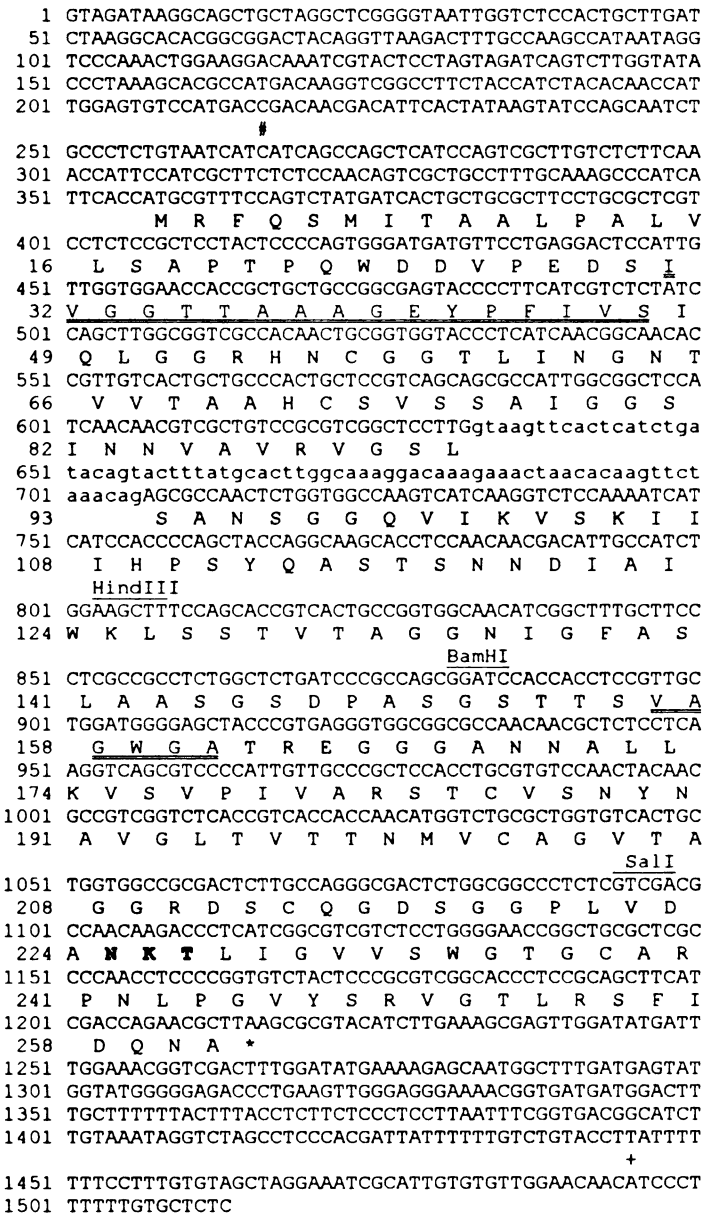
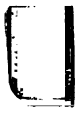


Figure 4. Sequence of *ALP1*. The amino acid sequence of the mature N-terminus obtained directly from Alp1a, IVGGTTAAAGEYPFIVS, is indicated by double-underlining. The sequence conserved among other trypsin-like proteases and used to design the second oligonucleotide primer for PCR amplification, VAGWGA, is also indicated by double-underlining. The intron is indicated in lower case letters. The indicated *Hind*III, *Sal*I, and *Bam*HI restriction sites are those used to construct and linearize the disruption vector pJM9. The transcriptional start site is indicated by # and the polyadenylation site by + (symbols refer to nucleotides underneath and amino acid codes refer to nucleotides above). The predicted N-glycosylation site (amino acid 225) is indicated in bold lettering.



kD, which is in agreement with the size of Alp1a estimated by SDS-PAGE. A single predicted N-glycosylation site (NKT) occurs at amino acid 225. Comparison of the predicted N-terminal amino acid sequence with the experimentally-determined sequence of the mature N-terminus (double-underlined in Figure 4) reveals a 30-amino acid signal peptide with a potential signal peptide cleavage site that violates the (-3, -1) rule (von Heijne 1986). Many trypsin-like proteases are synthesized as prepropeptides (trypsinogens) that undergo a second proteolytic processing after removal of the signal peptide, e.g., mammalian trypsins (Walsh and Wilcox 1970). Alp1 might also be synthesized as a prepropeptide.

The predicted amino acid sequence of Alp1a has a high degree of similarity with other trypsin-like proteases from a variety of species (Figure 5). Alp1a contains the catalytic triad common to all serine proteases (His-71, Asp-120, and Ser-217) (Neurath 1984). The sequence flanking the active site serine (CQGDSGGP) is completely conserved in the other trypsin-like proteases (Figure 5).

N-terminal sequencing of Alp2.

Fractions from the cation-exchange HPLC step that had Alp2 activity were run on SDS-PAGE, blotted, and three proteins that were visible with Coomassie R-250 staining of the blot were excised and sequenced from their N-termini. Two of the proteins had N-terminal sequences with no strong similarity to any sequences in the non-redundant databases, whereas one of the proteins, which showed the best correlation between staining intensity and Alp2 activity among the HPLC fractions, gave the sequence

```
                1                                                #          50
C. carbonum     ......MRFQ SMITAALPAL VLSAPTPQWD DVPEDSIVGG TTAAAGEYPF
F. oxysporum    .......... ..MVKFASVV ALVAPLAAAA PQEIPNIVGG TSASAGDFPF
B. mori         .......... MTNSLLICFT ILGLAASSPK PIGDIRIVGG EDIVITEAPY
B. taurus       .......... .......... .......... VDDDDKIVGG YTCGANTVPY
S. griseus      MKHFLRALKR CSVAVATVAI AVVGLQPVTA SAAPNPVVGG TRAAQGEFP.
                                                        **** * * *** *
                51                                                100
C. carbonum     IVSIQLGGRH NCGGTLINGN TVVTAAHCSV SSAIGGSINN VAVRVGSLSA
F. oxysporum    IVSISRNGGP WCGGSLLNAN TVLTAAHCVS GYAQSG.... FQIRAGSLSR
B. mori         QVSVMFRGAH SCGGTLVAAD IVVTAAHCVM SFAPED.... YRIRVGSSFH
B. taurus       QVSLN.SGYH FCGGSLINSQ WVVSAAHCYK S........G IQVRLGEDNI
S. griseus      MVRLSMG... .CGGALYAQD IVLTAAHCVS GSGNNTS... ..ITATGGVV
                 **    * *  *** * *    *******   * *          * **
                101                                               150
C. carbonum     N...SGGQVI KVSKIIIHPS YQASTSNNDI AIWKLSSTVT AGGNIGFASL
F. oxysporum    T...SGGITS SLSSVRVHPS Y..SGNNNDL AILKLSTSIP SGGNIGYARL
B. mori         Q...RDGMLY DVGDLAWHPD FNFASMDNDI AILWLPKPVM FGDTVEAIEM
B. taurus       NVVEGNEQFI SASKSIVHPS YNSNTLNNDI MLIKLKSAAS LNSRVASISL
S. griseus      DLQSSSAVKV RSTKVLQAPG YN..GTGKDW ALIKLAQP.. ....INQPTL
                    * *      **   *** *     **** ** **       *  *    *
                151                                               200
C. carbonum     AASGSDPASG STTS.VAGWG ATREGGGANN .ALLKVSVPI VARSTCVSNY
F. oxysporum    AASGSDPVAG SSAT.VAGWG ATSEGGSSTP VNLLKVTVPI VSRATCRAQY
B. mori         VETNSEIPDG DITI.VTGWG HMEEGGG.NP SVLQRVIVPK INEAACAEAY
B. taurus       PTSCA..SAG TQCL.ISGWG NTKSSGTSYP DVLKCLKAPI LSDSSCKSAY
S. griseus      KIATTTAYNQ GTFTGVAGWA NR.EGGSQQR Y.LLKANVPF VSDAACRSAY
                  * *    *      *****    ***       **** *** *    * * *
                201                                               250
C. carbonum     NAVGLTVTTN MVCAGV.TAG GRDSCQGDSG GPLVDANKT. ...LIGVVSW
F. oxysporum    GTSA..ITNQ MFCAGV.SSG GKDSCQGDSG GPIVDSSNT. ...LIGAVSW
B. mori         SPI.YAITPR MLCAGT.PEG GKDACQGDSG GPLVH.KKK. ...LAGIVSW
B. taurus       PG...QITSN MFCAGY.LEG GKDSCQGDSG GPVV.CSGK. ...LQGIVSW
S. griseus      GNE.LVANEE I.CAGYPDTG GVDTCQGDSG GPMFRKDNAD EWIQVGIVSW
                      *   * ***    * * ******** ** *          * * ***
                251                                   290
C. carbonum     GTGCARPNLP GVYSRVGTLR SFIDQNA$.. ..........
F. oxysporum    GNGCARPNYS GVYASVGALR SFIDTYA$.. ..........
B. mori         GLGCARPEYP GVYTKVSALR EWVDENITNL RLKHILRRF$
B. taurus       GSGCAQKNKP GVYTKVCNYV SWIKQTIASN $.........
S. griseus      GYGCARPGYP GVYTEVSTFA SAIASAARTL $.........
                * ****** * ***  *  ** * **  *
                                %Similarity  %Identity
F. oxysporum                         80          57
B. mori                              60          39
B. taurus                            62          41
S. griseus                           55          33
```

Figure 5. Comparison of the predicted amino acid sequences of *ALP1* and four related trypsin-like proteases using PILEUP (Devereux et al. 1984). Sequence references: *Fusarium oxysporum*, SwissProt P35049, Rypniewski et al. (1993); *Bombyx mori*, PIR S32794, Ikeda et al. (1991); *Bos taurus*, PIR A90164, Walsh and Neurath (1964); *Streptomyces griseus*, PIR JQ1302, Olafson et al. (1975). Amino acids conserved between Alp1 and at least two of the other proteins are indicated by asterisks. Putative signal peptides were not included in the PILEUP analysis. The sequence for *B. taurus* is trypsinogen. The mature N-terminus of Alp1a is indicated by # and the stop codons by $.

AYTTQSSAPWGLARISSQXRGTTGYXXDD, where X indicates an unknown amino acid. Analysis of this sequence by TBLASTN (Gish and States 1993) showed that it has strong similarity to numerous serine proteases, including subtilisin-like proteases from the fungi *Trichoderma album* (GenBank M54901) (69% identity), *Paecilomyces lilacinus* (GenBank L29262) (57% identity) and *Beauveria bassiana* (GenBank U16305) (58% identity). We are currently testing the hypothesis that this protein is Alp2 by cloning and disrupting the corresponding gene.

Transformation-mediated gene disruption of ALP1.

Plasmid pJM9 containing an internal 250-bp *Hind*III/*Sal*I fragment of *ALP1* (Figures 3 and 6A) plus the gene for hygromycin resistance was linearized at a unique *Bam*HI restriction site and transformed into *C. carbonum* wild type strain 367-2A. Two single-spored, hygromycin-resistant transformants (T515-1A and T515-3A) were analyzed. The restriction map of the wild type *ALP1* locus and the predicted map resulting from integration of a single copy of pJM9 at *ALP1* are shown in Figures 6A and 6B, respectively. The pattern of hybridization of pC8-6.1 indicates that pJM9 has integrated at *ALP1* in single and multiple copies in T515-1A and T515-3A, respectively (Figure 6C).

Both *ALP1* mutants were analyzed for their protease profiles and pathogenicity. Total extracellular protease activity was reduced by 35 to 45% in the *ALP1* mutants. Growth of the mutants on 1% collagen was not significantly altered based on appearance or yield of DNA and total extracellular protein (data not shown). Therefore, we conclude that *ALP1* by itself is not required for growth in vitro on collagen. Activities of other extracellular

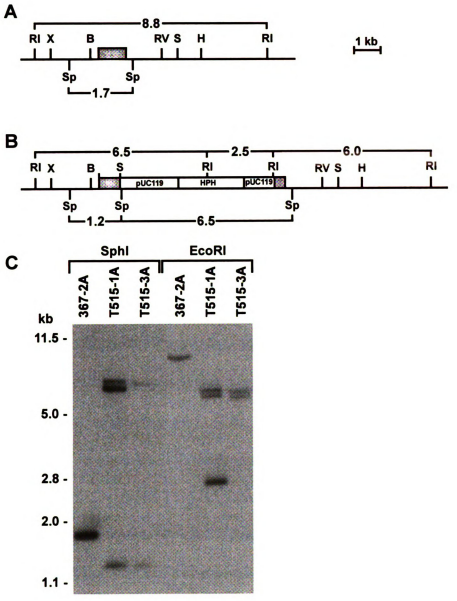


Figure 6. **A**, Restriction map of wild type locus of *ALP1* showing the location of the *ALP1* transcript. **B**, Predicted restriction map of *ALP1* with insertion of a single copy of pJM9. Predicted *Eco*RI and *Sph*I fragment sizes are indicated (in kb). **C**, DNA blot comparing wild type (367-2A) and two transformants (T515-1A and T515-3A). Isolated DNA was cut with *Sph*I (lanes 1 to 3) or *Eco*RI (lanes 4 to 6) and the blot was probed with a cDNA copy of *ALP1* (C8-6.1). The extra bands in the digests of T515-1A are as predicted for tandem multiple insertions of pJM9. The shaded boxes indicate *ALP1* sequences. RV, *Eco*RV; RI, *Eco*RI; X, *Xba*I; B, *Bam*HI; Sp, *Sph*I; H, *Hin*dIII; S, *Sal*I. Not all sites are shown. Additional sites within *ALP1* are shown in Figure 4.

enzymes of *C. carbonum* (cellulase, endo-1,4-xylanase, endo-polygalacturonase, 1,3-1,4-glucanase and exo-1,3-glucanase - Scott-Craig et al. 1990; Apel et al. 1993; Sposato et al. 1995; unpublished results) were unaltered in the *ALP1* mutant, indicating that *ALP1* is also not required for functional processing of these enzymes.

Protease activities from wild type and *ALP1* mutant strains were purified as described above through cation exchange HPLC. The *ALP1* mutants lacked two peaks of activity and UV absorption corresponding to Alp1a and Alp1b (shown for mutant T515-1A in Figure 7B). Taking into account that (1) Alp1b is ca. 5 kDa larger than Alp1a, (2) Alp1b but not Alp1a is glycosylated, (3) Alp1a and Alp1b are similarly inhibited by aprotinin and leupeptin, (4) the product of *ALP1* has one predicted glycosylation site, and (5) disruption of *ALP1* results in the disappearance of both Alp1a and Alp1b, we conclude that Alp1a and Alp1b are products of the same gene, *ALP1*. Since Alp2 is not affected by disrupting *ALP1* (Figure 7), we conclude that it is the product of another gene.

Pathogenicity of T515-1A and T515-3A were compared to 367-2A on both resistant (cv. Great Lakes) and susceptible (Pr x K61) cultivars of maize in the greenhouse. Rate of lesion development, lesion size, and lesion morphology were examined daily for 14 d, at which point the plants had been killed by both the wild type and the *ALP1* mutants. No differences in lesion morphology, size, color, or rate of formation between the wild type and the two mutants were observed (data not shown). Leaves with both low and high lesion densities were observed. The fungus was reisolated from the maize leaves and tested for hygromycin sensitivity. Twenty-six isolates from wild type lesions were all

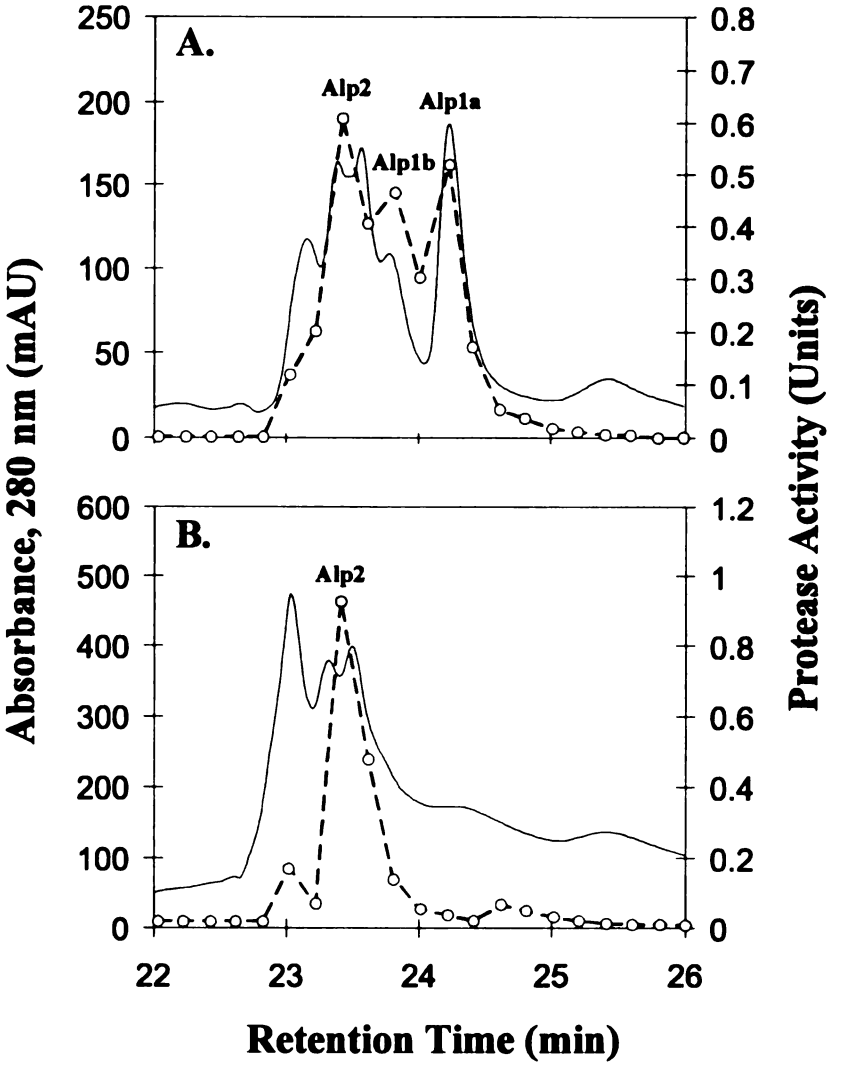


Figure 7. Cation exchange HPLC analysis of proteins from culture filtrates of (A) wild type and (B) *ALP1* mutant T515-1A. Prior to HPLC analysis, crude culture filtrates were passed over a low-pressure anion-exchange column to remove acidic proteins and pigments. Solid lines: OD_{280}; dashed lines: protease activity.

hygromycin-sensitive, 50 isolates from T515-1A lesions were all resistant to hygromycin, and 35 isolates from T515-3A lesions were all resistant to hygromycin. Thus we conclude that the pathogenicity of T515-1A and T515-3A was not due to restoration of *ALP1* activity by simple excision of pJM9.

We conclude that *ALP1* is neither an essential pathogenicity factor nor a major virulence factor for *C. carbonum* race 1. Since this pathogenicity assay is not quantitative, we cannot exclude the possibility that *ALP1* makes a small contribution to virulence. Since *C. carbonum* makes at least one additional extracellular protease, it cannot be concluded from this study that proteases have no role in pathogenicity or virulence. A similar conclusion was drawn from studies of the role of *ALP*, encoding an alkaline protease, in murine respiratory mycosis caused by *Aspergillus fumigatus*; *ALP* mutants were still fully pathogenic but residual protease activity remained (Monod et al. 1993; Tang et al. 1993).

Materials and Methods

Fungal culture growth and maintenance

Conidia of *C. carbonum* race 1, strain 367-2A, were stored at -80°C in 25% glycerol and grown on V-8 juice agar plates. For enzyme production, approximately 5 x 10^5 spores were inoculated into a 1-L Erlenmeyer flask containing 200 ml of mineral salts supplemented with 0.1% yeast extract and trace elements (van Hoof et al. 1991). Substrate supplements were: Type I collagen (Sigma C-9879), casein (Sigma C-0376), maize cell walls (Sposato et al. 1995), Type A gelatin (Sigma G-2625), bovine serum

albumin (Sigma A-7906), corn bran (Country Life Natural Foods, Pullman, Michigan), and pectin (Sigma P-9135). For routine protease production, the fungus was grown on 1.0% collagen. Cultures were incubated at 21 to 23°C with shaking at 125 rpm for 3.5 to 4 d.

Enzyme assays

Proteases were assayed using azocasein (Sigma A-2765) (Ansari and Stevens 1983). Azocasein (0.5 ml of a 25 mg/ml solution in 50 mM sodium phosphate, pH 7.5) was incubated with 5 to 10 µl of enzyme fraction at 45°C for 30 min. To stop the enzyme reaction, 20 µl of 50% (w/v) trichloroacetic acid was added and the solution vortexed vigorously for ca. 5 sec. The sample was centrifuged for 5 min at 14,000 x g in a microcentrifuge and the OD_{410} of 200 µl measured in an ELISA plate reader (Bio-Tek). Protease inhibitors were from Sigma. Units of enzyme activity are defined as OD_{410} per 10 µl enzyme fraction under the conditions described.

Protein purification and characterization

Culture filtrates (typically 200 to 300 ml per batch) were concentrated to ca. 10% of the original volume by rotary evaporation under vacuum at 37°C. After centrifugation to remove insoluble material, the filtrates were dialyzed for 16 hr at 4°C in cellulose dialysis tubing (SpectraPor, MWCO 12,000 to 14,000) against 25 mM sodium acetate, pH 5.0 and applied to a column (10 to 20 ml bed volume in a 60 cc disposable syringe) of DEAE cellulose (Sigma) equilibrated in 25 mM sodium acetate, pH 5.0. The column was washed

with one bed volume of 25 mM sodium acetate, pH 5.0, and all material that was eluted from the column was pooled. The samples were again concentrated by rotary evaporation to ca. 5 ml and dialyzed against 25 mM sodium acetate, pH 5.0. After clarification by centrifugation, the samples were fractionated on a cation exchange HPLC column (polysulfoethylaspartamide, The Nest Group, Southboro, Massachusetts). Running conditions were a linear gradient of buffer A (25 mM sodium acetate, pH 5.0) to buffer B (25 mM sodium acetate, pH 5.0, plus 0.4 M KCl) in 20 min at 1 ml/min. The peak of UV (280 nm) absorbance corresponding to Alp1a activity was collected and further purified by hydrophobic interaction chromatography (Biogel TSK-Phenyl-5PW, Biorad, Richmond, California) after adding ammonium sulfate to a final concentration of 1.7 M. Proteins were eluted with a 20 min linear gradient of 0.1 M KH_2PO_4, pH 7.0, plus 1.7 M ammonium sulfate to water at a flowrate of 0.9 ml/min. Fractions containing Alp1a were desalted, lyophilized, and sequenced by automated Edman degradation. Alp2 was purified using the same procedure as for Alp1a through cation exchange HPLC, taking 0.2 ml fractions (Figure 7), and then separated on SDS-PAGE. The proteins in the gel were transferred to ProBlot (Applied Biosystems, Foster City, California) (Matsudaira 1987) and the blot was stained briefly with 0.1% Coomassie R-250 in 40% methanol and destained with 50% methanol. Three proteins were visible on the blot; all three were excised and sequenced.

SDS-PAGE was carried out in 12% (w/v) acrylamide resolving gel with 5% (w/v) stacking gels (Hames and Rickwood 1981). Glycoproteins were detected by periodic acid/Schiff staining (Strömqvist and Gruffman 1992).

The pH optima for the purified enzymes was measured using buffers composed of 10 mM citric acid/20 mM sodium phosphate (pH range 5 to 7), 50 mM Tris-HCl (pH range 7 to 9), and 50 mM CAPS (3-cyclohexylamino-1-propane-sulfonic acid)-HCl (pH range 9 to 11).

Extensin was purified from maize stylar tissue as described (Murphy and Hood, 1993) and incubated with Alp1a, Alp1b, and Alp2 alone and in combination for 60 min or overnight at 45°C. Degradation was evaluated by SDS-PAGE. Casein was used as a control.

Nucleic acid manipulations and sequencing

DNA was isolated as described by Pitkin et al. (1996) and RNA was isolated as described by Chomczynski and Sacchi (1987). The transcription start site of *ALP1* was determined using the Amplifinder RACE Kit (Clontech, Palo Alto, California) (Frohman et al. 1988). Reverse transcription was primed with the oligonucleotide CGTCGCTGTCCGCGTCGG (starting at nucleotide 608, see Figure 4). PCR amplification was done using the primer sequence GTTGGTGGAACCACCGCT GCTGCCG (starting at nucleotide 450, see Figure 4) and the "anchor" primer supplied with the RACE Kit. *ALP1* was sequenced using specific oligonucleotides spaced ca. 250-bp apart. Sequencing was performed by automated fluorescent sequencing at the MSU-DOE-PRL Plant Biochemistry Facility using an Applied Biosystems (Foster City, California) Catalyst 800 for Taq cycle sequencing and an Applied Biosystems 373A Sequencer for analysis of the products.

Isolation of ALP1

PCR amplification of the 330-bp 5' region of *ALP1* was performed as follows: 1x PCR reaction buffer (Gibco-BRL, Gaithersburg, Maryland); 0.15 mM each of dATP, dCTP, dATP, and dGTP; 1.5 mM $MgCl_2$; 2 U *Taq* Polymerase (Gibco-BRL); 100 μM oligonucleotide primer; 50 ng DNA isolated from phage lysate of a cDNA library prepared from polyA$^+$-RNA extracted from *C. carbonum* grown on maize cell walls (Pitkin et al. 1996). PCR reactions were performed in a Perkin-Elmer thermocycler model 480 under the following conditions: one min denaturation at 94°C; 35 cyles of 1 min denaturation at 94°C, 2 min annealing at 55°C, and 3 min primer extension at 72°C; 7 min primer extension at 72°C. PCR primers used were GAGTAYCCNTTYATHGT and GTNGCNGGNTGGGGNGC (N=any nucleotide; Y=T or C; H=A,T, or C) corresponding to the amino acid sequences EYPFIV and VAGWGA, respectively (Figure 4). After one extraction with one volume chloroform, PCR products were precipitated with two volumes ethanol plus one-tenth volume 3 M sodium acetate, pH 5.2, by incubating for 10 min at 20°C and centrifuging 10 min in a microcentrifuge (14,000 x g). The resulting pellet was dried, redissolved in water, treated with T4 DNA Polymerase (Boehringer-Mannheim, Indianapolis, Indiana) in the presence of 1 mM dNTP's for 5 min at 37°C, and fractionated on a 1.0% agarose gel in TAE buffer (Maniatis et al. 1982). The unique product corresponding to *ALP1* was excised from the gel and purified using Gene-Clean (Bio 101, Vista, California). The blunt-ended *ALP1* PCR product was ligated into pBluescript II SK+ at the *Sma*I site and sequenced.

Screening of the cDNA and genomic libraries, DNA blotting, probe labelling, and hybridization have been described (Scott-Craig et al. 1990; Sposato et al. 1995). DNA blotting was done with Nytran (Schleicher & Schuell, Keene, New Hampshire) and hybridizations were done in in 5x SSPE (Maniatis et al. 1982), 7% SDS, and 0.5% non-fat dry milk at 65°C for 16 hr. Blots were washed twice at 22°C with 2x SSPE and 0.1% SDS for 15 min each time, and twice at 65°C in 0.1x SSPE and 0.1% SDS for 15 min each time.

Disruption of ALP1

The transformation vector was constructed by cloning the 2.5-kb *SalI/Hind*III fragment of pHYG1 (Apel et al. 1993) containing the *C. heterostrophus* promoter 1 driving the expression of the *hph* gene encoding hygromycin phosphotransferase (Schäfer et al. 1989) into the *Aat*II site of pUC119 to create pHYG3. Concurrently, the plasmid harboring the *ALP1* cDNA clone, pC8-6.1, was digested with *Hind*III, treated with T4 polymerase, and digested with *Sal*I. The resulting 290-bp internal fragment of *ALP1* (Figure 4) was ligated into pHYG3 that had been digested with *Sma*I and *Sal*I to create pJM9. This vector was linearized at the unique *Bam*HI site located within the 290-bp *ALP1* sequence (Figure 4) and used to transform strain 367-2A of *C. carbonum*.

Preparation and transformation of protoplasts was as described (Scott-Craig et al. 1990; Apel et al. 1993). Transformants able to grow on V-8 juice agar containing 100 units/ml hygromycin (Calbiochem) were single-spored twice to obtain nuclear homogeneity. Pathogenicity was evaluated by inoculating leaves of 2-week-old maize

cultivars Pr x K61 (susceptible) and Great Lakes (resistant) with 10^4 conidia/ml in 0.1% Tween-20. The whole plants as well as different leaves with different infection densities were observed daily for two weeks. After one week, individual lesions were excised and after surface sterilization in 10% (v/v) commercial bleach plus 0.1% Tween-20 plated on V-8 juice agar. After two days growth, the fungi were transferred to V-8 juice agar plates containing hygromycin and evaluted for resistance to hygromycin.

CHAPTER 2

Jenifer M. Görlach, Esther van der Knaap, and Jonathan D. Walton. 1997. Three mixed-linked glucanases from the filamentous fungus *Cochliobolus carbonum:* cloning and targeted disruption of *MLG1*. Eur. J. Biochem. (submitted).

Chapter 2

THREE MIXED-LINKED GLUCANASES FROM THE FILAMENTOUS FUNGUS *COCHLIOBOLUS CARBONUM:* CLONING AND TARGETED DISRUPTION OF *MLG1*

Abstract

Three extracellular enzymes (MLGases) able to hydrolyze β-1,3-1,4-glucans (mixed-linked glucans or β-glucans) were purified and characterized from culture filtrates of the plant pathogenic fungus *Cochliobolus carbonum*. Total MLGase activity was stimulated by substrates containing mixed-linked glucans and by 0.2% sucrose and was partially repressed by 2.0% (w/v) sucrose. Maize bran induced higher total MLGase activity in culture than oat bran. The three MLGases, called Mlg1a, Mlg1b, and Mlg2, were resolved by cation exchange and hydrophobic-interaction HPLC. Mlg1a and Mlg1b also hydrolyze β-1,3-glucan, whereas Mlg2 does not degrade β-1,3-glucan but does degrade β-1,4-glucan to a slight extent. Mlg1a, Mlg1b, and Mlg2 have monomer molecular masses of 33.5-kD, 31-kD, and 29.5-kD, respectively. The N-terminal amino acid sequences of Mlg1a and Mlg1b are identical (AAYNLI). Mlg1a is glycosylated whereas Mlg1b is not. The gene encoding Mlg1b, *MLG1*, was isolated using PCR primers based on amino acid sequences of Mlg1b obtained from the purified protein. The product of *MLG1* has no close similarity to any known protein but does contain a motif (EIDI)

that occurs at the active site of MLGases from several prokaryotes. An internal fragment of *MLG1* was used to create *mlg1* mutants by transformation-mediated gene disruption. Total MLGase and β-1,3-glucanase activities in culture filtrates of the mutants are reduced by approximately 50% and 40%, respectively. When analyzed by cation exchange HPLC, the mutants are missing the two peaks of mixed-linked glucanase activity corresponding to Mlg1a and Mlg1b. Together, the data indicate that Mlg1a and Mlg1b are products of the same gene, *MLG1*. Growth of *mlg1* in culture medium supplemented with macerated maize cell walls or maize bran and disease symptoms on maize are identical to wild type.

Introduction

Monocot cell walls are composed of a variety of macromolecules including cellulose, arabinoxylan, xyloglucan, pectin, and proteins. One of the major hemicelluloses of the walls of plants in the Poaceae is mixed-linked glucan (also called β-1,3-1,4-glucan or β-glucan), in which unbranched chains of β-1,4-glucose are disrupted by periodic β-1,3-linkages in the ratio of about 2:1. Several lines of evidence suggest that this polysaccharide is particularly important for the control of plant cell expansion, and therefore it might have a critical role in maintaining the structural integrity of the wall (Carpita, 1996).

Enzymes that can degrade mixed-linked glucan are called mixed-linked glucanases (here abbreviated MLGase), β-1,3-1,4-glucanases, β-glucanases, or lichenases. Some MLGases can also degrade other glucans, for example, β-1,3-glucans and β-1,4-glucans (Sakellaris et al., 1993, Schimming et al., 1992, Spilliaert et al., 1994, Høj et al., 1989).

Genes encoding MLGases have been cloned from a number of bacteria (e.g., Schimming et al., 1992; Teather and Erfle, 1990; Spilliaert et al., 1994) and higher plants (e.g., Slakeski et al., 1990; Yun et al., 1993). An MLGase has been purified from the fungus *Rhizopus arrhizus* (Clark et al., 1978) but to our knowledge no genes encoding MLGases have previously been isolated from fungi.

Cochliobolus carbonum, an ascomycetous pathogen of maize, penetrates into and ramifies through intact leaves, in the process obtaining nutrients for its growth from the plant cell cytoplasm and walls. For penetration, ramification, and nutrient assimilation, both as a pathogen and during the saprophytic phase of its life cycle, *C. carbonum* produces a variety of extracellular enzymes, including pectinases, xylanases, β-1,3-glucanases, cellulases, β-xylosidase, α-arabinosidase, and proteases, that can degrade the polymers of the plant cell wall.

A common feature of the extracellular degradative enzymes of microorganisms is redundancy, that is, most microorganisms make two or more chromatographically separable proteins that have the same or similar enzymatic activities. *C. carbonum* is no exception to this rule, making, for example, at least four endo-β-1,4-xylanases (Apel-Birkhold et al., 1996) and three proteases (Murphy and Walton, 1996). Enzymatic redundancy can be due to multiple genes encoding proteins with similar or overlapping enzymatic activities, differential RNA processing (e.g., Boel et al., 1984), and/or differential post-translational modification. Apparent redundancy can also be caused by artifacts such as proteolytic nicking during fermentation or purification. Although it is difficult to establish the ontogenetic relationships between isozymes by purely biochemical

methods, it is feasible to establish the relationship between isozymes, and between isozymes and their encoding genes, by comparing the enzymatic profiles of wild type strains and strains that have been specifically mutated. Targeted mutation of genes is facile in prokaryotes, but due to the greater technical difficulties of molecular genetic manipulation of eukaryotes such as fungi much less is known about the relationship between the multiple forms of extracellular degradative enzymes and their genes in these organisms.

In this study, we describe the identification and characterization of three extracellular enzymes that degrade β-glucan from the filamentous fungus *C. carbonum* and demonstrate by cloning and targeted gene disruption experiments that one gene encodes two of the three MLGases. The two MLGases that are derived from the same gene differ in glycosylation.

Results and Discussion

Characterization and purification of Mlg1a, Mlg1b, and Mlg2

Several carbon sources were tested for optimal production of extracellular MLGase by *C. carbonum*. Maize bran is a better inducer than two commercial oat bran products (data not shown). Similar to endopolygalacturanase, exo-β-1,3-glucanase, xylanase, β-xylosidase, α-arabinosidase, and protease, adding 0.2% sucrose enhances production of MLGase. Unlike xylanase, exo-β-1,3-glucanase, or endopolygalacturanase, however, some MLGase activity is still produced when *C. carbonum* is grown on 2% (w/v) sucrose

as sole carbon source (data not shown) (Holden and Walton, 1992; Murphy and Walton, 1996; Ransom and Walton, 1997; van Hoof et al., 1991; Walton and Cervone, 1990).

After concentration by rotary evaporation, dialysis, and passage through an anion-exchange column to remove acidic proteins and pigments, MLGase activities were fractionated by cation-exchange HPLC. One major (Peak 1) and one minor (Peak 2) peak of activity were resolved (Figure 8A). Both peaks were then separately applied to HI-HPLC for further purification (Figs. 8B and 8C). Cation-exchange HPLC peak 1 (Figure 8A) was thereby resolved into two peaks of activity, called Mlg1a and Mlg1b (Figure 8B). Peak 2 (Figure 8A) remained as a single peak of MLGase activity, called Mlg2 (Figure 8C). Mlg1a and Mlg1b were subsequently chromatographed by gel filtration to purify them to electrophoretic homogeneity.

The molecular masses of Mlg1a, Mlg1b, and Mlg2, as determined by SDS-PAGE, are 33.5, 31, and 29.5-kD, respectively. Mlg1a and Mlg1b are endo-acting enzymes as determined by their ability to rapidly reduce the viscosity of a β-glucan solution relative to the simultaneous appearance of reducing sugars (data not shown). The temperature and pH optima for all three enzymes are approximately 55°C and 5.0, respectively. The activity of Mlg1a and Mlg1b against β-1,3-glucan is comparable to that against β-glucan. Neither Mlg1a nor Mlg1b has activity in long-term assays (17 h) against any of the β-1,4-glucan substrates tested. Based on their HI-HPLC retention times and their ability to degrade β-1,3-glucan, Mlg1a and Mlg1b are probably responsible for the two peaks of β-1,3-glucanase activity remaining in culture filtrates of *EXG1* (exo-β-1,3-glucanase) mutants of *C. carbonum* (Schaeffer et al., 1994). Mlg2 has no detectable activity against

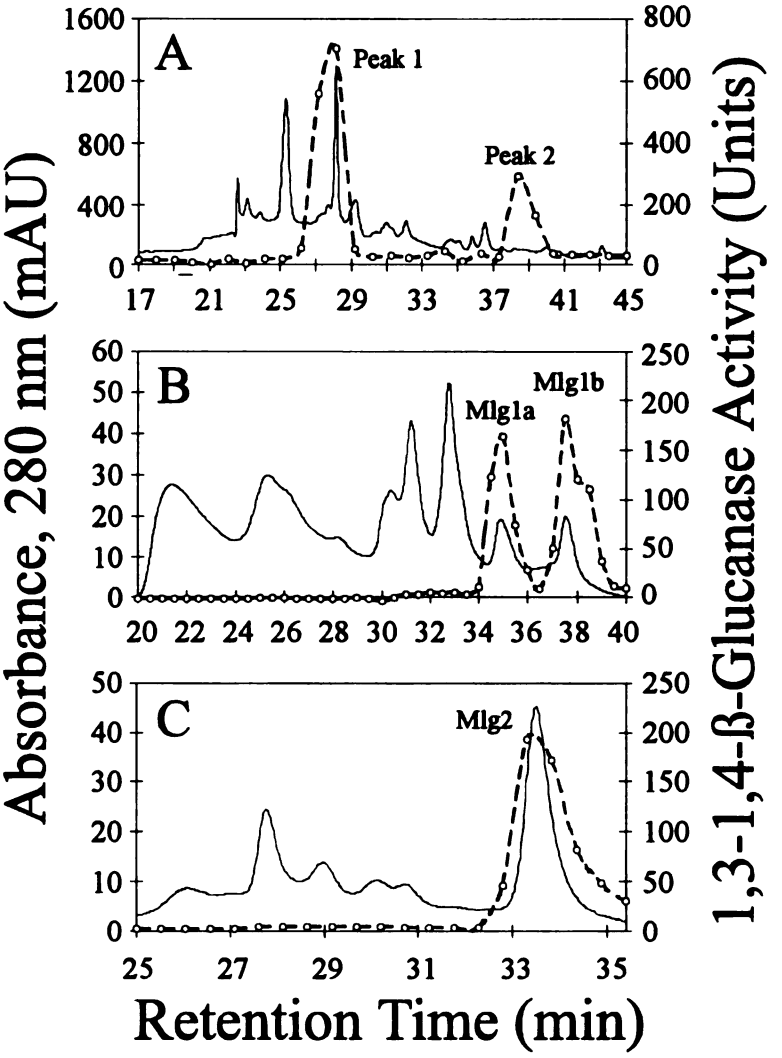


Figure 8. Purification of Mlg1a, Mlg1b, and Mlg2. (A) Cation exchange chromatography of culture filtrates. (B) HI-HPLC of Peak 1 from (A). (C) HI-HPLC of Peak 2 from (A). Solid lines, absorbance at 280 nm; dashed lines, MLGase activity.

β-1,3-glucan but in long (17h) incubations shows some ability to degrade several β-1,4-glucans (low viscosity CM-cellulose, Whatman cellulose, and Avicel). Mlg2 is 180 to 340 times less active against these β-1,4-glucans than it is against β-glucan (data not shown). In a 17 h incubation, Mlg2 shows no ability to degrade two other β-1,4-glucan substrates, high viscosity CM-cellulose and α-cellulose.

On the basis of their substrate preferences, Mlg1a and Mlg1b can be considered as bifunctional β-1,3-1,4/ -1,3-glucanases and Mlg2 as a β-1,3-1,4-glucanase.

Mlg1a is glycosylated whereas Mlg1b is not (Figure 9). Glycosylation can alter the pH optima, temperature optima, or thermostability of enzymes (Doan and Fincher, 1992; Meldgaard and Svendsen, 1994), but Mlg1a and Mlg1b have similar temperature optima and thermostability (data not shown).

At least the first five amino acids of the mature Mlg1a and Mlg1b proteins are identical (Table 2). Analysis of the N-terminal sequence of Mlg1b (22 amino acids) by BLASTP (Gish and States, 1993) found no strong similiarity to any sequence in the nonredundant databases. Two internal tryptic peptides of Mlg1b were sequenced. One (peptide 3) overlaps with the N-terminal peptide (Table 2). Peptide 2 from Mlg1b has 77% identity to an MLGase from the prokaryote *Rhodothermus marinus* (PIR S48201).

For the final purification of Mlg2, proteins in the HI-HPLC fractions containing MLGase activity were separated by SDS-PAGE, blotted, and Mlg2 was excised from the blot for sequencing (Matsudaira, 1987). As the N-terminus was blocked, internal amino acid sequences were obtained from three tryptic peptides (Table 2). BLASTP analysis indicates that peptide 2 of Mlg2 (Table 2) is 56% identical to a cellulase, EglS, from the

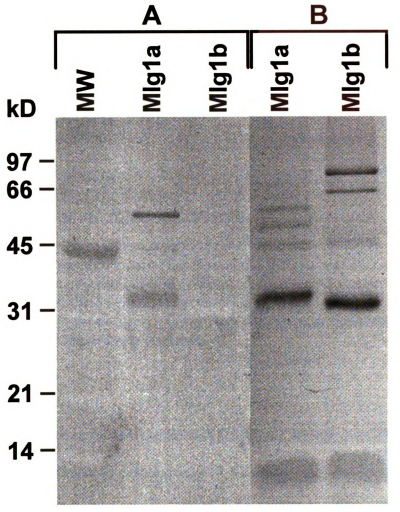


Figure 9. Glycosylation of Mlg1a and Mlg1b. (A) Protein blot from SDS-PAGE gel of HI-HPLC purified Mlg1a and Mlg1b stained with periodic acid/Schiff reagent (Strömqvist and Gruffman, 1992). (B) SDS-PAGE of the same samples in (A) stained with Coomassie R-250. Mlg1a and Mlg1b are the bands at 33.5 and 31 kD, respectively. Molecular weight standards are shown on the left; ovalbumin (45 kDa) is a glycoprotein.

TABLE 2. Experimentally determined amino acid sequences from Mlg1a, Mlg1b, and Mlg2, and comparison to the sequences deduced from the nucleotide sequence of *MLG1*.

Protein	*Peptide sequence*	
	Experimental	*Deduced from DNA Sequence*
Mlg1a	1. AAYNLI	1. AAYNLI
Mlg1b	1. AAYNLIDTYDA(A)N(W)AAKFNFED	1. AAYNLIDTYDAsNWAsKFNFED
	2. GPNWP(A)QGE(I)D(I)	2. GPNWPnQGEIDI
	3. FNFEDIADPDT	3. FNFEDIADPT
Mlg2	1. FTVNQ(C)SANAY	
	2. YDVYPIGSSQGMVNVAGR	
	3. GFPINSQNLITYQFGTEAFTGGP	

- Purified proteins were sequenced at the N-terminus (Mlg1a peptide 1 and Mlg1b peptide 1) or from tryptic peptides (all of the others).
- Amino acids in parentheses indicate uncertainty.
- Lower case letters in the deduced sequence indicate discrepancies between the experimental and the deduced sequences.

prokaryote *Streptomyces rochei* (GenBank X73953) and peptide 3 is 78% identical to the F1 CM-cellulase of *Aspergillus aculeatus* (PIR S12610). EglS (Perito et al., 1994) and F1 CM-cellulase (Ooi et al., 1990) are members of cellulase family H (Gilkes et al., 1991) or glycosyl hydrolase family 12 (Henrissat and Bairoch, 1993). The cloning and sequencing of the gene for Mlg2 is in progress.

Isolation and characterization of MLG1

Two 96-fold degenerate oligonucleotides based on the amino acid sequences IDTYDA and QGEIDI (Table 2) were used in PCR to amplify a fragment of the encoding gene.

This primer combination yielded a single, 340-bp PCR product when DNA from a *C. carbonum* cDNA library was used as a template and a single, 460-bp PCR product when *C. carbonum* genomic DNA was used as a template. To confirm that these PCR products encoded Mlg1b, they were blotted and probed with a third 36-fold degenerate oligonucleotide based on the amino acid sequence KFNFED (Table 2, Figure 10). Both products hybridized to the third oligonucleotide and were therefore cloned and sequenced. Sequencing indicated that the PCR products are identical except for the presence of two introns of 57- and 64-bp in the PCR product amplified from genomic DNA (see Figure 10).

Using the cDNA-derived PCR fragment as a probe, *C. carbonum* cDNA and genomic libraries were screened. From the cDNA library, a 1.34-kb *MLG1* cDNA (C4-2.1) was isolated and sequenced. A 7.0-kb *Bam*HI fragment (see Figure 12) of DNA (MLG1-2B) containing the *MLG1* genomic locus was subcloned and sequenced on both strands. Figure 10 shows the sequence of *MLG1* and the deduced amino acid sequence. The transcription start site of *MLG1* was determined by analyzing the sequence of three independent RACE products (Frohman et al., 1988). The *MLG1* transcript has a 64-bp 5' untranslated region. The context of the deduced translation start site (CACTCATGTCT, Figure 10) conforms with the consensus sequence for *Neurospora crassa* translation initiation (CAMMATGGCT where M = C or A) (Edelmann and Staben, 1994). Three introns (Figure 10) were identified by comparing the sequences of the cDNA and the RACE products with the genomic clone. The 5' and 3' splice sites, splice branch sites, and lengths of the introns are consistent with introns in *N. crassa* and in other genes of *C.*

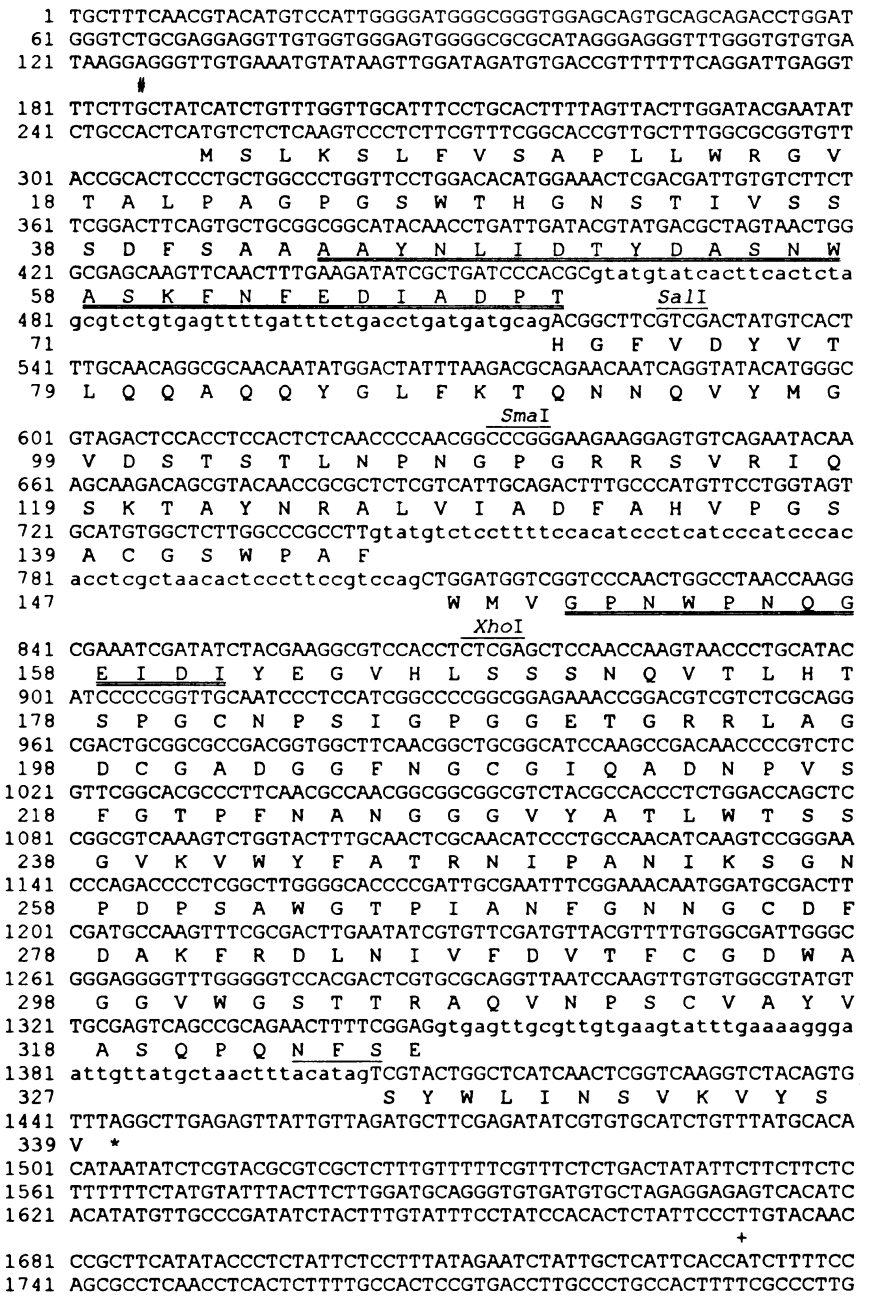


Figure 10. Nucleotide sequence and deduced amino acid sequence of *MLG1*. Amino acids are placed below the corresponding codons. The amino acid sequences of the mature N-terminus and of the internal tryptic peptide are indicated by double underlining. The three introns are indicated in lower case letters. The indicated *Sal*I, *Sma*I, and *Xho*I restriction sites were used to construct and linearize pJM5 for the gene disruption experiments. The transcription start site is indicated by # and the polyadenylation site by + (symbols refer to the nucleotides below). The predicted N-glycosylation site (amino acid 323) is single-underlined. The nucleotide sequence of *MLG1* has been deposited in GenBank with accession number U81606.

carbonum and *N. crassa* (Apel et al., 1993; Apel-Birkhold and Walton, 1996; Murphy and Walton, 1996; Scott-Craig et al., 1990; Sposato et al., 1995; Pitkin et al., 1996; Edelmann and Staben, 1994). Like other *C. carbonum* genes, no obvious AATAAA polyadenlyation signal sequence precedes the polyadenylation site (Gurr et al., 1987).

The open reading frame of the cDNA C4-2.1 is predicted to encode a mature protein of 31.8-kD which is in good agreement with the size of Mlg1b, 31-kD, as determined by SDS-PAGE. The predicted pI of the mature protein is 4.99. The program SignalP v1.1 (Nielsen et al., 1997) predicts a signal peptide cleavage site between amino acids 19 and 20 (VTA/LPA); if this is the true cleavage site then Mlg1 must undergo additional processing to generate the mature protein. One predicted N-glycosylation site (NFS), which therefore probably accounts for the differential glycosylation of Mlg1a and Mlg1b, occurs near the C-terminal end (Figure 10).

The predicted amino acid sequence of Mlg1b has little overall sequence similarity to that of any other gene in the nonredundant databases. The best matches are to two prokaryotic glucanases, a β-1,3-glucanase from *Oerskovia xanthineolytica* (BLAST score 51, P=0.84) and an MLGase from *R. marinus* (BLAST score 50, P=0.92) (Spilliaert et al., 1994; Ferrer et al., 1996). The overall amino acid similarity and identity of Mlg1 to the MLGase of *R. marinus* are 50% and 22%, respectively. The longest stretch of identity is a motif of five amino acids (GEIDI) surrounding a Glu residue which is at the active site of MLGases from *Bacillus* and other prokaryotes (Figure 11) (Keitel et al., 1993; Planas et al., 1992; Spilliaert et al., 1994). This similarity to the bacterial MLGases classifies Mlg11 as a member of the family 16 glycosyl hydrolases (Henrissat and Bairoch, 1993).

```
            1                                                    50

C. carbonum .......AAY NLI..DTYDA SNWASKFNFE DIADPTHGFV DYVTLQQAQQ
R. marinus  SDRSDKAPHW ELVWSDEFDY SGLPDPEKW. DYDVGGHGWG N....QELQY
                  . . .:   : .:  :  .  : .  :   ..:::.  .    :. :
            51                                                  100
C. carbonum YGLFKTQNNQ VYMGVDSTST LNPNGPGRR. .SVRIQSKTA YNRALVIADF
R. marinus  YTRARIENAR VGGGVLIIEA RHEPYEGREY TSARLVTRGK ASWTYGRFEI
            :.  . .:   :  ::    .       ::    : :. ...     .    .
            101                                  #              150
C. carbonum AHVPGSACGS WPAFWMV... ....GPNWPN QGEIDIYEGV HLSSS.NQVT
R. marinus  RARLPSGRGT WPAIWMLPDR QTYGSAYWPD NGEIDIMEHV GFNPDVVHGT
                .:. :. :::.::.        .. ::. .:::::: : :    .     :
            151                                                 200
C. carbonum LHTSPGCNPS IGPGGETGRR LAGDCGADGG FNGCGIQADN PVSFGTPFNA
R. marinus  VHTK.AYNHL LGTQRGGSIR VP...TARTD FH........ ..........
            .::  . :   .:.   .. : ..   .: .  :
            201                                                 250
C. carbonum NGGGVYATLW TSSGVKVWYF ATRNIPANIK SGNPDPSAWG TPIANFGNNG
R. marinus  ....VYAIEW TPEE..IRWF VDDSLYYRFP NER.....LT DPEADWRHWP
                :::  : :.    . .:     .               .  : :..   .
            251                                                 300
C. carbonum CDFDAKFRDL NIVFDVTFCG DWAGGVWGST TRAQVNPSCV AYVASQPQNF
R. marinus  FD.....QPF HLIMNIAVGG AWGG...... ...QQGVDPE AFPAQLVVDY
             :          .. ...  .  :.:          :       :. :    ..
            301          316
C. carbonum SESYWLINSV KVYSV*
R. marinus  VRVYRWVE*. ......
               :  ..
```

Figure 11. Comparison of the amino acid sequences of *C. carbonum* Mlg1 and *Rhodothermus marinus* β-glucanase. Alignment was done using PILEUP (Program Manual, 1994). The probable glutamic acid residue of the active site, deduced by sequence comparison to the known active site of *Bacillus* MLGases (Keitel et al., 1993; Planas et al., 1992), is indicated by #. Identical and similar amino acids are indicated by colons and periods, respectively. Stop codons are indicated by *. Putative signal peptides are not shown.

The sequence of *MLG1* has no detectable similarity to any known plant MLGases nor to any β-1,3-glucanases, including *EXG1* from *C. carbonum* (Schaeffer et al., 1994).

Transformation-mediated gene disruption of MLG1

A 345-bp *SalI/Xho*I fragment, within the open reading frame of *MLG1* (Figure 10), was subcloned into the *Cochliobolus* transformation vector pHYG1 (Sposato et al. 1995) conferring hygromycin B resistance. The resulting plasmid, pJM5, was linearized with *Sma*I and introduced into wild type *C. carbonum* strain 367-2A by transformation of protoplasts. Hygromycin B-resistant transformants were characterized by DNA gel blot analysis. Figure 12A depicts the wild type *MLG1* locus whereas Figure 12B shows the predicted map for a single integration event of pJM5 into *MLG1*. The DNA gel blot analysis of strain T503-4A (Figure 12C, lanes 3 and 6) is consistent with the predicted single insertion event shown in Figure 12B. The pattern of hybridization seen for strain T503-1A (Figure 12C, lanes 2 and 5) is consistent with a tandem integration event of the 6-kb pJM5 vector. Total MLGase and β-1,3-glucanase activities in culture filtrates of Mlg1b mutants T503-1A and T503-4A grown on maize bran are reduced by approximately 50 and 40%, respectively. However, growth (i.e., mycelial mat dry weight) of T503-1A and T503-4A are similar to wild type (data not shown); apparently the residual MLGase activity as well as other enzymes capable of degrading other substrates in maize bran are sufficient to support normal growth of the *mlg1* mutants.

MLGase activities from wild type and the *mlg1* mutant T503-1A were purified in parallel through HI-HPLC. When analyzed by cation exchange HPLC, the *mlg1* mutant is

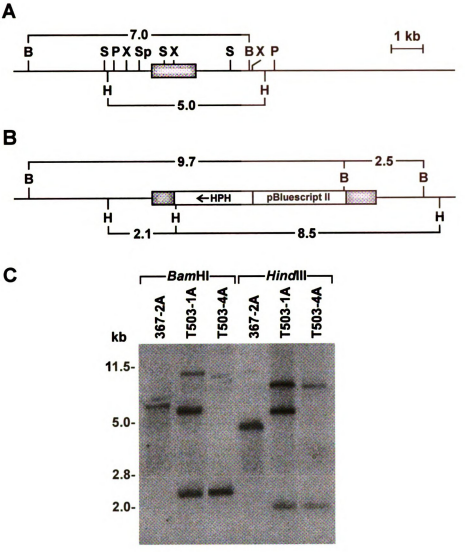


Figure 12. Analysis of the *MLG1* locus in wild type and *mlg1* mutants. (A) Restriction map of the wild type *MLG1* locus showing the location of the *MLG1* transcript (shaded box). (B) Predicted restriction map of the *MLG1* locus with a single insertion of the transforming plasmid pJM5. (C) DNA blot of wild type (367-2A) and two transformants (T503-1A and T503-4A). Total genomic DNA was digested with *Bam*HI (lanes 1 to 3) or *Hind*III (lanes 4 to 6), fractionated by agarose gel electrophoresis, blotted, and probed with the *MLG1* cDNA (C4-2.1). The disappearance of a *Bam*HI band of 7.0 kb and a *Hind*III band of 5.0 kb, and the appearance of *Bam*HI bands of 9.7 kb and 2.5 kb and *Hind*III bands of 2.1 kb and 8.5 kb, are as predicted from homologous integration of pJM5. The additional bands in digests of T503-1A are as predicted for homologous integration of more than one copy of pJM5. The shaded areas in (A) and (B) indicate *MLG1* sequences. B, *Bam*HI; S, *Sal*I; P, *Pst*I; X, *Xho*I; Sp, *Sph*I; H, *Hind*III.

missing Peak 1; Peak 2, corresponding to Mlg2, is still present (Figs. 13A and 13B). Thus, Mlg2 is not encoded by *MLG1*. HI-HPLC analysis (Figs. 13C and 13D) indicates that both Mlg1a and Mlg1b are missing in the *mlg1* mutant (Figure 13D).

Because Mlg1a and Mlg1b have the same substrate specificities and the same N-terminal amino acid sequences, and because *mlg1* mutants have neither Mlg1a nor Mlg1b activity, we conclude that *MLG1* encodes both Mlg1a and Mlg1b. The different chromatographic behavior of Mlg1a and Mlg1b is probably due to differential glycosylation, because Mlg1a is glycosylated whereas Mlg1b is not, Mlg1a is 2.5-kD larger than Mlg1b, and the *MLG1* gene product has one predicted N-glycosylation site. The two products of *MLG1* are probably not due to differential intron splicing of the *MLG1* transcript (Boel et al., 1984) because all three introns of *MLG1* contain a stop codon or frame shift (Figure 10).

Pathogenicity of mlg1 mutants

As the highest amounts of β-glucan are in young maize seedlings (Carpita, 1984), we tested whether infection of young seedlings by *C. carbonum* was impeded by mutating *MLG1*. There were no measurable differences in lesion morphology and development or percent of seedlings that germinated when inoculated with either wild type strain 367-2A or *mlg1* mutant strains T503-1A or T503-4A. Wild type and *mlg1* mutants were also indistinguishable in regard to lesion size, color, and rate of lesion formation when spray-inoculated onto leaves of 14-day old maize seedlings. Thus, *MLG1* does not by itself make a significant contribution to the virulence of *C. carbonum*.

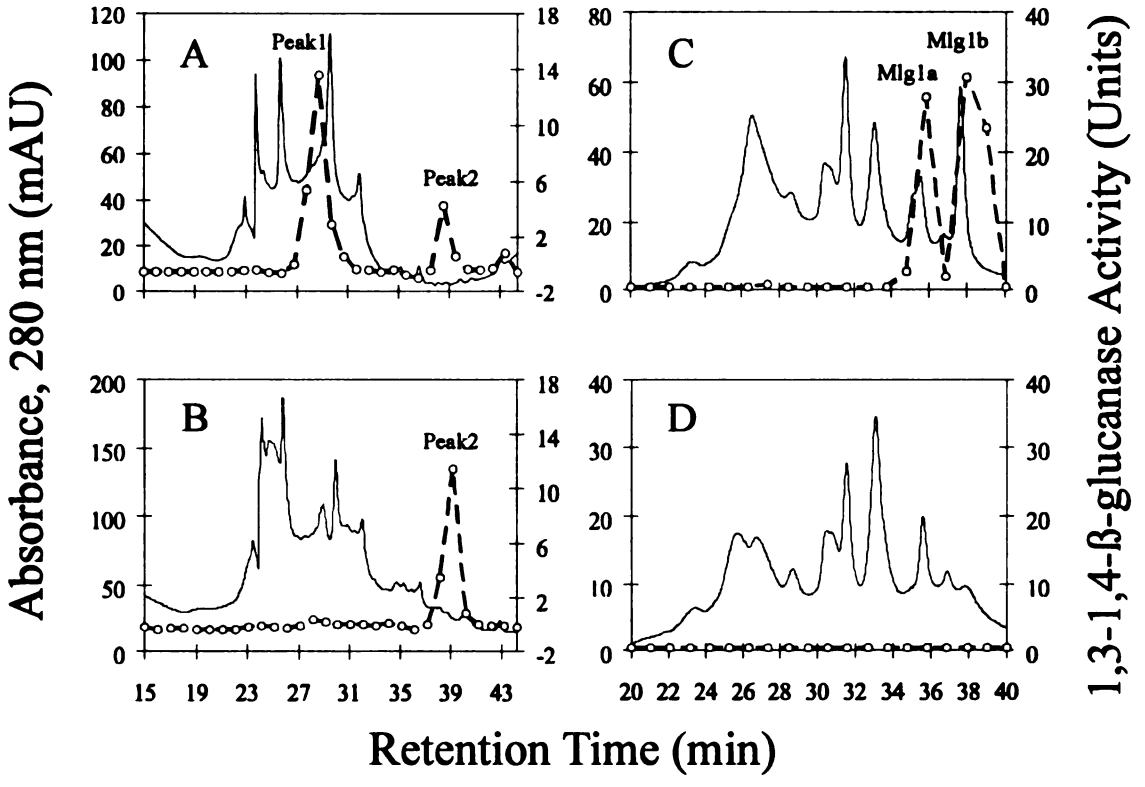


Figure 13. HPLC analysis of MLGase from *mlg1* mutant. (A,B) cation exchange HPLC analysis of MLGase from wild type (A) and *mlg1* mutant T503-1A (B). (C,D) HI-HPLC analysis of Peak 1 from cation exchange HPLC of wild type (C) and from cation exchange HPLC of *mlg1* mutant (D). Solid lines, absorbance at 280 nm; dashed lines, MLGase activity.

Materials and Methods

Fungal culture and maintenance

C. carbonum race 1 strain 367-2A, which is a progeny of strain SB111 (American Type Culture Collection 90305) was grown on V8 juice agar plates. For MLGase production, two fungal plugs (5 mm^2) were inoculated into a 1000-ml Erlenmeyer flask containing 125 ml mineral salts, 0.2% yeast extract, and trace elements (van Hoof et al., 1991) and grown in still culture for 9 d at 21 to 23°C. Supplemental carbon sources tested were Country Life maize bran (Country Life Natural Foods, Pullman, Michigan), Mother's Oat Bran cereal, and Quaker Oat Bran cereal (both from The Quaker Oats Company, Chicago, Illinois). For routine enzyme production, cultures were grown on 1% maize bran plus 0.2% sucrose.

Enzyme assays

Routine enzyme assays were done using a reducing sugar assay (Lever 1972) and barley β-glucan (Sigma G6513) as substrate. Laminarin (Sigma L9634) was used to test for β-1,3-glucanase activity and Avicel PH-101 (Fluka 11365), high viscosity CM-cellulose (Sigma C5013), low viscosity CM-cellulose (Sigma C5678), α-cellulose (Sigma C8002), and microgranular Whatman cellulose were used for β-1,4-glucanase activities. Assays were performed using 0.2% substrate, except laminarin which was used at 0.1%, in 50 mM sodium acetate buffer, pH 5.0, at 37°C for 30 min with 10 to 20 µl enzyme. When cellulosic substrates were used the assay was performed for 17 h at 37°C. After heating the reaction mixtures at 100°C for 10 min, 200 µl of each reaction was placed in a 96-well

microtiter plate, cooled to 22°C, and the absorbance at 410 nm read in an ELISA plate reader (Bio-Tek). One unit of activity is defined as one nmol glucose released per µl enzyme per min at 37°C.

Viscometric assays were performed with a number 200 tube viscometer and 0.5% barley β-glucan in 50 mM sodium acetate, pH 5.0, at 37°C. Viscometry readings were taken every 3 min for 20 min.

Protein purification

Concentration and purification of MLGase activities from culture filtrates through low-pressure DEAE-cellulose chromatography and dialysis was by the method of Murphy and Walton (1996) except that the 25 mM sodium acetate buffer was adjusted to pH 4.0. Fractionation on a polysulfylethyl aspartamide cation exchange HPLC column (The Nest Group, Southboro, Massachusetts) was with a 30 min linear gradient from buffer A (25 mM sodium acetate, pH 4.0) to buffer B (25 mM sodium acetate, pH 4.0, plus 0.4 M KCl) at 1 ml/min. The peak of UV absorption (280 nm) containing Mlg1a and Mlg1b was collected, adjusted to 1.7 M ammonium sulfate, and applied to a hydrophobic-interaction HPLC (HI-HPLC) column (Biogel TSK-Phenyl-5PW, BioRad, Richmond, California) (Murphy and Walton, 1996). Fractions containing Mlg1a and Mlg1b activity were then individually passed over a gel filtration HPLC column (Beckman Ultraspherogel SEC3000, 7.5 x 300 mm). Purified Mlg1a and Mlg1b were lyophilized and sequenced directly from the N-terminus, as well as after digestion with trypsin and separation of peptides by microbore HPLC, by automated Edman degradation. Mlg2 was purified using

the same methods as for Mlg1a and Mlg1b through HI-HPLC. The fractions containing Mlg2 activity were then fractionated by SDS-PAGE (12% acrylamide), transferred to ProBlot (Applied Biosystems, Foster City, California) (Matsudaira, 1987), stained with 0.1% Coomassie R-250 in 40% methanol, and destained with 50% methanol. Mlg2 was excised from the blot and digested with trypsin. Resulting peptides were separated by HPLC and sequenced by automated Edman degradation.

Methods of SDS-PAGE and glycoprotein detection by periodic acid/Schiff staining were as described (Hames and Rickwood, 1981; Strömqvist and Gruffman, 1992). Determination of pH optima for the three enzymes was as described (Murphy and Walton, 1996).

Nucleic acid manipulations

DNA and RNA were isolated as described by Pitkin et al. (1996) and Chomczynski and Sacchi (1987), respectively. Genomic and cDNA library screening, probe labeling, DNA blotting, and hybridization have been described (Scott-Craig et al., 1990; Murphy and Walton, 1996). Sequencing with gene-specific primers was performed by automated fluorescent sequencing at the MSU-DOE-PRL Plant Biochemistry Facility using an Applied Biosystems 373A Sequencer for analysis of the products. The transcription start site of *MLG1* was determined using the Amplifinder RACE kit (Clonetech, Palo Alto, California) (Frohman et al., 1988). First strand cDNA synthesis was primed with the reverse complement oligonucleotide GAAGGCGGGCCAAGAGCC (starting at nucleotide 727, Figure 10). PCR primer CGTGCGTGGGATCAGCGATATCTTC

(reverse complement) (starting at nucleotide 439, Figure 10) and the "anchor" primer provided with the RACE kit were used to amplify the 5' end of the *MLG1* transcript.

Cloning of MLG1

PCR conditions used to amplify *MLG1* and the protocol used to clone the PCR fragments were as described (Murphy and Walton, 1996). Template DNA that generated the 340-bp product was DNA isolated from phage lysate of a cDNA library prepared from mRNA from *C. carbonum* grown on maize cell walls (Pitkin et al., 1996). Total genomic DNA was used as a template for the reaction that yielded the 460-bp product. The PCR primers ATHGAYACNTAYGAYGC and ATRTCDATYTCNCCYTG (H = A,C, or T; Y = C or T; N = any nucleotide; R = A or G; D = A,G, or T) corresponding to the sequences IDTYDA and QGEIDI (Table 2 and Figure 10), respectively, were used at an annealing temperature of 55°C for PCR amplification of a fragment of *MLG1*. Oligonucleotide sequence AARTTYAAYTTYGARGA, corresponding to amino acid sequence KFNFED (Table 2, Figure 10), was end-labeled (Sambrook et al., 1989) and hybridized at 45°C to the PCR products for confirmation that a fragment of *MLG1* had been amplified. The *MLG1* PCR products were cloned into pBluescript II SK+ at the *Sma*I restriction site and sequenced. A 7.0-kb *Bam*HI *MLG1* genomic fragment from a EMBL3 phage that hybridized to the *MLG1* cDNA was subcloned into pBluescript II SK+.

Targeted gene disruption of MLG1

The transformation vector was made by digesting the *MLG1* cDNA clone, pC4-2.1, with *Xho*I/*Sal*I to liberate a 345-bp fragment internal to the *MLG1* locus (Figure 10), treating the fragment with T4 DNA polymerase, and ligating it into the *Sma*I restriction site of pHYG1 (Sposato et al., 1995). The resulting vector, pJM5, was linearized at the unique *Sma*I restriction site (Figure 10) and used to transform wild type *C. carbonum* strain 367-2A.

Protoplast isolation and transformation have been described (Scott-Craig et al., 1990; Apel et al., 1993). Transformants were selected for their ability to grow on 100 units per ml of hygromycin B (Calbiochem, La Jolla, California). Two rounds of single spores were isolated to ensure nuclear homogeneity.

For pathogenicity tests on germinating young seedlings, ten seeds of susceptible maize cultivar Pr (genotype *hm/hm*) and ten seeds of resistant cultivar Pr1 (genotype *Hm/Hm*) were surface sterilized 10 min in 10% (v/v) commercial sodium hypochlorite (household bleach), washed five times with water, imbibed with water for 17 h, and planted at a depth of 2 cm in soil in 13-cm diameter clay pots. The pots were watered with 100 ml of 10^5 fresh conidia per ml. Germination and growth were monitored daily for 10 days. Pathogenicity tests on 14-day old maize seedlings were performed by inoculating leaves of susceptible hybrid Pr X K61 (genotype *hm/hm*) and resistant cultivar Great Lakes (genotype *Hm/Hm*) with a fine mist of 10^4 conidia per ml suspended in 0.1% Tween 20. Disease symptoms were observed daily until the plants were dead.

CHAPTER 3

Jenifer M. Görlach, John W. Pitkin, and Jonathan D. Walton. 1997. Investigation of the involvement of two proteases in virulence of the fungal pathogen, *Cochliobolus carbonum*, on maize (in preparation).

Chapter 3

INVESTIGATION OF THE INVOLVEMENT OF TWO PROTEASES IN VIRULENCE OF THE FUNGAL PATHOGEN, *COCHLIOBOLUS CARBONUM*, ON MAIZE

Abstract

This paper describes the cloning and disruption of *ALP2*, the gene encoding a subtilisin-like protease secreted by *Cochliobolus carbonum*. The *ALP2* gene was isolated by PCR amplification using primers based on N-terminal amino acid sequence and conserved amino acid sequences of subtilisins. A genomic and partial cDNA clone were isolated and sequenced. Four introns were found which conform with 5' and 3' splice site sequences from other *C. carbonum* genes. Transformation-mediated gene replacement of *ALP2* was performed in wild type *C. carbonum* and in an *alp1* mutant strain. Total protease activity in crude culture filtrates of the *alp2* and *alp1/alp2* mutants grown on 1% collagen were approximately 130% and 41%, respectively, of wild type activity, yet fungal mat dry weights were similar to wild type. Chromatographic analysis of culture filtrates of *alp2* mutants indicated that one peak of protease activity, corresponding to Alp2, was missing. The *alp1/alp2* double mutant lacked three peaks of protease activity, corresponding to Alp1a, Alp1b, and Alp2. Disease symptoms on maize of the *alp2* and *alp1/alp2* were similiar to wild type.

Introduction

Proteases have been implicated in a number of plant-fungal (Rauscher et al 1995, Hislop et al. 1982, Ries and Albersheim 1973, Choi et al. 1993, Ball et al. 1991), plant-bacterial (Dahler et al. 1990, Dow et al. 1990), human-fungal (Monod et al. 1993, Tang et al. 1993, Larcher et al. 1996), human-bacterial (Grenier 1996), and fungal-insect (St. Leger 1995, Bonants et al. 1995) host-pathogen interactions. A possible role for proteases during pathogenesis of these organisms on their respective hosts has been demonstrated in only a few cases (Dow et al. 1990, Ball et al. 1991, St. Leger 1995).

The functions of these proteases and their proposed involvement in virulence may vary depending on the pathogen and host. Proteases may degrade host proteinase inhibitors (Grenier 1996) or proteinaceous substrates as a means of invasion or nutrient gathering for proliferation of growth. Alternatively, proteases could have a role in the propagation of the pathogen. Conidial discharge of the fungus *Conidiobolus coronatus* is regulated by a secreted subtilisin-like protease (Phadatare et al. 1992, Phadatare et al. 1989). Protease inhibitors affected conidial discharge, induction of the protease by casein promoted early conidial discharge, and a mutant with reduced conidial discharge had reduced protease activity (Phadatare et al. 1989).

To evaluate the role that proteases play in virulence of the fungal pathogen *Cochliobolus carbonum* on maize, we have constructed null mutants deficient in protease production. In a previous report, Murphy and Walton (1996) showed that there were at least three alkaline proteases secreted by *C. carbonum* and that a gene disruption of *ALP1*, which encodes two of these proteases, has no significant effect on virulence or growth of

this fungus. As these proteases may have redundant functions during growth and host invasion, we have cloned the gene encoding the third protease (Alp2) secreted by *C. carbonum* and generated *alp2* and *alp1/alp2* mutants.

Results and Discussion

Isolation and characterization of ALP2

Purification and characterization of the Alp2 subtilisin-like protein has been described (Murphy and Walton 1996). The N-terminal amino acid sequence of Alp2 was determined to be AYTTQSSAPWGLARISSQXRGTTGYXXDD (Figure 14), where X was an unknown amino acid. Four degenerate oligonucleotides were designed to amplify by PCR the DNA containing the gene encoding Alp2, *ALP2*. A 256-fold degenerate oligonucleotide based on the N-terminal amino acid sequence AYTTQS (see Figure 14) was used in combination with oligonucleotides based on highly conserved internal amino acid sequences of fungal subtilisin-like proteases. The three additional 256-fold degenerate oligonucleotides were based on the sequences TYGVAK (a sense and anti-sense oligonucleotide were designed) and MATPH$^{I}/_{V}$ (see Figure 14). The primers, in appropriate combinations, were used in PCR with either genomic DNA or DNA isolated from a *C. carbonum* cDNA library as templates. No PCR products were amplified when the N-terminus-derived oligonucleotide was used in combination with any other primer. However, a 450-bp cDNA-derived PCR product was obtained when the combination of primers based on the internal amino acid sequences TYGVAK and MATPH$^{I}/_{V}$ were used. BLASTX analysis of the PCR product sequence showed a 78% identity to a *Paecilomyces*

lilacinus serine protease, 74% identity to a *Fusarium* alkaline protease, and 67% identity to proteinase K. *C. carbonum* genomic and cDNA libraries were hybridized with the 450-bp PCR fragment. A 1.45-kb partial cDNA clone (C2-1.1) and a 8.0-kb *Pst*I/*Eco*RI genomic fragment (ALP2G1)(see Figure 16) were isolated and sequenced. Even though the PCR product was derived from conserved internal amino acid sequences, the predicted amino acid sequence of the cloned gene and the mature N-terminus of Alp2 were nearly identical.

Figure 14 shows the nucleotide sequence of *ALP2* and its deduced amino acid sequence. Three independent RACE products (Frohman et al. 1988) were sequenced and compared to determine the *ALP2* transcription start site. The context of the codon for the first methionine (CAACATGAAG) (Figure 14) is similar to the consensus for *Neurospora crassa* translation initiation (CAMMATGGCG where M = C or A) (Edelmann and Staben 1994). Typical lower eukaryotic promoter sequences, TATAA and CAAC (Gurr et al. 1987) are located 33 and 59 bp, respectively, upstream of the transcription start site (Figure 14). Four introns were found when genomic and cDNA clones of *ALP2* were compared (Figure 14). The intron borders, splice junction sites and lengths are consistent with those from other genes of *C. carbonum* and filamentous fungi (Figure 14 and Table 3). A characteristic polyadenylation signal sequence, AATAAA (Gurr et al. 1987), could be found 33 bp upstream of the polyadenylation site (Figure 14).

The *ALP2* gene is predicted to encode a 40.4-kD polypeptide, which is slightly larger than the mature protein (38-kD) as determined by SDS-PAGE (Murphy and Walton 1996). Alp2 is predicted to contain one N-glycosylation site ($\underline{N}^{242}$-M-S) based on the

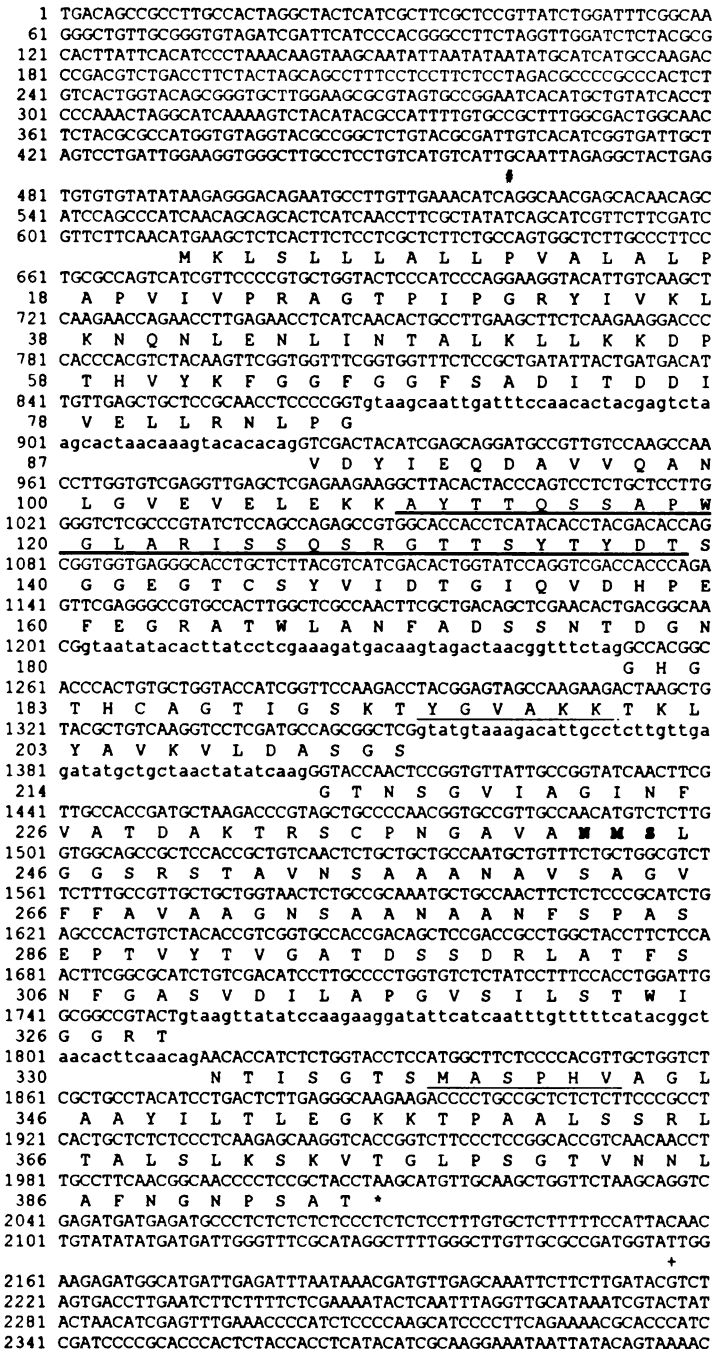


Figure 14. Sequence of *ALP2*. The experimentally determined N-terminal amino acid sequence of Alp2 is double-underlined. Sequences conserved among other fungal subtilisin-like proteases, used to design degenerate oligonucleotides for PCR amplification of *ALP2*, are indicated by single-underlining. Introns are designated by lower case lettering. The transcription start site is labeled with # and the polyadenylation site by +. Symbols refer to nucleotides below and amino acid codes refer to the nucleotides above. One predicted N-glycosylation site (starting at amino acid 242) is indicated by bold letters.

TABLE 3. Intron features *C. carbonum* genes.

Gene	*Intron #*	*5' splice site*	*branch site*	*3' splice site*	*length (bp)*
ALP2[a]	1.	T^GTAAGC	CACTAAC	CAG	54
	2.	G^GTAATA	GACTAAC	TAG	50
	3.	G^GTATGT	TGCTAAC	AAG	50
	4.	T^GTAAGT	GGCTAAC	CAG	62
ALP1[b]	1.	G^GTAAGT	AACTAAC	CAG	74
MLG1[c]	1.	C^GTATGT	TTCTGAC	CAG	57
	2.	T^GTATGT	CGCTAAC	CAG	64
	3.	G^GTGAGT	TGCTAAC	TAG	55
XYL1[d]	1.	G^GTATGG	CACTAAC	CAG	53
XYL2[e]	1.	G^GTAGGT	TACTAAT	TAG	53
	2.	G^GTAAGT	GACTGTT	TAG	56
XYL3[e]	1	G^GTAAGC	TACTAAC	CAG	60
CEL1[f]	1.	G^GTAAGT	TTCTAAC	CAG	55
PGN1[g]	1.	G^GTAAGC	GACTAAC	CAG	57
PGX1[h]	1.	G^GTTCGT	TACTAAC	CAG	51
	2.	G^GTGAGT	AACTGAC	TAG	63
	3.	G^GTACGT	TGCTAAC	TAG	70
EXG2[i]	1.	C^GTAAGT	TGCTGAC	TAG	70
	2.	C^GTAAGT	TGCTAAC	CAG	55
	3.	G^GTAAGC	TGCTAAT	CAG	47
	4.	G^GTATGT	TACTGAC	CAG	53
	5.	C^GTGAGC	AACTGAC	CAG	56
TOXA[j]	1.	A^GTAAGT	TACTAAT	TAG	61
	2.	A^GTAAGT	TTCTAAC	TAG	74
	3.	T^GTTAGT	TACTGAT	TAG	81
TOXC[k]	1.	G^GTAGAG	AGCTGAC	CAG	52
fungal consensus[l]	typically 0-4	G^GTAAGT	TRCTAAC	YAG	50-700

- R = A or G, Y = C or T
- a, Görlach and Walton 1997b; b, Murphy and Walton 1996; c, Görlach et al. 1997a; d, Apel et al. 1993; e, Apel-Birkhold and Walton 1996; f, Sposato et al. 1995; g, Scott-Craig et al. 1990; h, Scott-Craig et al. 1996; i, J.M. Görlach unpublished data; j, Pitkin et al. 1996, k, Ahn and Walton 1997; l, Ballance 1991, Edelmann and Staben 1994.

information compiled by Gavel and von Heijne (1990) (N-X-$^{T}/_{S}$-Y, where X and Y are typically not proline). Comparison of the experimentally determined N-terminus with the predicted N-terminal amino acid sequence (Figure 14) reveals that the Alp2 precursor is probably 109 amino acids longer than the mature protein. A signal peptide cleavage site, as predicted by SignalP v1.1 (Neilsen et al. 1997), is ALA↓LP (residues 13 to 17, Figure 14).

The signal peptide is followed by a 94 amino acid propeptide sequence characteristic of subtilisins. Interestingly, when the deduced amino acid sequence of *ALP2* is compared to other subtilisins (Figure 15), Alp2 has an additional 10 amino acids ending in KK preceding the mature N-terminus. Out of 27 fungal and bacterial subtilisins retrieved from GenBank, only three enzymes had a dibasic peptide preceding the experimentally determined N-terminus (Geremia et al. 1993, Sato et al. 1994, Davidow et al. 1987). Instead of, or in addition to, autocatalytic processing for activation (Power et al. 1986), the dibasic amino acid (KK) sequence may indicate that Alp2 is further processed by an enzyme similar to the endoproteases Mkc1, Kex2p, and Yap3p found in the secretory pathway of *Saccharomyces cerevisiae* (Komamo and Fuller 1995, Bourbonnais et al. 1993, Julius et al. 1984). An additional KK processing site may lie between residues 55 and 56. Multiple precursor processing sites has been described by Matoba et al. (1988) for a subtilisin secreted by *Yarrowia lipolytica*.

A comparison of the mature protein sequence of Alp2, deduced from the *ALP2* sequence, with subtilisins from other filamentous fungi (Figure 15) showed 63% identity (75.8% similarity) with a *Metarhizium anisopliae* cuticle-degrading protease (PIR

```
                1                                                    50
C. carbonum     MKLSLLLALL PVALALP... ......APVI VPRAGTPIPG RYIVKLKNQN
M. anisopliae   MHLSALLTLL PAVLAAPATI GRRAEPAPLF TPQAESIIAD KYIVKFKDDI
Fusarium sp.    MRLSI.IAVL PLALAAPV.L ....EPAPLL EARGSQPIAG KYIVKLKDTA
T. album        MRLSVLLSLL PLALGAPA.V EQRSEAAPLI EARG.EMVAN KYIVKFKEGS
                :.:: :...: :  :.::  .   :.:.::.   . .   ..  .:::: :.
                51                                                  100
C. carbonum     LENLINTALK LLKKDPTHVY KFGGFGGFSA DITDDIVELL RNLPGVDYIE
M. anisopliae   ARIATDDTVS ALTSKADFVY E.HAFHGFAG SLTKEELKML REHPGVDFIE
Fusarium sp.    KIGIMEATAK ..VANPERVY Q.NVIKGFSA SLCKEEVERL RHDPDVESIE
T. album        ALSALDAAME KISGKPDHVY K.NVFSGFAA TLDENMVRVL RAHPDVEYIE
                     . .    .   .  ::       ::..  .  . .  : :  : :. ::
                101                                                 150
C. carbonum     QDAVVQANLG VEVELEKKAY TTQSSAPWGL ARISSQSRGT TSYTYDTSGG
M. anisopliae   KDAVMRIS.. ........GI TEQSGAPWGL GRISHRSKGS TTYRYDDSAG
Fusarium sp.    QDAIISIN.. ........AI TQQQGATWGL TRISHRQRGS TAYAYDTTAG
T. album        QDAVVTIN.. ........A. .AQTNAPWGL ARISSTSPGT STYYYDESAG
                 ::..             .  . :  :.::: .:::    :. ..: :: ..:
                151     ↓                                ↓        200
C. carbonum     EGTCSYVIDT GIQVDHPEFE GRATWLANFA DSSNTDGNGH GTHCAGTIGS
M. anisopliae   QGTCVYIIDT GIEASHPEFE GRATFLKSFI SGQNTDGHGH GTHCAGTIGS
Fusarium sp.    QGACAYVIDT GVEDTHPEFE GRAKQIKTFA ST.ARDGNGH GTHCSGTIGS
T. album        QGSCVYVIDT GIEASHPEFE GRAQMVKTYY YS.SRDGNGH GTHCAGTVGS
                .:.: :.::: :..  ::::: ::: . .   .  :: :: ::::.::.::
                201                                                 250
C. carbonum     KTYGVAKKTK LYAVKVLDAS GSGTNSGVIA GINFVATDAK TRSCPNGAVA
M. anisopliae   KTYGVAKKAK LYGVKVLDNQ GSGSYSGIIS GMDYVAQDSK TRGCPNGAIA
Fusarium sp.    KTYGVAKKVS IFGVKVLDDN GSGSLSNVIA GMDFVASDYR SRNCPRGVVA
T. album        RTYGVAKKTQ LFGVKVLDDN GSGQYSTIIA GMDFVASDKN NRNCPKGVVA
                .:::::::   ...:::::   ::: : .:. :...:: :    : :: : .:
                251                                                 300
C. carbonum     NMSLGGSRST AVNSAAANAV SAGVFFAVAA GNSAANAANF SPASEPTVYT
M. anisopliae   SMSLGGGYSA SVNQGAAALV NSGVFLAVAA GNDNRDAQNT SPASEPSACT
Fusarium sp.    SMSLGGGYSA TVNQAAARLQ SSGVFVAVAA GNDNRDAANT SPASEPSVCT
T. album        SLSLGGGYSS SVNSAAARLQ SSGVMVAVAA GNNNADARNY SPASEPSVCT
                 .:::::. :. .:: .::     .::  :::: ::   .: :  :::::::.  :
                301                                               ↓  350
C. carbonum     VGATDSSDRL ATFSNFGASV DILAPGVSIL STWIGGRTNT ISGTSMASPH
M. anisopliae   VGASAENDSR SSFSNYGRVV DIFAPGSNVL STWIVGRTNS ISGTSMATPH
Fusarium sp.    VGATDSSDRR SSFSNYGRAL DIFAPGTDIT STWIGGRTNT ISGTSMATPH
T. album        VGASDRYDRR SSFSNYGSVL DIFGPGTSIL STWIGGSTRS ISGTSMATPH
                :::.   :   ..::::.:  . :: .::  .  :::: : : . ::::::::.::
                351                                                 400
C. carbonum     VAGLAAYILT LEGKKTPAAL SSRLTALSLK SKVTGLPSGT VNNLAFNGNP
M. anisopliae   IAGLAAYLSA LQGKTTPAAL CKKIQDTATK NVLTGVPSGT VNYLAYNGA*
Fusarium sp.    IAGLGAYLLA LEG.GSASTI CARIQTLSTK NAISGVPSGT VNYLAFNNAT
T. album        VAGLAAYLMT L.GKTTAASA CRYIADTANK GDLSNIPFGT VNLLAYNNYQ
                .:::.::. . : :: ....     .   . :   .. .: :: :: ::.:
                401
C. carbonum     SAT*
M. anisopliae   ....
Fusarium sp.    *...
T. album        A*..
                .
```

Figure 15. Comparison of *ALP2* predicted amino acid sequence with other fungal subtilisin-like proteases. The sequences of *C. carbonum*, *Metarhizium anisopliae* (St. Leger et al. 1992), *Fusarium* sp. (Morita et al. 1994), *Tritirachium album* (Gunkel and Gassen 1989) were compared using PILEUP (Program Manual, 1994). Similar and identical amino are indicated by . and :, respectively. The mature N-terminal amino acids are underlined, the putative dibasic endopeptidase processing site is in italics, residues in the catalytic triad are indicated by ↓, and stop codons are designated with *.

S22387, St. Leger et al. 1992), 62% identity (72.5% similarity) with a *Fusarium* sp. S-19-5 alkaline protease (PIR JC2142, Morita et al. 1994), and 60% identity (71.7% similarity) with *Tritirachium album* proteinase K (PIR S02142, Gunkel and Gassen 1989). The catalytic triad found in serine proteases is conserved in Alp2 (Asp-159, His-190, Ser-345; Figure 15).

Transformation-mediated gene replacement of ALP2

To investigate whether proteases have a role in the virulence of *C. carbonum*, we generated *alp2* and *alp1/alp2* mutants. A gene replacement plasmid, JMM80, containing *amdS*, a gene required for acetamide utilization, flanked by 5' and 3' *ALP2* sequences was constructed and used to transform both *C. carbonum* wild type (367-2A) and *alp1* mutant (T515-3A, Murphy and Walton 1996) strains. Transformants able to use acetamide as the sole nitrogen source were single-spore isolated. The mutant lacking Alp2, designated T650-1A, and the *alp1/alp2* mutant, designated T651-2A, were further analyzed. Figure 16 depicts the restriction map for the wild type *ALP1* locus and *ALP1* gene disruption (Figure 16A) and the wild type *ALP2* locus and *ALP2* gene replacement (Figure 16B). DNA blot analysis of genomic DNA isolated from wild type (367-2A), an *alp1* mutant (T515-3A, Murphy and Walton 1996), an *alp2* mutant (T650-1A), and an *alp1/alp2* mutant (T651-2A) indicates that the *ALP1* locus is intact in 367-2A and T650-1A and disrupted in T515-3A and T651-2A (Figure 16C). Likewise, the *ALP2* locus is intact in 367-2A and T515-3A and replaced by *amdS*, as indicated by the absence of the internal *Sal*I fragments, in T650-1A and T651-2A (Figure 16C).

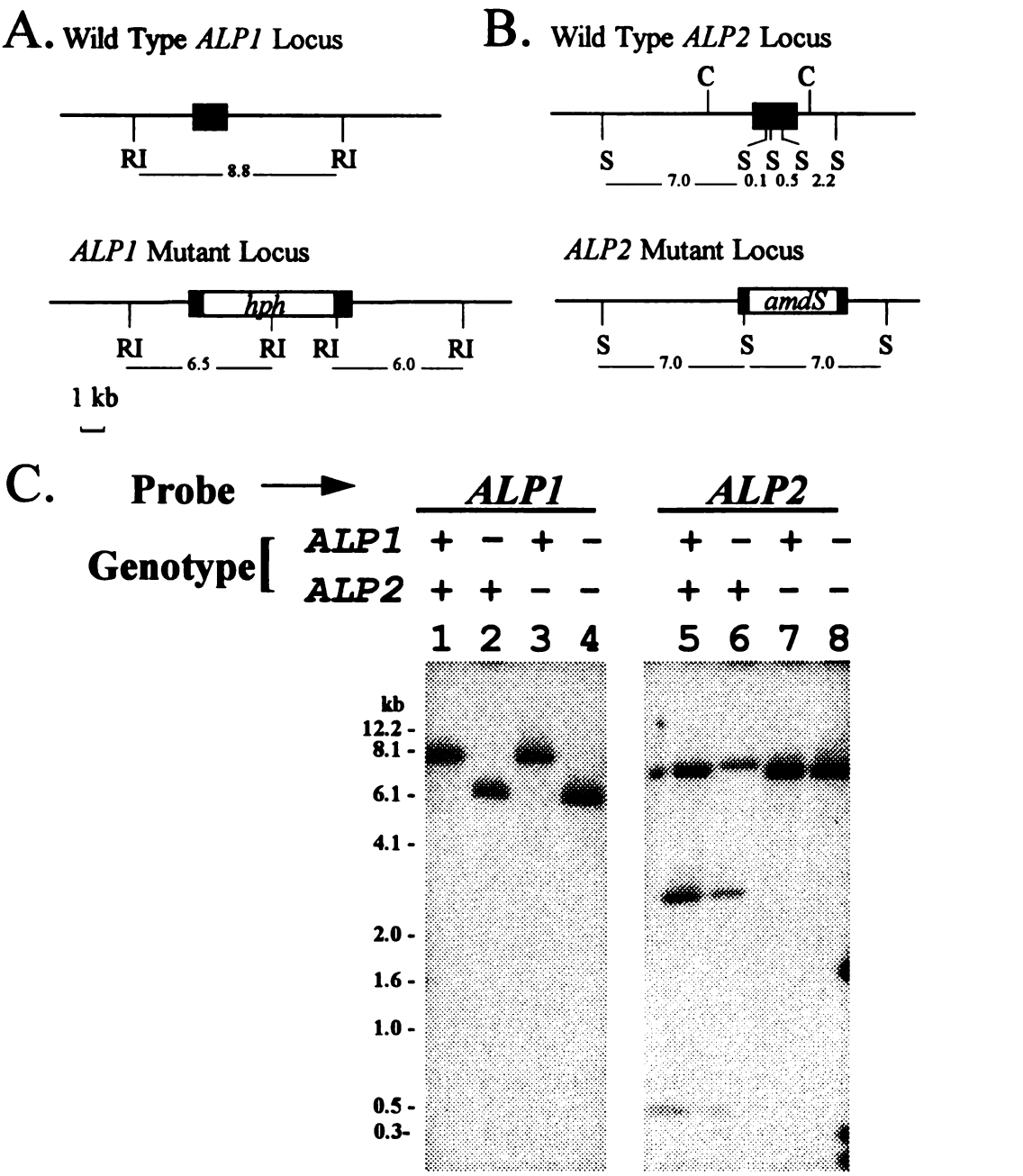


Figure 16. Restriction maps of the wild type and mutant loci of *ALP1* and *ALP2*. **A,** *Eco*RI restriction map of the *ALP1* wild type and mutant loci depicting the result of a gene disruption of *ALP1*. **B,** *Sal*I restriction map of the *ALP2* wild type and mutant loci depicting the result of an *ALP2* gene replacement with *amdS*. Predicted *Eco*RI and *Sal*I fragment sizes are indicated (in kb). *ALP1* and *ALP2* open reading frames are indicated by shaded boxes. **C,** DNA blot analyses comparing wild type (Lanes 1 and 5), *alp1* mutant (Lanes 2 and 6), *alp2* mutant (Lanes 3 and 7), and *alp1/alp2* double mutant (Lanes 4 and 8). Identical DNA blots were hybridized with a cDNA of *ALP1* and the 4.5 kb *Cla*I fragment of *ALP2*. S, *Sal*I; RI, *Eco*RI; C, *Cla*I.

Protease production in culture was measured for *alp2* and *alp1/alp2* mutants. Total protease activity in crude culture filtrates of the *alp2* and *alp1/alp2* (T651-2A) mutants grown on 1% collagen was approximately 130% and 41%, respectively. The increase in total protease activity in the *alp2* mutant may be due to an increase in other protease activities to compensate for the loss of Alp2. Growth of *alp2* and *alp1/alp2* mutants, as determined by dry weight, in liquid culture containing 1% collagen or 1% macerated maize cell walls was comparable to wild type; therefore, Alp1 and Alp2 are not required for *in vitro* growth on collagen or maize cell walls. There was also no observable delay in sporulation in culture as a result of the mutations.

In addition to their involvement in catabolism, secreted proteases may be involved in activating other secreted cell wall degrading enzymes (Rypniewski et al. 1993, Moormann et al. 1993, Drapeau 1978). Therefore, for both the *alp2* and *alp1/alp2* mutants we investigated the activities of other *C. carbonum*-secreted enzymes. Like *alp1* mutants (Murphy and Walton 1996), total exo-1,3-β-glucanase and polygalacturonase activities were not significantly changed in *alp2* and *alp1/alp2* mutants. However, total cellulase, endo-β1,4-xylanase, and 1,3-1,4-β-glucanase activities were reduced to 8%, 85%, and 53%, respectively, in the *alp1/alp2* double mutant but not in *alp1* and *alp2* single mutants Therefore, Alp1 and Alp2 may be functionally redundant enzymes involved in precursor processing.

Wild type and *alp2* and *alp1/alp2* mutant strains were grown in liquid culture supplemented with 1% collagen; protease activities from each were purified in parallel as described (Murphy and Walton 1996). The *alp2* mutants lacked one peak of protease

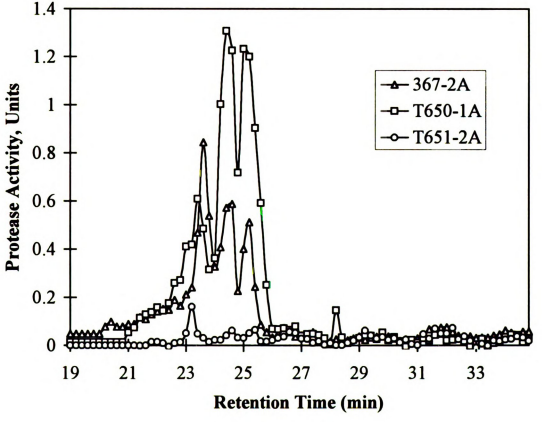


Figure 17. Protease activity in fractions collected from cation exchange HPLC analysis of proteins from culture filtrates of wild type (367-2A), an *alp2* mutant (T650-1A), and an *alp1/alp2* mutant (T651-2A). Prior to HPLC analysis, proteins were passed over a low pressure anion exchange column, concentrated, and dialyzed. One unit of protease activity is defined as 1.0 OD_{410} per 15 µL enzyme fraction at 45°C for 45 min.

activity corresponding to Alp2 (Figure 17) and therefore *ALP2* encodes Alp2. The *alp1/alp2* mutants lacked all three peaks of protease activity corresponding to Alp2, Alp1a, and Alp1b (Figure 17). One substantial residual protease activity was detected (Alp3, retention time 23.2 min, Figure 17) which, based on protease inhibition assays, appears to be another subtilisin-like protease.

To test the potential role that proteases may play during pathogenesis of *C. carbonum* on maize, 21-day-old maize seedlings were inoculated with 367-2A and *alp1*, *alp2*, and *alp1/alp2* mutants. There were no distinguishable differences in lesion onset or morphology between wild type and any of the mutants on PR (resistant) or PR1 (susceptible) maize cultivars. As Alp1a, Alp1b, and Alp2 constitute the majority of proteases secreted by *C. carbonum in vitro* we conclude that proteases alone probably do not significantly contribute to the overall virulence of *C. carbonum* on maize.

Materials and Methods

Fungal growth and maintenance

Fungal strains were routinely grown on V8-juice agar for production of conidia. For protease production, approximately 4.5×10^5 spores were inoculated into a 1-L Erlenmeyer flask containing 200 mL mineral salts medium plus 0.1% yeast extract, trace elements, and 1% type 1 collagen (Sigma C-9879) or 1% macerated maize cell walls (Sposato et al. 1995) (van Hoof et al. 1991). For protein purification cultures were incubated at 22 to 25°C with continuous shaking at 130 rpm for 3.5 days. For growth studies cultures were incubated for seven days at 22 to 25°C without shaking.

Protein purification and enzyme assays

Purification of secreted proteases during mutant analysis was previously described (Murphy and Walton 1996). Serine protease activity was measured as described (Murphy and Walton 1996). Units of enzyme activity are defined as OD_{410} per 15 μL enzyme fraction at 45°C for 45 min.

Nucleic acid manipulation and sequence analysis

Fungal genomic DNA was isolated as described by Pitkin et al. (1996). RNA was isolated as described (Chomczynski and Sacchi 1987). The 5' end of the *ALP2* transcript was determined using the Amplifinder RACE kit (Clonetech, Palo Alto, CA) (Frohman et al. 1988). The oligonucleotide used for reverse transcription was GCTCGAACACT GACGGC (starting at nucleotide 1182, see Figure 14). The PCR amplification step was performed using the "anchor" primer provided by the RACE kit in combination with the primer CGGTGGTTTCGGTGGTT (starting at nucleotide 799, see Figure 14).

ALP2 sequence was generated by automated fluorescent sequencing at the MSU-DOE-PRL Plant Biochemistry Facility using an Applied Biosystems (Foster City, CA) Catalyst 800 for Taq cycle sequencing and an Applied Biosystems 373A Sequencer for analysis of the products.

Isolation of ALP2

The reaction mixure and PCR conditions for amplifying DNA containing the *ALP2* gene were as described (Murphy and Walton 1996), with the exception that annealing was

performed at 45°C. Template DNA was genomic DNA and DNA isolated from phage lysate of a *C. carbonum* cDNA library (Pitkin et al. 1996). PCR primers were as follows: GCNTAYACNACNCARTC, corresponding to the Alp2 N-terminal amino acid sequence AYTTQS, and ATGGCNACNCCNCAYRT and TAYGGNGTNGCNAARAA, corresponding to the subtilisin internal amino acid sequences MAPTH$^{I}/_{V}$ and TYGVAK, respectively (where N = any nucleotide, R = A or G, and Y = T or C). PCR products were cloned as described by Murphy and Walton (1996) into the *Sma*I restriction site of pBluescriptII SK^{+}.

Screening of the genomic and cDNA libraries, DNA blotting, probe labeling, and hybridization protocols have been described (Scott-Craig et al. 1990, Sposato et al. 1995). DNA was blotted to Nytran (Schleicher and Schuell, Keene, New Hampshire).

Disruption of ALP2

The *ALP2* gene replacement vector was generated by first subcloning the 4.5-kb *Cla*I fragment (Figure 16A) into *Acc*I-digested pBluescriptII SK^{+} lacking *Kpn*I, resulting in pJMM78. The gene encoding acetamidase, *amdS*, was liberated from pAMD-72 (Pitkin et al. 1996) with *Kpn*I and *Sal*I and introduced into pJMM78 digested with *Kpn*I and *Sal*I such that 900-bp of the ALP2 open reading frame was replaced with a 4.0-kb fragment of *amdS* (see Figure 16A). The resulting plasmid, pJMM80, was than digested with *Xho*I and *Eco*RI to liberate vector sequence from the insert and the entire digest was introduced

into both a *C. carbonum* wild type strain, 367-2A, and a hygromycin B-resistant (Hyg^R) *alp1* mutant strain, T515-3A (Murphy and Walton 1996).

Isolation and transformation of protoplasts has been described (Scott-Craig et al. 1990, Apel et al. 1993). Transformants were selected for their ability to utilize acetamide (Ace^+) as a sole nitrogen source (Pitkin et al. 1996). The *alp1/alp2* mutants were selected for their ability to grow on acetamide plates (Hynes et al. 1983) and V8-juice agar plates containing 100 units/mL of hygromycin B (Calbiochem). To ensure nuclear homogeneity, Ace^+ (*alp2* mutants) transformants and Ace^+/Hyg^R (*alp1/alp2* mutants) transformants were single-spore isolated as described by Pitkin et al. (1996).

Pathogenicity was measured by inoculating 21-day-old PR1 (susceptible) and PR (resistant) maize seedlings with 10^4 conidia/ml in 0.1% Tween-20. Disease symptoms were analyzed until the plants were dead (approximately 4 days).

CONCLUSION

Microscopic and genetic evidence suggests that *Cochliobolus carbonum* may require cell wall depolymerases to penetrate the maize cell wall. However, which cell wall degrading enzyme is (are) essential to the process of cell wall degradation is not known.

This study focused on investigating the role that several different plant cell wall degrading enzymes have during the colonization of maize by *C. carbonum*. Three proteases (Alp1a, Alp1b, Alp2) and three 1,3-1,4-β-glucanases (Mlg1a, Mlg1b, Mlg2) were purified and the corresponding genes cloned. Gene disruption experiments were performed to generate *alp1*, *alp2*, *alp1/alp2*, and *mlg1* mutants. Each mutant was tested for its ability to cause lesions on maize leaves. The result of these experiments indicated that each enzyme or enzyme class (for proteases only) is not required for this fungus to penetrate the maize wall. One can not say, however, that these mutations do not have some effect that we are unable to detect in the virulence assays we conduct under artificial greenhouse conditions. These mutations may, over generations, decrease the fitness of this organism in the field.

Future Objectives

One hurdle that has been encounted in the search for the key enzyme(s) involved in host wall penetration is redundancy. The second has been the uncertainty of whether the

enzymes being investigated in *in vitro* studies are being expressed *in planta*. Several approaches could be implemented to overcome these problems. One approach would be to identify and disrupt genes encoding transcription factors required for cell wall degrading enzyme expression. It is possible that one transcription factor may either regulate all genes encoding one enzyme class or genes induced upon exposure to a particular carbon source. As *XYL3* from *C. carbonum* is expressed only *in planta*, one could identify transcription factors specific for *in planta* gene expression. Gene disruptions in several of these transcription factors could eliminate the expression of a significant number of wall depolymerases.

A second approach is to alter the processing of the secreted enzymes such that once secreted they are inactive. When one compares the experimentally determined mature N-termini of the cell wall degrading enzymes purified from *C. carbonum* (Table 4, indicated by #) to the mature N-termini predicted by SignalP v1.1 (Nielsen et al. 1997), one sees that several of these enzymes do not have typical signal peptides sequences. In addition, there are *C. carbonum* enzymes for which the predicted mature N-termini indicate that they would also lack a typical signal peptide sequence (Table 4, indicated by *). The extra amino acids may, however, correspond to processing signals required for enzyme activation prior to secretion. The basic residues preceding the mature N-termini of Alp2, Exg2, Pgn1, and Pme1 and the putative N-termini of Pgx1, Xyl1, Xyl2, Xyl3, and Xyl4 (Table 4) conform with the processing signal sequence recognized by the *Saccharomyces cerevisiae* processing enzymes Kex2p, Yap3p, and Mkc7p (Julius et al. 1984, Komano and Fuller 1995, Bourbonnais et al. 1993). Disruption of *C. carbonum*

TABLE 1. Features of *C. carbonum* cell wall degrading enzyme N-termini.

Protein	*Putative Signal Peptide Sequence*	*N-Terminus*
Alp1	[a]MRFQSMITAALPALVLSAPTPQWDDVPEDS	#IVG
Alp2	[b]MKLSLLLALLPVALALPAPVIVPRAGTPIPGRYIVKLKNQNL ENLINTALKLLKKDPTHVYKFGGFGGFSADITDDIVELLR NLPGVDYIEQDAVVQANLGVEVELEKK	#AYT
Exg1	[c]MRFSSLLACLGAVGIQAAAIPFQRRVDNTTDSGSLDAAQAAA	#AIV
Exg2	[d]MILTKLVSTLSLCAAVLAAPAQQKR	#AVG
Mlg1	[e]MSLKSLFVSAPLLWRGVTALPAGPGSWTHGNSTIVSSSDFSAA	#AAY
Pgn1	[f]MVAYALTSMLLSAGALVAAAPSGLDAR	#DGC
Pme1	[l]MHPTLVFFLSLVAATAAAPAENVLRR	#ASF
Pgx1	[g]MRVTDIISCALLQASIALSTPVEELGAKAVVAKR	*FPP
Xyl1	[h]MVSFTSIITAAVAATGALAAPATDVSLVAR	*QNT
Xyl2	[i]MVSFKSLLLAAVATTSVLAAPFDFLRERDDVNATALLEKR	*QST
Xyl3	[i]MVAFTSVLLGLSAIGSAFAAPVADVPDFEFSGPKHLAAR	*QDY
Xyl4	[j]MKFSLITILSASALVAASPFAEPEAFLEER	*QAA
Cel1	[k]MYRTLAFASLSLYGAARA	*QQV
Cut1	[l]MKFLTLSMMTALAVASPITTRSETA	*LEA

- # Indicates that the N-terminus was experimentally determined
- * Indicates that N-terminus was determined by sequence homology with other enzymes having experimentally determined N-termini.
- Single-underlined amino acids precede putative signal peptide cleavage sites as predicted by SignalP v1.1 (Nielsen et al. 1997).
- Double-underlined amino acids are residues possibly processed by Yap3p, Mkc7p, and Kex2p homologues (Julius et al. 1984, Bourbonnais et al. 1993, Komano and Fuller 1995).
- Bold letters indicate the first three amino acids in the (putative) mature form of the enzyme.
- a, Murphy and Walton 1996; b, Görlach et al. 1997b; c, Nikolskaya et al. 1996; d, C. Caprari unpublished data; e, Görlach et al. 1997a; f, Scott-Craig et al. 1990; g, Scott-Craig et al. 1996; h, Apel et al. 1993; i, Apel-Birkhold and Walton 1996; j, P.C. Apel-Birkhold unpublished data; k, Sposato et al. 1995; l, J.S. Scott-Craig unpublished data.

KEX2, *MKC7*, and *YAP3* homologues may block the activation of several secreted cell wall degrading enzymes. Because of redundancy, not all enzyme activities in one class may be eliminated but the overall reduction of active wall depolymerases may have an affect on host invasion by delaying infection or reducing the total number of successful penetrations. One possible risk with this approach is that mutations in any one of these three loci might be lethal. *S. cerevisiae* mutants deficient in Kex2p are viable (Rogers et al. 1979); however, a mutation in the *KEX2*-homologue of *Schizosaccharomyces pombe*, *KRP*, is lethal (Davey et al. 1994). Another consideration is that a mutation in *KEX2*, *YAP3*, or *MKC7* may alter the processing of a protein, other than a cell wall degrading enzyme, that is essential for growth and/or pathogenesis, making pathogenicity tests difficult to interpret.

To address the concern about whether the genes encoding *C. carbonum* cell wall degrading enzymes are expressed *in planta*, one could use these genes to probe RNA blots of *C. carbonum*-infected maize tissue. With this information, it could be determined which enzymes are being expressed in the compatible (susceptible) or incompatible (resistance) interaction during the early and late stages of the infection process. If there is temporal regulation, one could evaluate whether gene expression correlates with spore germination or differentiation of hyphae into appressoria, penetration pegs, or conidia. Possible common themes of expression may emerge revealing shared upstream regulatory sequences which govern expression *in planta*.

As an alternative one could examine what plant or fungal-derived signals induce the expression of genes such as *XYL3*. One could then identify the fungal receptor which

starts the signal transduction cascade leading ultimately to cell wall degrading enzyme gene expression. Furthermore, one could investigate which proteins are involved in the signal transduction cascade and whether the pathogenicity factor, HC-toxin, is also regulated by this pathway.

As many phytopathology laboratories focus on one particular class of wall depolymerase or have identified many degradative enzymes without obtaining the corresponding genes, little is known about how cell wall degrading enzymes fit into the process of disease. Completing one of the approaches outlined above would increase the collective knowledge about *C. carbonum* wall depolymerases; getting us closer to the goal of understanding the role that cell wall degrading enzymes play in pathogenicity

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PURIFICATION OF A PUTATIVE EXO-1,3-β-GLUCANASE AND MOLECULAR CLONING OF THE GENE, *EXG2*, FROM *COCHLIOBOLUS CARBONUM*

Introduction

The cell walls of ascomycetous fungi are composed of chitin, protein, 1,3-β-glucan, 1,6-glucan, and melanin. During growth, chitin and 1,3-β-glucan are extruded from the cell and cross-linked together (for a review see Wessels 1994). Cell expansion or differentiation might then involve the secretion of hydrolytic enzymes capable of degrading these cell wall components. Both endo-1,3-β-glucanases and exo-1,3-β-glucanases have been described from fungi (van Hoof et al. 1991, Chambers et al. 1993, Hien and Fleet 1983). In *Saccharomyces cerevisiae* these enzymes are synthesized during different stages of development (Larriba et al. 1995, Rey et al. 1979). EXGI and EXGII are constitutively expressed exo-1,3-β-glucanase activities secreted by *S. cerevisiae*. SPR1 (also called SSG1) is a sporulation-specific exo-1,3-β-glucanase (Muthukumar et al. 1993, San Segundo et al. 1993). The *spr1* mutants have mild phenotypes such as reduced ascospore thermoresistance and delayed ascus maturation.

In phytopathogenic organisms, exo-1,3-β-glucanases might be involved in degrading callose deposits that the plant has synthesized in defense against invading pathogens. In an attempt to investigate this, Schaeffer et al. (1994) generated an exo-1,3-β-glucanase

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mutant and evaluated its ability to penetrate maize tissue. The results indicated that the mutant was as pathogenic as wild type *C. carbonum*. However, it must be noted that residual activity remained in the mutant, probably due to a 1,3-1,4-β-glucanase which was able to hydrolyze 1,3-β-glucans.

This paper describes the identification and cloning of a second exo-1,3-β-glucanase secreted by *C. carbonum*. This enzyme was identified as being a major protein in culture filtrate (C. Caprari, unpublished data). N-terminal amino acid sequence identified it as a homolog of EXG1 from yeast. The gene was cloned and sequence data were obtained.

Results and Discussion

Protein purification and characterization

One method to investigate the role of cell wall degrading enzymes in host colonization is to purify proteins that are secreted at high levels, obtain N-terminal amino acid sequence, clone the corresponding gene, and generate null mutants which can be tested for pathogenicity. Using this approach, *C. carbonum* was grown for nine days on macerated corn cell walls, crude culture filtrate was harvested, and proteins were separated by cation-exchange HPLC. A major peak of 1,3-β-glucanase activity was measured in the column flow through. This fraction was further purified by hydrophobic interaction HPLC resulting in three peaks of 1,3-β-glucanase activities. The third peak was identified from its N-terminus as the already characterized Exg1 protein (van Hoof et al. 1991, Schaeffer

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et al. 1994). The second peak, in addition to having 1,3-β-glucanase activity, appeared to contain β-glucosidase activity. Two abundant proteins, 50- and 31-kD, from the second peak were resolved on SDS-PAGE and sequenced at the N-terminus. The N-terminal amino acid sequence of the 31-kD protein, AAYNLIDTYDASNWASKFNFEDIAD, was identical to Mlg1 (Görlach et al. 1997a). The N-terminal amino acid sequence of the 50-kD protein, called p50, VGFNWGSEKIRGVNIGGXLVLEPXITPSI where X is unknown, had 51% identity with an exo-1,3-β-glucanase, XOG1, from *Candida albicans* (Chambers et al. 1993), 73% identity with the sporulation-specific exo-1,3-β-glucanase, SPR1, from *S. cerevisiae* (Muthukumar et al. 1993), and 77% identity with EXG1 from *S. cerevisiae* (Vazquez de Aldana et al. 1991). The first peak of 1,3-β-glucanase activity was a 65-kD protein as determined by SDS-PAGE. The N-terminal amino acid sequence was identified as FVGSATVSSTVLVIARDAISALN, which had no sequence homology to any protein in the database.

Isolation and characterization of the gene encoding p50, EXG2

Based on part of the N-terminal amino acid sequence (VGFNWG, Figure 18) and sequence from the homologous exo-1,3-β-glucanases (QNGFDN, Figure 19) described, two degenerate PCR primers were synthesized. Using genomic DNA and DNA from a *C. carbonum* cDNA library as templates, two PCR products, 550-bp and 430-bp, were amplified, respectively. Sequence analysis of the PCR products confirmed that they

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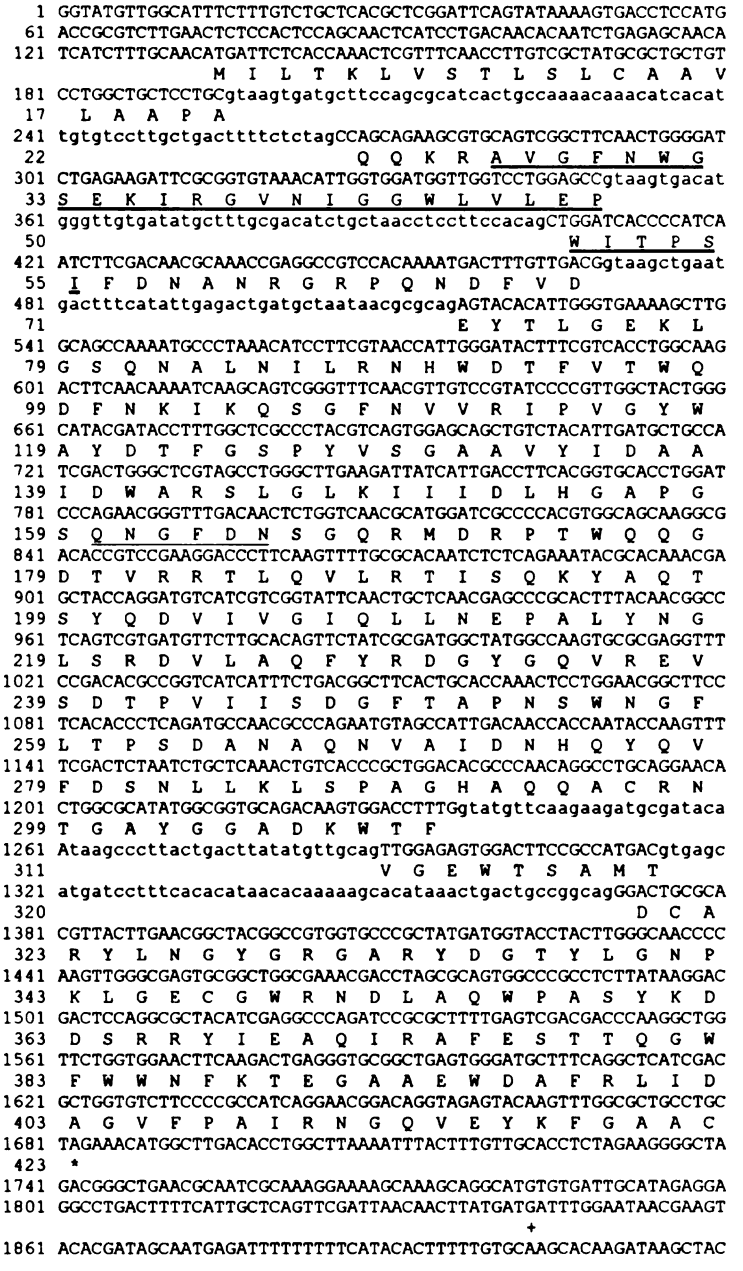

Figure 18. Sequence of *EXG2*. The N-terminal amino acid sequence of the mature protein is indicated by double-underlining. The amino acid residues conserved amongst exo-1,3-β-glucanases and used to design a degenerate oligonucleotide for amplification of *EXG2* are indicated by single-underlining. The introns are designated by lower case letters. The polyadenylation site is labeled with + (symbols refer to the nucleotides below and amino acid codes refer to the nucleotide above). There are no predicted N-glycosylation sites.

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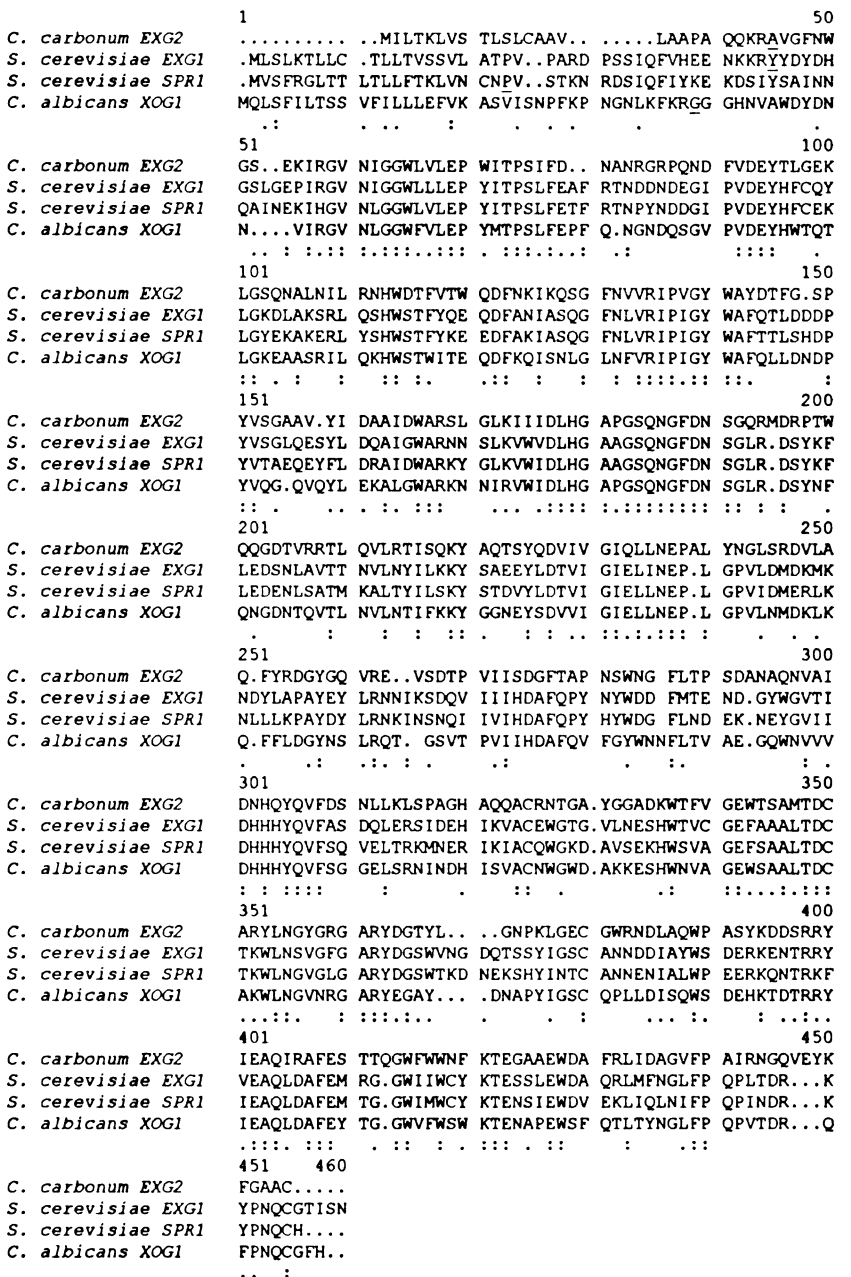

Figure 19. Comparison of the predicted amino acid sequence of *EXG2* with other known exo-1,3-β-glucanases. The sequences Exg2 of *C. carbonum* (this study), EXGI of *S. cerevisiae* (SwissProt P23776, Vazquez de Aldana et al. 1991), SPR1 of *S. cerevisiae* (SwissProt P32603, Muthukumar et al. 1993), and XOG1 of *C. albicans* (SwissProt P29717, Chambers et al. 1993) were compared by PILEUP (Devereux et al. 1984). Amino acid located at the mature N-terminus is underlined. Identical (:) and similar (.) amino acids are indicated. The conserved region used to design a degenerate oligonucleotide primer for PCR-amplification of *EXG2* is indicate in bold letters. Stop codons are designated by *.

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encoded the purified p50 (Figure 18). The size difference between the two PCR products was later established to be due to two introns (Figure 18).

Genomic and cDNA libraries were screened with the 430-bp cDNA-derived PCR product. A 1.7-kb cDNA clone, C2-1.1, was isolated and sequenced. To obtain sequence of the genomic copy of *EXG2*, two *Pst*I fragments, 2.4-kb and 1.2-kb, were subcloned (Figure 20). For confirmation that the 2.4-kb and 1.2-kb fragments overlapped at the same *Pst*I restriction site, sequence was obtained from a 650-bp *Bam*HI/*Kpn*I fragment which spans the *Pst*I restriction site (Figure 20). When cDNA and genomic sequences

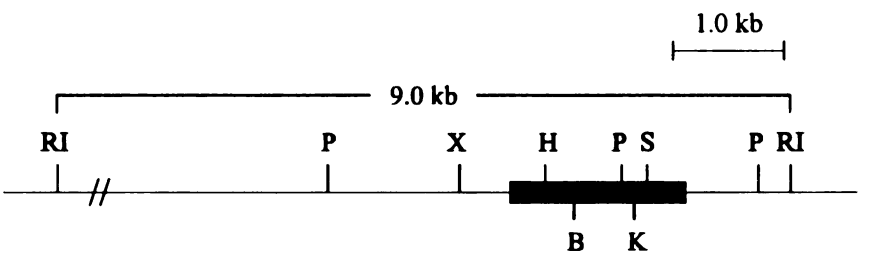

Figure 20. Restriction map of the wild type *EXG2* locus showing the location of the *EXG2* transcript (shaded box). The *Pst*I and *Bam*HI/*Kpn*I fragments sequenced are indicated (in kb). P, *Pst*I; X, *Xho*I; RI, *Eco*RI ; H, *Hind*III; B, *Bam*HI; K, *Kpn*I; S, *Sal*I.

were compared, five introns were identified; three within the first 380-bp of the open reading frame and two closer to the C-terminal end of the protein (Figure 18). The 5' and

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3' intron borders, splice branch sites, and lengths are consistent with introns in other genes of *C. carbonum* (Görlach et al. 1997b).

The deduced amino acid sequence (Figure 18) predicts that *EXG2* encodes a mature polypeptide of 44,525 Daltons, which is smaller than the SDS-PAGE estimated size of 50-kD for this protein, and has a pI of 5.51. The context of the predicted translation start site (CAACATGATT) (Figure 18) conforms with the *Neurospora crassa* transcription start site consensus sequence (CAMMATGGCT, where M = C or A, Edelmann and Staben 1994). The amino acids residues preceding the mature N-terminus (KR) do not agree with the (-3, -1) rule described by von Heijne (1986). Instead these charged amino acids are indicative of a Kex2p processing site (Julius et al. 1984). A comparison of the deduced amino acid sequence of *EXG2* with other exo-1,3-β-glucanase genes (Figure 19) indicated that several of these enzymes have the dibasic peptide sequence KR preceding the mature N-terminus. It has been shown that EXGI of *S. cerevisiae* is processed by Kex2p (Basco et al. 1996). The significance of this processing is not known as the enzyme is active without being processed . If the exo-1,3-β-glucanases are involved in fungal cell wall metabolism, they may be regulated by this post-translational processing.

The similarity between the deduced amino acid sequence of protein encoded by *EXG2* and that of known exo-1,3-β-glucanases is 61 to 63% (Figure 19). However, this protein is not similar to Exg1 from *C. carbonum* which shares slight homology with an α-amylase from *Bacillus licheniformis* (van Hoof et al. 1991). The homologies with the yeast

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proteins indicate that the protein encoded by *EXG2* is a member of family 5 of the glycosylhydrolases (Henrissat and Bairoch 1993) As p50 co-purified with Mlg1, it is not clear whether the 1,3-β-glucanase activity detected was associated with p50 or Mlg1. Therefore, it is not known whether the gene product of *EXG2* of *C. carbonum* is an active exo-1,3-β-glucanase or not.

Materials and Methods

Isolation of EXG2

PCR conditions for *EXG2* amplification and cloning of the resulting PCR fragment have been described (Murphy and Walton 1996). Template DNA was isolated *C. carbonum* genomic DNA and DNA extracted from phage lysate of a *C. carbonum* cDNA library (Pitkin et al. 1996). The PCR primers used were GTNGGNTTYAAYTGGGG and CARAAYGGNTTYGAYAA (where N = any nucleotide, R = A or G, Y = C or T) which correspond to the amino acid sequences VGFNWG and QNGFDN, respectively. The final concentration of $MgCl_2$ in the reaction mixture was 1.5 mM. The reaction conditions for 35 cycles were: denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and primer extension at 72°C for 3 min. The PCR products were separated on a 1.2% agarose gel and putative *EXG2*-containing PCR fragments were excised from the gel and cloned in the pBluescriptII SK^+ at the *Sma*I restriction site as described (Murphy and Walton 1996).

Screening of the genomic and the cDNA libraries, genomic DNA isolation, DNA

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blotting, probe labeling, and hybridization protocols have been described (Scott-Craig et al. 1990, Pitkin et al. 1996, Sposato et al. 1995). DNA was blotted to Nytran (Schleicher and Schuell, Keene, New Hampshire).

EXG2 sequence was generated by automated fluorescent sequencing at the MSU-DOE-PRL Plant Biochemistry Facility.

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CLONING OF THE GENE ENCODING A 1,3-1,4-β-GLUCANASE, *MLG2*, FROM *COCHLIOBOLUS CARBONUM*

Introduction

As redundancy contributes to the problem of interpreting gene disruption experiments for any one enzyme activity, each overlapping activity should be identified, the corresponding gene cloned, and a mutant generated lacking all overlapping activities. Apel et al. (1996) and Murphy and Walton (1996) have taken this approach with xylanases and proteases, respectively, from *Cochliobolus carbonum*. In addition, all pectin hydrolyzing activities are being characterized from *C. carbonum* with the ultimate goal of a mutant deficient in pectin-degrading enzymes (Scott-Craig et al. 1990, 1996). As there are two detectable 1,3-1,4-β-glucanase activities secreted by *C. carbonum* (Görlach et al. 1997a), it is necessary to clone the corresponding genes and generate null mutants to eliminate all 1,3-1,4-β-glucanases. The major 1,3-1,4-β-glucanase has already been cloned and *mlg1* mutants generated (Görlach et al. 1997a). In this study, a cDNA encoding the second 1,3-1,4-β-glucanase (Mlg2) was cloned, sequence was obtained, and the *MLG2* locus was mapped.

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Results and Discussion

Internal tryptic peptide sequence analysis of the purified Mlg2 protein yielded three sequences (Figure 21) (Görlach et al. 1997a). Mlg2 internal peptides II and III (Figure 21) shared homology with cellulases from *Streptomyces roche* and *Aspergillus aculeatus*. Utilizing the homologous sequences as a reference, the location of peptides II (starting at amino acid 170, Figure 22) and III (starting at amino acid 231, Figure 22) in the Mlg2 protein were determined. Using DNA from a *C. carbonum* cDNA library as a template, PCR primers corresponding to the amino

Peptide I. FTVNQ(C)SANAY

Peptide II. YDVYPIGSSQGMVNVAGR

Peptide III. GFPINSQNLITYQFGTEAFTGGP

Figure 21. Sequences of Mlg2 tryptic peptide fragments. The identity of the amino acid in parentheses was uncertain. Single-underlining indicates the location of the amino acids used to design PCR primers for amplification of the *MLG2* gene.

acid sequences QGMVNV and ITYQFG were used to amplify a DNA fragment containing the gene encoding Mlg2. The deduced amino acid sequence of the resulting 200 bp-cDNA-derived fragment had the peptide sequences used to make both primers and flanking sequences of peptides II and III (compare Figure 21 and 22).

A *C. carbonum* cDNA library was screened using the 200-bp PCR fragment as a

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```
                1                                                    50
C. carbonum     .......... .......... .......... .......... ..........
A. aculeatus    .......... .......... .MKAFHLLAA LAGAAVAQQA Q..LCDQYA.
S. lividans     MRTLRPQARA PRGLLAALGA VLAAFALVSS LVTAAAPAQA DTTICEPFGT
                   ::  .:.  :.. ::: .    :  :... : .:. . ::  ::.:. ...
                51                                                   100
C. carbonum     .......... NNIWGRSSAT SGSQCTY... VNSVSQTGAK WTSNWQWQGG
A. aculeatus    TYTGGVYTIN NNLWGKDAGS  GSQCTT... VNSASSAGTS WSTKWNWSGG
S. lividans     TTIQGRYVVQ NNRWGSTAPQ CVTATDTGFR VTQADGSAPT NGAPKSYPSV
                :   : : .. :: ::  ..    .    ::: :     . .   ..   . .
                101                                                  150
C. carbonum     RDNVKSYVYS GPSNHKKPVS QYSNLETEAY WVYDTSNIRC NVAYDLFTSA
A. aculeatus    ENSVKSYANS GLTFNKKLVS QISQIPTTAR WSYDNTGIRA DVAYDLFTAA
S. lividans     FNGCHYTNCS PGTDLPVRLD TVSAAPSSIS YGFVDGAVYN ASYDIWLDPT
                 .  .    : . .     .    :   .    . .    .           ..
                151                                                  200
C. carbonum     NVNHPTSSGD YELMVWLGRY .DVYPIGSSQ GMVNVAGRQW DFFYGLNGNM
A. aculeatus    DINHVTWSGD YELMIWLARY GGVQPIGSQI ATATVDGQTW ELWYGANGSQ
S. lividans     ARTDGV..NQ TEIMIWFNRV GPIQPIGSPV GTASVGGRTW EVWSAANGSN
                      :    :.:.:  :  : . ::::   .   : :  : . . . ::
                201                                                  250
C. carbonum     KVYSFVTPSG PIYNFKASMK DFFQYLANNK GFPINSQNLI TYQFGTEAFT
A. aculeatus    KTYSFVAP.T PITSFQGDVN DFFKYLTQNH GFPASSQYLI TLQFGTEPFT
S. lividans     DVLSFVAPSA .ISGWSFDVM DFVR.ATVAR GLAENDWYLT SVQAGFEPWQ
                   :::.::. :.  .   .  ::  : .  . : .     :  . : : :..
                251                                                  300
C. carbonum     GGPAKFTVNQ WSANAY*.... .......... .......... ..........
A. aculeatus    GGPATLSVSN WSASVQ*... .......... .......... ..........
S. lividans     NG.AGLAVNS FSSTVETGTP ....GGTDPG DPGGPSACAV SYGTNVWQDG
                 :::  .:   .:.   ::..      .  :: :::.:.::.: ::.:::::  :
                301                                                  350
C. carbonum     .......... .......... .......... .......... ..........
A. aculeatus    .......... .......... .......... .......... ..........
S. lividans     FTADVTVTNT GTAPVDGWQL AFTLPSGQRI TNAWNASLTP SSGSVTATGA
                :::.:::::  :.:::::: : :::::::: .   :::::..: :::.::::::.
                351                                     390
C. carbonum     .......... .......... .......... ..........
A. aculeatus    .......... .......... .......... ..........
S. lividans     SHNARIAPGG SLSFGFQGTY GGAFAEPTGF RLNGTACTTV*
                .  ::::.:: : :::::::.: .:.::.:..:  ::::::.::
```

Figure 22. Deduced amino acid sequence of Mlg2 compared to homologous protein sequences. PILEUP (Devereux et al. 1984) of *C. carbonum* Mlg2, *A. aculeatus* FI-CMCase (EMBL X52525, Ooi et al. 1990), and *S. lividans* CelB (GenBank U04629, Wittmann et al. 1994) Single-underlining indicates tryptic peptide sequences from the Mlg2 protein. Similar (.) and identical (:) amino acids are labeled. Stop codons are designated by *.

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probe. A partial 900-bp *MLG2* cDNA clone, C2-1, was isolated and sequenced obtained for one strand. A comparison of the deduced amino acid sequence of Mlg2 with other proteins in the databases, indicated that Mlg2 was highly similar to cellulases from *Streptomyces lividans* and *Aspergillus aculeatus* (Figure 22).

To map the *MLG2* locus, *C. carbonum* genomic DNA was digested with various restriction enzymes, size-separated on an 0.8% agarose gel, blotted, and hybridized with the 200-bp PCR fragment. The resulting map is shown in figure 23.

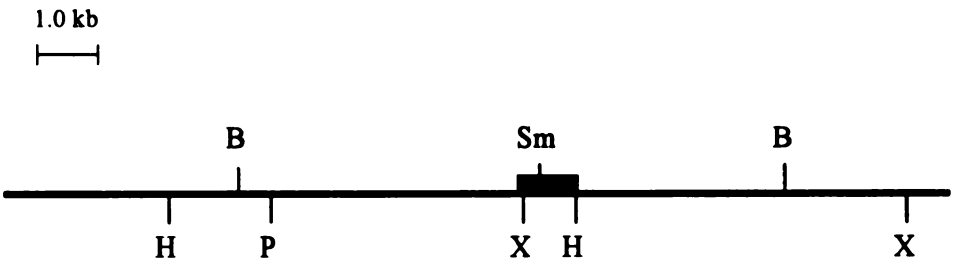

Figure 23. Restriction map of the *MLG2* locus showing the location of the *MLG2* transcript (shaded box). P, *Pst*I; Sm, *Sma*I; X, *Xho*I; B, *Bam*HI; H, *Hind*III.

Materials and Methods

Nucleic acid manipulation

Genomic DNA was isolated as described by Pitkin et al. (1996). The cDNA library was synthesized from PolyA$^+$-RNA isolated from *C. carbonum* grown on macerated corn cell walls (Pitkin et al. 1996). Sequencing was performed by automated fluorescent

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sequencing at the MSU-DOE-PRL Plant Biochemistry Facility.

Isolation of MLG2

PCR conditions for the amplification of *MLG2* by PCR and the method used to clone the resulting PCR fragment were as described by Murphy and Walton (1996). Template DNA was isolated from phage lysate of a *C. carbonum* cDNA library (Pitkin et al. 1996). The PCR primers used were CCRAAYTGRTNAGTRAT (where R = A or G, Y = C or T, N = any nucleotide), corresponding to ITYGFG, and CARGGNATGGTNAAYGT, corresponding to QGMVNV. The final concentration of $MgCl_2$ in the PCR reaction was 4 mM. The reaction conditions for 35 cycles were as follows: denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and primer extension for 2 min at 72°C. The PCR products were size separated on a 1.2% agarose gel and potential *MLG2* PCR fragments were excised from the gel and cloned into the *Sma*I site of pBluescript II SK+ as described (Murphy and Walton 1996).

Screening of the genomic and the cDNA libraries, genomic DNA isolation, DNA blotting, probe labeling, and hybridization protocols have been described (Scott-Craig et al. 1990, Pitkin et al. 1996, Sposato et al. 1995). DNA was blotted to Nytran (Schleicher and Schuell, Keene, New Hampshire).

BIBLIOGRAPHY

BIBLIOGRAPHY

1. **Ahn, J. -H., and J. D. Walton.** 1996. Chromosomal organization of *TOX2*, a complex locus controlling host-selective toxin biosynthesis in *Cochliobolus carbonum*. Plant Cell **8:**887-897.

2. **Ahn, J. -H., and J. D. Walton.** 1997. A fatty acid synthase gene in *Cochliobolus carbonum* required for production of HC-toxin, cyclo(D-prolyl-L-alanyl-D-Alanyl-L-2-amino-9,10-epoxi-8-oxodecanoyl). Mol. Plant-Microbe Interact. **10** (in press).

3. **Alexander, D., K. Lawton, S. Uknes, E. Ward, J. and Ryals.** 1994. Defense-related gene induction in plants. Pages 195-212 in: Genetic engineering, Vol. 16, J.K. Setlow, ed. Plenum Press, New York.

4. **Allen, E. A., B. E. Hazen, H. C. Hoch, Y. Kwon, G. M. E. Leinhos, R. C. Staples, M. A. Stumpf, and B. T. Terhune.** 1991. Appressorium formation in response to topographical signals by 27 rust species. Phytopathology. **81:**323-331.

5. **Ansari, H., and L. Stevens.** 1983. Purification and properties of two neutral proteinases from *Aspergillus nidulans*. J. Gen. Microbiol. **129:**1637-1644.

6. **Apel, P. C., D. G. Panaccione, F. R. Holden, and J. D. Walton.** 1993. Cloning and targeted gene disruption of *XYL1*, a β1,4-xylanase gene from themaize pathogen *Cochliobolus carbonum*. Mol. Plant-Microbe Interact. **6:**467-473.

7. **Apel-Birkhold, P. C. and J. D. Walton.** 1996. Cloning, disruption, and expression of two endo-β1,4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. Appl. Environ. Microbiol. **62**.

8. **Bailey, B. A., J. F. D. Dean, and J. D. Anderson.** 1990. An ethylene biosynthesis-inducing endoxylanase elicits electrolyte leakage and necrosis in *Nicotiana tabacum* cv. Xanthi leaves. Plant Physiol. **94:**1849-1854.

9. **Baker, C. J., and D. F. Bateman.** 1978. Cutin degradation by plant pathogenic fungi. Phytopathology **68:**1577-1584.

10. **Ball, A. M., A. M. Ashby, M. J. Daniels, D. S. Ingram, and K. Johnstone.** 1991. Evidence for the requirement of extracellular protease in the pathogenic interraction of *Pyrenopeziza brassicae* with oilseed rape. Physiol. Mol. Plant Pathol. **38:**147-161.

11. **Ballance, D. J.** 1991. Transformation systems for filamentous fungi and an overview of fungal gene structure. Pages 1-29 in: Molecular Industrial Mycology; Systems and Applications for Filamentous Fungi. S. A. Leong and R. M. Berka, eds. Marcel Dekker, New York.

12. **Basco, R. D., R. Cueva, E. Andaluz, and G. Larriba.** 1996. In vivo processing of the precursor of the major exoglucanase by KEX2 endoprotease in the *Saccharomyces cerevisiae* secretory pathway. Biochim. Biophys. Acta **1310:**110-118.

13. **Bateman, D. F., and H. G. Basham.** 1976. Degradation of plant cell walls and membranes by microbial enzymes. Pages 316-355 in: Encyclopedia of Plant Physiology, New Series, Vol. 4, R. Heitefuss and P.H. Williams, eds. Springer-Verlag, NY.

14. **Bateman, D. F., T. M. Jones, and O. C. Yoder.** 1973. Degradation of corn cell walls by extracellular enzymes produced by *Helminthosporium maydis* race T. Phytopathology **63:**1523-1529.

15. **Becker, A., A. Kleickmann, W. Arnold, and A. Pühler.** 1993. Analysis of the *Rhizobium meliloti exoH/exoK/exoL* fragment: ExoK shows homology to excreted endo-β-1,3-1,4-glucanases and ExoH resembles membrane proteins. Mol. Gen. Genet. **238:**145-154.

16. **Boccara, M., A. Diolez, M. Rouve, A. Kotoujansky.** 1988. The role of individual pectate lyases of *Erwinia chrysanthemi* strain 3937 in pathogenicity on saintpaulia plants. Physiol. Mol. Plant Pathol. **30:**95-104.

17. **Boel, E., M.T. Hansen, I. Hjort, I. Høegh, & N.P. Fiil.** 1984. Two different types of intervening sequences in the glucoamylase gene from *Aspergillus niger*. EMBO J. **3:**1581-1585.

18. **Bonants, P. J. M., P. F. L. Fitters, H. Thijs, E. den Belder, C. Waalwijk, and J. W. D. M. Henfling.** 1995. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology **141:**775-784.

19. **Bourbonnais, Y., J. Ash, M. Daigle, and D. Y. Thomas.** 1993. Isolation and characterization of *S. cerevisiae* mutants defective in somatostatin expression: cloning and functional role of a yeast gene encoding an asparytl protease in precursor processing at monobasic cleavage sites. EMBO J. **12:**285-294.

20. **Brosch, G., R. Ransom, T. Lechner, J. D. Walton, and P. Loidl.** 1995. Inhibition of maize histone deacetylases by HC-toxin, the host-selective toxin of *Cochliobolus carbonum*. Plant Cell **7**:1941-1950.

21. **Carpita, N. C.** 1984. Cell wall development in maize coleoptiles. Plant Physiol. **76**:205-212.

22. **Carpita, N. C.** 1996. Structure and biogenesis of the cell walls of grasses. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47**:445-476.

23. **Choi, G. H., D. M. Pawlyk, B. Rae, R. Shapira, and D. L. Nuss.** 1993. Molecular analysis and overexpression of the gene encoding endothiapepsin, and aspartic protease from *Cryphonectria parasitica*. Gene **125**:135-141.

24. **Chomczynski, P., and N. Sacchi.** 1987. Single step method of RNA extraction by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156-159.

25. **Chumley, F. G., and B. Valent.** 1990. Genetic analysis of melanin-deficient nonpathogenic mutants of *Magnaporthe grisea*. Mol. Plant-Microbe Interact. **3**:135-143.

26. **Ciuffetti, L. M., M. R. Pope, L. D. Dunkle, J. M. Daly, and H. W. Knoche.** 1983. Isolation and structure of an inactive product derived from the host-selective toxin produced by *Helminthosporium carbonum*. Biochemistry **22**:3507-3510.

27. **Clark, D. R., J. Johnson, Jr., K. H. Chung, and S. Kirkwood.** 1978. Purification, characterization, and action-pattern studies on the endo-(1→3)-β-D-glucanase from *Rhizopus arrhizus* QM1032. Carbohydrate Res. **61**:457-477.

28. **Comstock, J. C., and R. P. Scheffer.** 1973. Role of host-selective toxin in colonization of corn leaves by *Helminthosporium carbonum*. Phytopathology **63**: 24-29.

29. **Cooper, R. M.** 1983. The mechanisms and significance of enzymic degradation of host cell walls by parasites. Pages 101-135 in: Biochemical Plant Pathology, J.A. Callow, ed. John Wiley & Sons, NY.

30. **Cooper, R. M.** 1984. The role of cell wall-degrading enzymes in infection and damage. In: Plant Diseases: Infection, Damage, and Loss. pp. 13-21. R. K. S. Wood and G. J. Jellis (eds). Blackwell Scientific Publications, Oxford, UK.

31. **Cooper, R. M., and R. K. S. Wood.** 1980. Cell wall degrading enzymes of vascular wilt fungi. III. Possible involvement of endo-pectin lyase in *Verticillium* wilt of tomato. Physiol. Plant Pathol. **16**:285-300.

32. **Côté, F., and M. G. Hahn.** 1994. Oligosaccharins: structures and signal transduction. Plant Mol. Biol. **26:**1379-1411.

33. **Dahler, G. S., F. Barras, and N. T. Keen.** 1990. Cloning of genes encoding extracellular metalloproteases from *Erwinia chrysanthemi* EC16. J. Bacteriol. **172:**5803-5815.

34. **Davidow, L. S., M. M. O'Donnell, F. S. Kaczmarek, D. A. Pereira, J. R. DeZeeuw, and A. E. Franke.** 1987. Cloning and sequencing of the alkaline extracellular protease gene of *Yarrowia lipolytica*. J. Bacteriol. **169:**4621-4629.

35. **Davey, J., K. Davis, Y. Imai, M. Yamamoto, and G. Matthews.** 1994. Isolation and characterization of krp, a dibasic endopeptidase required for cell viability in the fission yeast *Schizosaccharomyces pombe*. EMBO J. **13:**5910-5921.

36. **Deising, H., R. L. Nicholson, M. Haug, R. J. Howard, K. Mendgen.** 1992. Adhesion pad formation and the involvement of cutinase and esterases in the attachment of uredospores to the host cuticle. Plant Cell **4:**1101-1111.

37. **Deshpande, K. S., and K. B. Deshpande.** 1968. Pectic enzyme production and morphogenesis in *Helminthosporium atypicum*. Biol. Plant. (Praha) **10:**245-250.

38. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387-395.

39. **Dickman, M. B., G. K. Podila, P. E. Kolattukudy.** 1989. Insertion of cutinase gene into a wound pathogen enables it to infect intact host. Nature **342:**446-448.

40. **Doan, D. N. P., and G. B. Fincher.** 1992. Differences in the thermostability of barley (1→3, 1→4)-beta-glucanases are only partly determined by N-glycosylation. FEBS Lett. **309:**265-271.

41. **Dori, S., Z. Solel, and I. Barash.** 1995. Cell wall-degrading enzymes produced by *Gaeumannomyces graminis* var. *tritici in vitro* and *in vivo*. Physiol. Mol. Plant Pathol. **46:**189-198

42. **Dow, J. M., B. R. Clarke, D. E. Milligan, J. -L. Tang, and M. J. Daniels.** 1990. Extracellular proteases from *Xanthomonas campestris* pv. *campestris*, the black rot pathogen. Appl. Environ. Microbiol. **56:**2994-2998.

43. **Drapeau, G. R.** 1978. Role of metalloprotease in activation of the precursor of Staphylococcal protease. J. Bacteriol. **136:**607-613.

44. **Easton, C. J., and S. Rossall.** 1985. The production of certain cell wall-degrading enzymes by *Leptosphaeria maculans* in culture and in stem canker lesions of oilseed rape. Physiol. Plant Pathol. **26:**185-197.

45. **Edelmann, S. E., and C. Staben** 1994. A statistical analysis of sequence features within genes from *Neurospora crassa*. Exp. Mycol. **18:**70-81.

46. **Ferrer, P., T. Halkier, L. Hedegaard, D. Savva, L. Diers, & J. A. Asenjo.** 1996. Nucleotide sequence of a β-1,3-glucanase isoenzyme II_A gene of *Oersokovia xanthineolytica* LL G109 (*Cellulomonas cellulans*) and initial characterization of the recombinant enzyme expressed in *Bacillus subtilis*. J. Bacteriol. **178:**4751-4757.

47. **Flint, H. J., J. Martin, C. A. McPherson, A. S. Daniel, and J.-X. Zhang.** 1993. A bifunctional enzyme, with separate xylanase and β(1,3-1,4)-glucanase domains, encoded by the *xynD* gene of *Ruminococcus flavefaciens*. J. Bacteriol. **175:** 2943-2951.

48. **Frohman, M. A., M. K. Dush, and G. R. Martin.** 1988. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85:**8998-9002.

49. **Gavel, Y., and G. von Heijne.** 1990. Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. Prot. Engin. **3:**433-442.

50. **Gebhard, W., H. Tschesche, and H. Fritz.** 1986. Biochemistry of aprotinin and aprotinin-like inhibitors. Pages 375-388 in: Proteinase Inhibitors, A.J. Barrett and G. Salvesen, eds. Elsevier Science, Amsterdam.

51. **Geremia, R. A., G. H. Goldman, D. Jacobs, W. Ardiles, S. B. Vila, M. Van Montague, and A. Herrera-Estrella.** 1993. Molecular characterization of the proteinase-encoding gene, *prb1*, related to mycoparasitism by *Trichoderma harzianum*. Mol. Microbiol. **8:**603-613.

52. **Gilkes, N. R., B. Henrissat, D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren.** 1991. Domains in microbial β-1,4-glycanases: Sequence conservation, function, and enzyme families. Microbiol. Rev. **55:**303-315.

53. **Gish, W., and D. J. States.** 1993. Identificationof protein coding regions by database similarity search. Nature Genet. **3:**266-272.

54. **Görlach, J. M., E. van der Knaap, and J. D. Walton.** 1997a. Three mixed-linked glucanases from the filamentous fungus *Cochliobolus carbonum:* cloning and targeted disruption of *MLG1* (submitted).

55. **Görlach, J. M., J. W. Pitkin, and J. D. Walton.** 1997b. Investigation of the involvement of two proteases in virulence of the fungal pathogen, *Cochliobolus carbonum*, on maize. (in preparation).

56. **Grenier, D.** 1996. Degradation of host protease inhibitors and activation of plasminogen by proteolytic enzymes from *Porphyromonas gingivalis* and *Treponema denticola*. Microbiol. **142:**955-961.

57. **Gunkel, F. A., and H. G. Gassen.** 1989. Proteinase K from *Tritirachium album* Limber. Characterization of the gene and expression of the cDNA in *Escherichia coli*. Biochem. **179:**185-194.

58. **Gurr, S. J., S. E. Unkles, and J. R. Kinghorn.** 1987. The structure and organization of mucear genes of filamentous fungi. Pages 93-139 in: Gene Structure in Eukaryotic Microbes. J.R. Kinghorn, ed. IRL Press, London.

59. **Hames, B. D., and D. Rickwood, D.** (eds.) 1981. Gel electrophoresis, a practical approach. IRL Press, Oxford.

60. **Henrissat, B., and A. Bairoch.** 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. **293:**781-788.

61. **Hislop, E. C., J. L. Paver, and J. P. R. Keon.** 1982. An acid protease produced by *Monilinia fructigena in vitro* and in infected apple fruits, and its possible role in pathogenesis. J. Gen. Microbiol. **128:**799-807.

62. **Høj, P. B., E. B. Rodriguez, R. V. Stick, B. A. Stone.** 1989. Differences in active site structure in a family of β-glucan endohydrolases deduced from the kinetics of inactivation by epoxyalkyl β-oligoglucosides. J. Biol. Chem. **264:**4939-4947.

63. **Holden, F. R., and J. D. Walton.** 1992. Xylanases from the fungal maize pathogen *Cochliobolus carbonum*. Physiol. Mol. Plant Pathol. **40:**39-47.

64. **Howard, R. J., and M. A. Ferrari.** 1989. Role of melanin in appressorium function. Exp. Mycol. **13:**403-418.

65. **Howard, S. P., and J. T. Buckley.** 1985. Activation of the hole-forming toxin aerolysin by extracellular processing. J. Bacteriol. **163:**336-340.

66. **Huang, H. C., and R. D. Tinline.** 1976. Histology of *Cochliobolus sativus* infection in subcrown internodes of wheat and barley. Can. J. Bot. **54:**1344-1354.

67. **Hynes, M. J., C. M. Corrick, and J. A. King.** 1983. Isolation of genomic clones containing the *amdS* gene of *Aspergillus nidulans* and their use in the analysis of structural regulatory mutations. Mol. Cell Biol. **3:**1430-1439.

68. **Ikeda, M., T. Yaginuma, M. Kobayashi, and O. Yamashita.** 1991. cDNA cloning, sequencing and temporal expression of the protease responsible for vitellin degradation in the silkworm, *Bombyx mori*. Comp. Biochem. Physiol. **99B:**405-411.

69. **Jennings, P. R., and A. J. Ullstrup.** 1957. A histological study of three *Helminthosporium* leaf blights of corn. Phytopathology **47:**707-714.

70. **Johal, G. S., and S. P. Briggs.** 1992. Reductase activity encoded by the *HM1* disease resistance gene in maize. Science **258:**985-987.

71. **Julius, D., A. Brake, L. Blair, R. Kunisawa, and J. Thorner.** 1984. Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-α-factor. Cell **37:**1075-1089.

72. **Keitel, T., S. Ortwin, R. Borriss, and U. Heinemann.** 1993. Molecular and active-site structure of a *Bacillus* 1,3-1,4-β-glucanase. Proc. Natl. Acad. Sci. USA **90:**5287-5291.

73. **Kim, S. -D., H. W. Knoche, and L. D. Dunkle.** 1987. Essentiality of the ketone function for toxicity of the host-selective toxin produced by *Helminthosporium carbonum*. Physiol. Mol. Plant Pathol. **30:**433-440.

74. **Komano, H, and R. S. Fuller.** 1995. Shared functions *in vivo* of a glycosyl-phosphatidylinositol-linked aspartyl protease, Mkc7, and the proprotein processing protease Kex2 in yeast. Proc. Natl. Acad. Sci. USA **92:**10752-10756.

75. **Kubo, Y., H. Nakamura, K. Kobayashi, T. Okuno, and I. Furusawa.** 1991. Cloning of a melanin biosynthetic gene essential for appressorial penetration of *Colletotrichum lagenarium*. Mol. Plant-Microbe Interact. **4:**440-445.

76. **Kubo, Y., M. Tsuda, I. Furusawa, and J. Shishiyama.** 1989. Genetic analysis of genes involved in melanin biosynthesis of *Cochliobolus miyabeanus*. Exp. Mycol. **13:**77-84.

77. **Laio, C. -H., H. -Y. Hung, A. K. Chatterjee.** 1988. An extracellular pectate lyase is the pathogenicity factor of the soft-rotting bacterium *Pseudomonas viridiflava*. Mol. Plant-Microbe Interact. **1:**199-206.

78. **Larcher, G., B. Cimon, F. Symoens, G. Tronchin, D. Chabasse, and J. -P. Bouchara.** 1996. A 33 kDa serine proteinase from *Scedosporium apiospermum*. Biochem. J. **315:**119-125.

79. **Lawton, M. A., and C. J. Lamb.** 1987. Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. Mol. Cell. Biol. **7:**335-341.

80. **Le Cam, B., P. Massiot, and F. Rouxel.** 1994. Cell wall Polysaccharide-degrading enzymes produced by isolates of *Mycocentrospora acerina* differing in aggressiveness on carrot. Physiol. Mol. Plant Pathol. **44:**187-198.

81. **Leonard, K. J.** 1978. Polymorphisms for lesion type, fungicide tolerance, and mating capacity in *Cochliobolus carbonum* isolates pathogenic to corn. Can. J. Bot. **56:**1809-1815.

82. **Leonard, K. J.** 1987. The host populations as a selective factor. In: Populations of Plant Pathogens: Their Dynamics and Their Genetics. pp. 163-179. M. S. Wolfe and C. E. Caten (eds). Blackwell Sci. Publ., Oxford.

83. **Lever, M.** 1972. A new reaction for colorimetric determination of carbohydrates. Anal. Biochem. **47:**273-279.

84. **Liesch, J. M., C. C. Sweeley, G. D. Staffeld, M. S. Anderson, D. J. Weber, and R. P. Scheffer.** 1982. Structure of HC-toxin, a cyclic tetrapeptide from *Helminthosporium carbonum*. Tetrahedron **38:**45-48.

85. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

86. **Matoba, S., J. Fukayama, R. A. Wing, and D. M. Ogrydziak.** 1988. Intracellular precursors and secretion of alkaline extracellular protease of *Yarrowia lipolytica*. Mol. Cell. Biol. **8:**4904-4916.

87. **Matsudaira, P.** 1987. Sequence from picomole quantitites of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:**10035-10038.

88. **McNeil, M., A. G. Darvill, S. C. Fry, and P. Albersheim.** 1984. Structure and function of the primary cell walls of plants. Annu. Rev. Biochem. **53:**625-663.

89. **Meeley, R. B., and J. D. Walton.** 1991. Enzymatic detoxification of HC-toxin, the host-selective cyclic peptide from *Cochliobolus carbonum*. Plant Physiol. **97:**1080-1086.

90. **Meeley, R. B., G. S. Johal, S. P. Briggs, and J. D. Walton.** 1992. A biochemical phenotype for a disease resistance gene of maize. Plant Cell **4:**71-77.

91. **Meldgaard, M., and I. Svendsen.** 1994. Different effects of N-glycosylation on the thermostability of highly homologous bacterial (1,3-1,4)-beta-glucanases secreted from yeast. Microbiology **140:**159-166.

92. **Monod, M., S. Paris, J. Sarfati, K. Jaton-Ogay, P. Ave, and J. -P. Latgé.** 1993. Virulence of alkaline protease-deficient mutants of *Aspergillus fumigatus*. FEMS Microbiol. Lett. **106:**39-46.

93. **Moormann, M., A. Schlochtermeier, and H. Schrempf.** 1993. Biochemical characterization of a protease involved in the processing of a *Streptomyces reticuli* cellulase (Avicelase). Appl. Environ. Microbiol. **59:**1573-1578.

94. **Morita, S., M. Kuriyama, K. Maejima, and K. Kitano.** 1994. Cloning and nucleotide sequence of the alkaline protease gene from *Fusarium* sp. S-19-5 and expression in *Saccharomyces cerevisiae*. Biosci. Biotechnol. Biochem. **58:**621-626.

95. **Murao, S., R. Sakamoto, and M. Arai.** 1988. Cellulases of *Aspergillus aculeatus*. Meth. Enzymol. **160:**274-299.

96. **Murphy, J. M., and E. E. Hood.** 1993. Molecular basis for extensin size heterogeneity in two maize varieties. Plant Mol. Biol. **21:**885-893.

97. **Murphy, J. M., and J. D. Walton.** 1996. Three extracellular proteases from *Cochliobolus carbonum*: cloning and targeted disruption of *ALP1*. Mol. Plant-Microbe Interact. **9:**290-297.

98. **Murray, G. M., and D. P. Maxwell.** 1975. Penetration of *Zea mays* by *Helminthosporium carbonum*. Can. J. Bot. **53:**2872-2883.

99. **Muse, R. R., H. B. Couch, L. D. Moore, and B. D. Muse.** 1972. Pectolytic and cellulolytic enzymes associated with *Helminthosporium* leaf spot on Kentucky bluegrass. Can. J. Microbiol. **18:**1091-1098.

100. **Muthukumar, G., S. -H. Suhng, P. T. Magee, R. D. Jewell, and D. A. Primerano.** 1993. The *Saccharomyces cerevisiae SPR1* gene encodes a sporulation-specific exo-1,3-β-glucanase which contributes to ascospore thermoresistance. J. Bacteriol. **175:**386-394.

101. **Nelson, O. E., and A. J. Ullstrup.** 1961. The inheritance of pathogenicity in *Cochliobolus carbonum*. Phytopathology **51:**1288-1291.

102. **Nelson, O. E., and A. J. Ullstrup.** 1964. Resistance to leafspot in maize. J. Hered. **55:**195-199.

103. **Nelson, R. R., and R. T. Sherwood.** 1968. Genetic control of endopolygalacturonase production in *Cochliobolus carbonum*. Phytopathology **58:**1277-1280.

104. **Neurath, H.** 1984. Evolution of proteolytic enzymes. Science **224:**350-357.

105. **Nielsen, H., J. Engelbrecht, S. Brunak, & G . von Heijne.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineer. **10** (in press).

106.**Nikolskaya, A., J. W. Pitkin, H. J. Schaeffer, and J. D. Walton.** 1996. (in preparation).

107.**North, M. J.** 1982. Comparative biochemistry of the proteinases of eukaryotic microorganisms. Microbiol. Rev. **46:**308-340.

108.**Olafson, R. W., L. Jurasek, M. R. Carpenter, and L. B. Smillie.** 1975. Amino acid sequence of *Streptomyces griseus* trypsin. Cyanogen bromide fragments and complete sequence. Biochemistry **14:**1168-1177.

109.**Ooi, T, A. Shinmyo, H. Okada, S. Hara, T. Ikenaka, S. Murao, and M. Arai.** 1990. Cloning and sequence analysis of a cDNA for cellulase (FI-CMCase) from *Aspergillus aculeatus*. Curr. Genet. **18:**217-222.

110.**Ottensen, M., and I. Svendsen.** 1970. The subtilisins. Page 199-215 in: Methods in Enzymology: Proteolytic Enzymes, Vol. 19, G.E. Perlmann and L. Lorand, eds. Academic Press, Orlando.

111.**Panaccione, D. G., J. S. Scott-Craig, J. -A. Pocard, and J. D. Walton.** 1992. A cyclic peptide synthetase gene required for pathogenicity of the fungus *Cochliobolus carbonum* on maize. Proc. Natl. Acad. Sci. USA **89:**6590-6594.

112.**Perito, B., E. Hanhart, T. Irdani, M. Iqbal, A.J. McCarthy, & G. Mastromei.** 1994. Characterization and sequence analysis of a *Streptomyces rochei* A2 endoglucanase-encoding gene. Gene **148:**119-124.

113.**Phadatare, S. U., M. C. Srinivasan, and V. V. Deshpande.** 1992. Evidence for controlled autoproteolysis of alkaline protease: a mechanism for physiological regulation of conidial discharge in *Conidiobolus coronatus*. Eur. J. Biochem. **205:**679-686.

114.**Phadatare, S., M. C. Srinivasan, and M. Deshpande.** 1989. Evidence for the involvement of serine protease in the conidial discharge of *Conidiobolus coronatus*. Arch. Microbiol. **153:**47-49.

115.**Pitkin, J. W., D. G. Panaccione, and J. D. Walton.** 1996. A putative cyclic peptide efflux pump encoded by the *TOXA* gene of the plant-pathogenic fungus *Cochliobolus carbonum*. Microbiology **142:**1557-1565.

116.**Planas, A., M. Juncosa, J. Lloberas, and E. Querol.** 1992. Essential catalytic role of Glu^{134} in endo-β-1,3-1,4-D-glucan 4-glucanohydrolase from *B. licheniformis* as determined by site-directed mutagenesis. FEBS Lett. **308:**141-145.

117.**Pope, M. R., L. M. Ciuffetti, H. W. Knoche, D. McCrery, J. M. Daly, and L. D. Dunkle.** 1983. Structure of the host-specific toxin produced by *Helminthosporium carbonum*. Biochemistry **22:**3502-3506.

118.**Porter, F. M.** 1966. Protease activity in diseased fruits. Phytopathology. **56:**1424-1425.

119.**Power, S. D., R. M. Adams, and J. A. Wells.** 1986. Secretion and autoproteolytic maturation of subtilisin. Proc. Natl. Acad. Sci. USA **83:**3096-3100.

120.**Powers, J. C., and E. A. Harper.** 1986. Inhibitors of serine proteases. Pages 55-152 in: Protease Inhibitors, A.J. Barrett and G. Salvesen, eds. Elsevier Science, Amsterdam.

121.**Pringle, R. B., and R. P. Scheffer.** 1967. Isolation of the host-specific toxin and a related substance with nonspecific toxicity from *Helminthosporium carbonum*. Phytopathology **57:**1169-1172.

122.Program Manual for the Wisconsin Package 1994. Version 8, Genetics Computer Group, University of Wisconsin, Madison, Wiscosin, U.S.A.

123.**Ransom, R., and J. D. Walton.** 1996. Purification and characterization of extracellular β-xylosidase and α-arabinosidase from the plant pathogenic fungus *Cochliobolus carbonum*. Carbohyd. Res. (in press).

124.**Rasmussen, J. B., and R. M. Hanau.** 1989. Exogenous scytalone restores appressorial melanization and pathogenicity in albino mutants of *Colletotrichum graminicola*. Can. J. Plant Pathol. **11:**349-352.

125.**Rasmussen, J. B., and R. P. Scheffer.** 1988a. Effects of selective toxin from *Helminthosporium carbonum* on chlorophyll synthesis in maize. Physiol. Mol. Plant Pathol. **32:**283-291.

126.**Rasmussen, J. B., and R. P. Scheffer.** 1988b. Isolation and biological activities of four selective toxins from *Helminthosporium carbonum*. Plant Physiol. **86:**187-191.

127.**Rast, D. M., H. Stüssi, H. Hegnauer, and L. E. Nyhlén.** 1981. Melanins. In: The Fungal Spore: Morphogenic Controls. pp. 507-531. G. Turian and H. R. Hohl (eds.). Academic Press, New York.

128.**Rauscher, M., K. Mendgen, H. Deising.** 1995. Extracellular proteases of the rust fungus *Uromyces viciae-fabae*. Experiment. Mycol. **19:**26-34.

129.**Reddy, M. N., D. L. Stuteville, and E. L. Sorensen.** 1971. Protease production during pathogenesis of bacterial leaf spot of alfalfa and by *Xanthomonas alfalfae* in vitro. Phytopathology **61:**361-365.

130.**Ries, S. M., and P. Albersheim.** 1973. Purification of a protease secreted by *Colletotrichum lindemuthianum*. Phytopathology **63:**625-629.

131.**Robertsen, B.** 1984. An alkaline protease produced by *Cladosporium cucumerinum* and its possible importance in the development of scab disease of cucumber seedlings. Physiol. Plant Pathol. **24:**83-92.

132.**Rogers, D. T., D. Saville, and H. Bussey.** 1979. *Saccharomyces cerevisiae* killer expression mutant *kex2* has altered secretory proteins and glycoproteins. Biochem. Biophys. Res. Comm. **90:**187-193.

133.**Ryan, C.A.** 1990. Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. Annu. Rev. Phytopathol. **28:**425-429.

134.**Rypniewski, W. R., S. Hastrup, C. Betzel, M. Dauter, Z. Dauter, G. Papendorf, S. Branner, and K. S. Wilson.** 1993. The sequence and X-ray structure of the trypsin from *Fusarium oxysporum*. Protein Engineer. **6:**341-348.

135.**Sakellaris, H., J. M. Pemberton, and J. M. Manners.** 1993. Characterization of an endo-1,3(4)-β-D-glucanase gene from *Cellvibrio mixtus*. FEMS Microbiol. Lett. **109:**269-272.

136.**Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

137.**San Segundo, P., J. Correa, C. R. Vazquez de Aldana, and F. del Rey.** 1993. *SSG1*, a gene encoding a sporulation-specific 1,3-β-glucanase in *Saccharomyces cerevisiae*. J. Bacteriol. **175:**3823-3837.

138.**Sato, S., H. Suzuki, U. Widyastuti, Y. Hotta, and S. Tabata.** 1994. Identification and characterization of genes induced during sexual differentiation in *Schizosaccharomyces pombe*. Curr. Genet. **26:**31-37.

139.**Schaeffer, H. J., J. Leykam, and J. D. Walton.** 1994. Cloning and targeted gene disruption of *EXG1*, encoding exo-β1,3-glucanase, in the phytopathogenic fungus *Cochliobolus carbonum*. Appl. Environ. Microbiol. **60:**594-598.

140.**Schäfer, W., D. Straney, L. Cuiffetti, H. D. Van Etten, and O. C. Yoder.** 1989. One enzyme makes a fungal pathogen, but not a saprophyte, virulent on a new host plant. Science **246:**247-249.

141.**Scheffer, R. P., and R. S. Livingston.** 1984. Host selective toxins and their role in plant diseases. Science **223:**17-21.

142.**Scheffer, R. P., R. R. Nelson, and A. J. Ullstrup.** 1967. Inheritance of toxin production and pathogenicity in *Cochliobolus carbonum* and *Cochliobolus victoriae*. Phytopathology **57:**1288-1291.

143.**Schimming, S., W. H. Schwarz, and W. L. Staudenbauer.** 1992. Structure of the *Clostridium thermocellum* gene *licB* and the encoded β-1,3-1,4-glucanase. Eur. J. Biochem. **204:**13-19.

144.**Scott-Craig, J. S., D. G. Panaccione, F. Cervone, and J. D. Walton.** 1990. Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. Plant Cell **2:**1191-1200.

145.**Scott-Craig, J. S., F. Cervone, G. de Lorenzo, and J. D. Walton.** 1996. (in preparation).

146.**Sharon, A., Y. Fuchs, and J. D. Anderson.** 1993. The elicitation of ethylene biosynthesis by *Trichoderma* xylanase is not related to cell wall degradation activity of the enyzme. Plant Physiol. **102:**1325-1329.

147.**Showalter, A. M.** 1993. Structure and function of plant cell wall proteins. Plant Cell **5:**9-23.

148.**Slakeski, N., D.C. Baulcombe, K.M. Devos, D.N.P. Doan, & G.B. Fincher.** 1990. Structure and tissue-specific regulation of genes encoding barley (1 3,1 4)-β-glucan endohydrolases. Mol. Gen. Genet. **224:**437-449.

149.**Spilliaert, R., G. O. Hreggvidsson, J. K. Kristjansson, G. Eggertsson, and A. Palsdottir.** 1994. Cloning and sequencing of a *Rhodothermus marinus* gene, *bglA*, coding for a thermostable β-glucanase and its expression in *Escherichia coli*. Eur. J. Biochem. **224:**923-930.

150.**Sposato, P., Ahn, J. -H., and J. D. Walton.** 1995. Characterization and disruption of a gene in the maize pathogen *Cochliobolus carbonum* encoding a cellulase lacking a cellulose binding domain and hinge region. Mol. Plant-Microbe Interact. **8:**602-609.

151.**St. Leger, R. J.** 1995. Role of cuticle-degrading proteases in fungal pathogenesis of insects. Can. J. Bot. **73:**S1119-S1125.

152.**St. Leger, R. J., D. C. Frank, D. W. Roberts, and R. C. Staples.** 1992. Molecular cloning and regulatory analysis of the cuticle-degrading protease structural gene for the entomopathogenic fungus *Metarhizium anisopliae*. Eur. J. Biochem. **204:**991-1001.

153.**Staples, R. C. and V. Macko.** 1980. Formation of infection structures as a recognition response in fungi. Exp. Mycol. **4:**2-16.

154.**Strömqvist, M., and H. Gruffman.** 1992. Periodic acid/Schiff staining of glycoproteins immobilized on a blotting matrix. BioTechniques **13:**744-746.

155.**Tang, C. M., J. Cohen, T. Krausz, S. Van Noorden, and D. W. Holden.** 1993. The alkaline protease of *Aspergillus fumigatus* is not a virulence determinant in two murine models of invasive pulmonary aspergillosis. Infect. Immun. **61:** 1650-1656.

156.**Teather, R., and J. D. Erfle.** 1990. DNA sequence of a *Fibrobacter succinogenes* mixed linkage β-glucanase (1,3-1,4-β-D-glucan 4-glucanohydrolase) gene. J. Bacteriol. **172:**3837-3841.

157.**Tseng, T. C., and M. S. Mount.** 1974. Toxicity of endopolygalacturonate trans-eliminase, phosphatidase, and protease to potato and cucumber tissue. Phytopathology **64:**229-236.

158.**Ullstrup, A. J.** 1941. Two physiologic races of *Helminthosporium maydis* in the corn belt. Phytopathology **57:**1288-1291.

159.**van den Ackerveken, G. F. J. M., P. Vossen, and P. J. G. M. De Wit.** 1993. The AVR9 race-specific elicitor of *Cladosporium fulvum* is processed by endogenous and plant proteases. Plant Physiol. **103:**91-96.

160.**van Hoof, A., J. Leykam, H. J. Schaeffer, and J. D. Walton.** 1991. A single β1,3-glucanase secreted by the maize pathogen *Cochliobolus carbonum* acts by an exolytic mechanism. Physiol. Mol. Plant Pathol. **39:**259-267.

161.**von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nuc. Acids Res. **14:**4683-4690.

162.**Walsh, K. A., and H. Neurath.** 1964. Trypsinogen and chymotrypsinogen as homologous proteins. Proc. Natl. Acad. Sci. U.S.A. **52:**884-889.

163.**Walsh, K. A., and P. E. Wilcox.** 1970. Serine proteases. Pages 31-63 in: Methods in Enzymology: Proteolytic Enzymes, Vol. 19, G.E. Perlmann and L. Lornad, eds. Academic Press, Orlando.

164.**Walton, J. D.** 1987. Two enzymes involved in biosynthesis of the host-selective phytoxin HC-toxin. Proc. Natl. Acad. Sci. USA **84:**8444-8447.

165.**Walton, J. D.** 1994. Deconstructing the cell wall. Plant Physiol. **104:**1113-1118.

166.**Walton, J. D., and E. D. Earle.** 1983. The epoxide in HC-toxin is required for activity against susceptible maize. Physiol. Plant Pathol. **22:**371-376.

167.**Walton, J. D., and F. Cervone.** 1990. Endopolygalacturonase from maize pathogen *Cochliobolus carbonum*. Physiol. Mol. Plant Pathol. **36:**351-359.

168. **Walton, J. D., and F. R. Holden.** 1988. Properties of two enzymes involved in biosynthesis of the fungal pathogenicity factor HC-toxin. Mol. Plant-Microbe Interact. **1:**128-134.

169. **Walton, J. D., E. D. Earle, and B. W. Gibson.** 1982. Purification and structure of the host-specific toxin from *Helminthosporium carbonum* race 1. Biochem. Biophys. Res. Comm. **107:**785-794.

170. **Walton, J. D., E. D. Earle, H. Stähelin, A. Grieder, A. Hirota, and A. Suzuki.** 1985. Reciprocal biological activities of the cyclic tetrapeptides chlamydocin and HC toxin. Experientia **41:**348-350.

171. **Wijesundera, R. L. C., J. A. Bailey, R. J. W. Byrde, and A. H. Fielding.** 1989. Cell wall degrading enzymes of *Colletotrichum lindemuthianum*: their role in the development of bean anthracnose. Physiol. Mol. Plant Pathol. **34:**403-413.

172. **Woloshuk, C. P., H. D. Sisler, M. C. Tokousbalides, and S. R. Dutky.** 1980. Melanin biosynthesis in *Pyricularia oryzae*: site of tricyclazole inhibition and pathogenicity of melanin deficient mutants. Pestic. Biochem. Physiol. **14:**256-264.

173. **Yoder, O. C., and R. P. Scheffer.** 1973a. Effects of *Helminthosporium carbonum* toxin on absorption of solutes by corn roots. Plant Physiol. **52:**518-523.

174. **Yoder, O. C., and R. P. Scheffer.** 1973b. Effects of *Helminthosporium carbonum* toxin on nitrate uptake and reduction by corn tissues. Plant Physiol. **52:**513-517.

175. **Yun, S.J., D.J. Martin, B.G. Gengenbach, H.W. Rines, and D.A. Somers.** 1993. Sequence of a (1-3,1-4)-β-glucanase cDNA from oat. Plant Physiol. **103:**295-296.

176. **Xu, H., and K. Mendgen.** 1997. Targeted cell wall degradation at the penetration site of cowtpea rust basidiosporelings. Mol. Plant-Microbe Interact. **10:**87-94.