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THE ROLE OF CELL WALL DEGRADING ENZYMES IN COCHLIOBOLUS CARBONUM PATHOGENICITY

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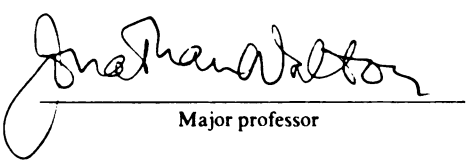

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THE ROLE OF CELL WALL DEGRADING ENZYMES IN *COCHLIOBOLUS CARBONUM* PATHOGENICITY

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ABSTRACT

THE ROLE OF CELL WALL DEGRADING ENZYMES IN *COCHLIOBOLUS CARBONUM* PATHOGENICITY

By

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Fungi secrete many cell wall degrading enzymes (CWDEs) for the penetration of host cell wall barrier, as well as for generation of simple molecules that can be assimilated for growth. Repression of six CWDE genes (*XYL1*, *XYL2*, *XYL3*, *XYL4*, *XYP1*, and *ARF1*) was observed in the maize pathogen *Cochliobolus carbonum* when it was grown on glucose as the sole carbon source, whereas expression was seen when the culture medium contained xylan. The *Saccharomyces cerevisiae* Mig1p and its fungal homolog (CreAp) has been implicated in glucose (catabolite) repression. Repression by Mig1p and CreAp is relieved by Snf1p which is encoded by *SNF1*, a gene first isolated from *S. cerevisiae*. An ortholog of *SNF1*, *ccSNF1*, was isolated from *C. carbonum* and *ccsnf1* mutants were created by targeted gene replacement. Growth of the *ccsnf1* mutant was reduced significantly on typical cell wall components, such as xylan, pectin, or purified maize cell walls. Extracellular CWDE activities, including β-1,3-glucanase, pectinase, and xylanase, as well as expression of several CWDE genes, were also significantly reduced in the *ccsnf1* mutant. The *ccsnf1* mutant was strongly reduced in virulence on susceptible maize, forming fewer spreading lesions, indicating that *ccSNF1*

is required for biochemical processes that are important for pathogenesis by *C. carbonum*. The *ARF1* gene is not expressed in the *C. carbonum ccsnf1* mutant. HPLC analysis of the culture filtrate of an *arf1* mutant constructed by gene replacement indicated that residual α-L-arabinofuranosidase activity remained high due to an additional two arabinofuranosidase activities. The growth and virulence of the *arf1* mutant were indistinguishable from the wild type. The disruption of a second arabinofuranosidase gene, *ARF2*, creating an *arf1/arf2* double mutant, led to the disappearance of the two major arabinofuranosidase activity peaks. However, the *arf1/arf2* double mutant had similar virulence as the wild type *C. carbonum*. The remaining activities were due to a bifunctional β-xylosidase/α-L-arabinofuranosidase (Ransom and Walton, 1997; Wegener et al., 1999). Thus a major decrease in virulence may require disruption of a regulatory gene (such as *ccSNF1*) that activates the expression of several CWDE genes. The CreA-Snf1 pathway may play an important role in the regulatory process that leads to cell wall degrading enzyme expression and virulence in pathogenic fungi.

For the Urhobos of West Africa.

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ARF1	α-L-arabinofuranosidase gene (1)
arf1	*Cochliobolus carbonum* mutant lacking functional *ARF1* gene
Arf1p	Protein product of *ARF1* gene
ARF2	α-L-arabinofuranosidase gene (2)
arf2	*Cochliobolus carbonum* mutant lacking functional *ARF2* gene
Arf2p	Protein product of *ARF2* gene
ccCREA	Cochliobolus carbonum CREA gene
ccCreAp	Protein product of *Cochliobolus carbonum CREA* gene
ccRPD3	*Cochliobolus carbonum* histone deacetylase gene
ccsnf1	*Cochliobolus carbonum* mutant lacking functional *ccSNF1* gene
ccSNF1	*Cochliobolus carbonum SNF1* gene
ccSnf1p	Protein product of *Cochliobolus carbonum SNF1* gene
CREA	*CREA* gene
CreAp	Protein product of *CREA* gene
CWDE(s)	Cell wall degrading enzyme(s)
EXG1	*Cochliobolus carbonum* exo-β-1,3-glucanase gene
GPD1	*Cochliobolus heterostrophus* glyceraldehyde-3-phosphate dehydrogenase gene
HPLC	High-perfomance liquid chromatography
MLG1	*Cochliobolus carbonum* β -1,3- -1,4-Glucanase gene
PGN1	*Cochliobolus carbonum* endopolygalacturonase gene
PGX1	*Cochliobolus carbonum* exopolygalacturonase gene

snf1	Mutant lacking functional *SNF1* gene
SNF1	*SNF1* gene
Snf1p	Protein product of *SNF1* gene
XYL1	*Cochliobolus carbonum* xylanase gene (1)
XYL2	*Cochliobolus carbonum* xylanase gene (2)
XYL3	*Cochliobolus carbonum* xylanase gene (3)
XYL4	*Cochliobolus carbonum* xylanase gene (4)
XYP1	*Cochliobolus carbonum* β-xylosidase gene

Chapter One

Enzymes and fungal pathogenicity

ABSTRACT

Plant pathogenic fungi secrete extracellular enzymes that are capable of degrading the cell walls of their host plants. These cell wall degrading enzymes (CWDEs) may be necessary for penetration of the cell wall barrier, as well as for generation of simple molecules that can be assimilated for growth. Most of these enzymes are substrate-inducible and their expression is controlled by both specific and global regulators. CWDE-inhibitors have been isolated from plants and some evidence indicates that they are components of general resistance.

INTRODUCTION

When microorganisms acquire the ability to enter and grow within healthy tissues, and subsequently circumvent plant host defense mechanisms, disease develops. Recent investigations on fungal pathogenicity have focused on defining the virulence factors produced by these pathogens, as well as the methods used to penetrate their hosts. Certain pathogenic fungi are known to produce host-specific toxins as agents of pathogenicity. In most cases, these toxins affect only a particular host plant. For example, *Fusarium graminearum* secretes trichothecene, a sesquiterpenoid involved in wheat head scab disease (Mirocha et al., 1994; O'Donnell et al., 2000), while pathotypes of *Alternaria alternata* produces several toxins specific for different plants such as AK-toxin for Japanese pear (Tanaka et al., 1999) and AAL-toxin for tomato (Abbas et al., 1995). The maize pathogen *Cochliobolus carbonum* makes HC-toxin, which is a critical determinant of virulence in its interaction with maize of genotype ***hm/hm*** (Ahn and Walton, 1996). Participation of these host-specific toxins in the establishment of plant diseases has been studied in the above and several other pathogenic fungi. Nevertheless, the ability of toxin-producing fungi to cause disease is dependent not only on their ability to produce a toxin, but also their fundamental ability to penetrate and grow within plant tissues.

Penetration into plant tissues and utilization of the nutrients found therein are requirements for all successful infections. Because cell wall constituents such as cellulose, xylan, pectin and proteins are typical among plants, the mechanism for penetration may be common across a variety of fungal pathogens. Most fungal pathogens and many non-pathogens produce large numbers of cell wall degrading enzymes (CWDEs) including pectinases, xylanases, cellulases, and proteases, which are capable of

depolymerizing the various components of the host cell walls. Consequently, they have been studied in relation to tissue maceration, penetration, or nutrient acquisition from the cell wall polymers. In pathogenic fungi, these CWDEs may be required not only for growth but for virulence as well. The question of whether CWDEs are important or necessary for disease development has been the subject of considerable research and debate over many decades (Annis and Goodwin, 1997; Mendgen et al, 1996; Walton, 1994).

FUEL, ENZYMES AND VIRULENCE

Generation of simple molecules

A major reason for microbial attack on plants is to obtain nutrients for growth. To achieve this, they must first overcome the physical barrier presented by the plant cell wall. Fungi utilize the food substances in their immediate vicinity to promote growth toward other areas of food availability. This continuing need to reach new food supplies for growth and reproduction may be the most significant driving force of fungal virulence. Generally, fungi secrete a wide variety of enzymes that can degrade various macromolecules to simple compounds that can be assimilated. Both the plant cell wall and the protoplasm contain nutrients that can be utilized by the growing fungus. Loosening and growing through the plant cell wall would allow the fungus access to

simple sugars, amino acids, minerals and nucleotides that are abundant within the cytosol of the plant. In addition, degradation of the cell wall macromolecules would generate simpler compounds such as xylose, glucose, and amino acids that could be absorbed for growth.

Adaptation

It could be that a microorganism becomes pathogenic if it can adapt its extracellular enzyme secretion both in proportion and kind to the cell wall constituents of a particular plant. This would occur if the rate of inhibition or degradation of these enzymes by plant substances is less than the rate of production of the enzymes by the microorganism. Additionally, the plant cell wall repair machinery may not be able to match the speed of the cell wall loosening activities of the fungal CWDEs. Investigations by St. Leger et al. (1997) indicate that fungi exhibit specific enzymatic adaptation to their various targets. The authors showed that plant pathogens (*Verticillium albo-atrum, Verticillium dahliae, Nectria haematococca*) produce high levels of enzymes capable of degrading pectic polysaccharides, cellulose, xylan and cutin, but secrete little or no chitinase and show no proteolytic activity against elastin or mucin. On the other hand insect pathogens (*Verticillium lecanii, Beauveria bassiana, Metarhizium anisopliae*) degrade a broad spectrum of proteins including elastin and mucin, but produce low levels of polysaccharidases. Saprophytes (*Neurospora crassa* and *Aspergillus nidulans*) and opportunistic pathogens (*Aspergillus fumigatus* and *Aspergillus flavus*) produce the broadest spectrum of protein and polysaccharide degrading enzymes, indicative of their less specialized nutritional status. The specialized adaptation of pathogenic fungi CWDEs

to their host polymers suggests a coevolved relationship between the fungi and their hosts (Cooper et al., 1988).

Redundancy

Fungal extracellular enzymes may have the potential to degrade the structural cell wall constituents of living plants, but proving that such interactions initiate or promote pathogenesis has been difficult. It is now well known that fungi often produce two or more distinguishable proteins with identical or similar enzymatic activity (Yao and Koller, 1995). Until a fungal genome is completely sequenced, it is impossible to determine the number of CWDE genes. Several isoforms of a particular enzyme can also occur and may be encoded by a single gene (Caprari et al., 1993). Although many other CWDE genes remain to be isolated, a search of the GENBANK database (www.ncbi.nlm.nih.gov) reveals that *Trichoderma reesei*, *C. carbonum*, *Magnaporthe grisea* and *Fusarium oxysporum* have multiple xylanase genes. As documented in Table 1, *C. carbonum* secretes numerous enzymes to degrade the complex web of carbohydrates and glycoproteins of the maize cell wall. Functional redundancy provides a means to adapt to different conditions, and is indicative of processes with vital importance to an organism. While some CWDEs are made at all times during the life cycle of the pathogen, others may be inducible only during plant infection, making it difficult to detect them *in vitro*. In the wheat scab fungus *F. oxysporum* f.sp. *lycopersici*, for example, two xylanase genes (*xyl2* and *xyl3*) are expressed *in vitro* during growth on oat spelt xylan or tomato vascular tissue. In contrast, RT-PCR revealed that *xyl3* is expressed in roots and in the lower stems of tomato plants infected by the fungus

Table 1. *C. carbonum* extracellular enzymes capable of degrading specific maize (*Zea mays*) cell wall components.

Cell Wall Component	Degrading Enzyme	Gene(s)	References
Arabinoxylan	endo-β-1,4-xylanase	*XYL1, XYL2, XYL3, XYL4*	Apel et al., 1993; Apel-Birkhold and Walton, 1996
	β-Xylosidase	*XYP1*	Wegener et al., 1999
	Arabinofuranosidase	*ARF1, ARF2*	Ransom and Walton, 1997; Tonukari et al., unpublished
Pectin	Endopolygalacturonase	*PGN1*	Scott-Craig et al., 1990
	Exopolygalacturonase	*PGX1*	Scott-Craig et al., 1998
	Pectin methylesterase	*PME1*	Scott-Craig et al., unpublished
Cellulose/ β-1,3-Glucan	Endo- β -1,4-glucanase (Cellulase)	*CEL1*	Sposato et al., 1995
	β -1,3- -1,4-Glucanase	*MLG1, MLG2*	Gorlach et al., 1998
	Exo-β-1,3 glucanase	*EXG1, EXG2*	Nikolskaya et al., 1998; Schaeffer et al., 1994
Protein	Protease	*ALP1, ALP2*	Murphy and Walton, 1996

throughout the whole disease cycle, whereas *xyl2* is only expressed during the final stages of disease (Ruiz-Roldan et al., 1999). Other CWDEs may be encoded by cryptic genes which may be only expressed under stress or unfavorable conditions, or when the products of the normal genes are compromised. *M. grisea xyl2* mutants secrete three additional xylanase activities that are not expressed in the parent strain (Wu et al., 1997). Similar secretion of previously undetected pectate lyases and an endo-β-1,3-glucanase following inactivation of related genes have been reported earlier by Kelemu and Collmer (1993) in bacteria and Beffa et al. (1993) in plants, respectively. Thus it is possible that additional genes are expressed during pathogenic growth, and some CWDEs may even exhibit overlapping activities. Therefore *in planta* studies are required to provide a more comprehensive knowledge of the full spectrum of enzymes that are produced during fungal pathogenesis. All these factors have made the assessment of the contribution of any individual CWDE to pathogenicity difficult.

Penetration

Penetration of the plant surface and cell walls is a crucial event in pathogenesis. Some fungal pathogens such as the rice blast fungus *M. grisea* form highly melanized appressoria (swellings at the end of germ tubes) inside which a high pressure builds up, allowing the penetration of the intact host cuticle by mechanical pressure (Howard et al., 1991; Money, 1997). Nevertheless, most fungal pathogens that do not form a large melanized appressorium probably need CWDEs for penetration (Mendgen et al., 1996). Even in fungal pathogens where the appressoria physically push through the plant cell wall, the contribution of enzymes to the softening of the wall cannot be ruled out.

Depolymerization or loosening of the components of the cell walls by enzymes would facilitate the penetration and passage through this barrier.

The ability to make knock-out mutants by DNA-mediated transformation now provides the means for the direct assessment of the role of CWDEs in disease development. Disruption of one or a few of the cell wall degrading enzyme genes may not result in any detectable decrease in the virulence of a fungus because of the redundancy of the enzymes. Such disruption may only eliminate a single component of the enzyme activity under investigation. Single mutations in each of 16 CWDE genes in *C. carbonum* have led to a reduction in specific enzyme activities in some cases but a corresponding decrease in virulence of the fungus was not observed (Apel et al., 1993; Apel-Birkhold and Walton, 1996; Gorlach et al., 1998; Murphy and Walton, 1996; Scott-Craig et al., 1990; Scott-Craig et al., 1998; Wegener et al., 1999). Multiple gene disruptions in *C. carbonum* have also been made including double and triple mutants (Apel-Birkhold and Walton, 1996; Scott-Craig et al., 1998) but these also had no effect in the virulence of the fungus. Despite this redundancy, however, single constitutive pectinase genes have been shown to contribute to the virulence of *Aspergillus flavus* on cotton bolls (Shieh et al., 1997) and *Botrytis cinerea* on tomato (ten Have et al., 1998). This evidence suggest that while a specific CWDE may be very important for virulence in one pathogenic fungus, others may likely require a combination of several CWDEs.

CWDE REGULATION

Substrate

The expression of most CWDEs depends on external conditions such as substrate availability or the specific stage of disease development. The majority of CWDEs are made at low basal levels on sugars such as glucose or sucrose, but are highly expressed when the fungus is grown on the appropriate substrate. In some cases, however, the products of CWDE digestion also induce expression. Northern analysis indicates that while *Trichoderma reesei xyn2* is induced in the presence of xylan and xylose (a product of xylan degradation), it is virtually silent in the presence of glucose (Zeilinger et al., 1996; Mach et al., 1996). Polygalacturonase, pectate lyase and pectin lyase activities are also induced in media supplemented with galactose or galacturonic acid (Crotti et al., 1998; Scott-Craig et al, 1990). Ilmen et al. (1997) also demonstrated that in *T. reesei* induction by cellulose and repression by glucose regulate cellulase expression in an actively growing fungus. However, derepression of the cellulase occurs without apparent addition of the inducer once glucose has been depleted from the medium. A similar conclusion on cellulase regulation was also obtained in *Agaricus bisporus* (Yague et al., 1997). Kolattukudy et al. (1995) reported that a cutinase present in spores of *F. solani pisi* releases small amounts of cutin monomers upon contact with the plant surface, which then trigger cutinase gene expression. This induction of CWDEs by cell wall components suggests a possible signaling pathway from the plant cell leading to the expression of CWDE-encoding genes inside the fungal cell.

Specific Regulators

Recent investigations indicate that both specific and global regulators control CWDEs (Table 2). XlnRp, a transcriptional activator of the xylanolytic system, has been identified in *A. niger* (van Peij et al., 1998). The *xlnR* gene encodes a polypeptide of 875 amino acids with a zinc binuclear cluster domain similar to the zinc clusters of the GAL4 superfamily of transcription factors. A 5'-GGCTAAA-3' consensus sequence, the binding site of XlnRp, is present within several xylanolytic promoters of various *Aspergillus* species and *Penicillium chrysogenum*. This sequence may be an important and conserved cis-acting element in the induction of xylanolytic genes in filamentous fungi. In contrast, the *Chaetomium gracile* xylanase A gene (*cgxA*) is repressed by binding of a protein designated AnRP. This protein binds specifically to a 5'TTGACAAAT-3' element in the promoter region of the *cgxA* gene (Mimura et al., 1999).

Global regulators

The involvement of CreAp, the carbon catabolite repressor, in the regulation of CWDEs has been reported in several fungi (Table 2). CreAp, which is the homolog of yeast Mig1p, binds to the promoter region of several genes and inhibits their expression (Dowzer and Kelly, 1991; Ronne, 1995). Deletion of this promoter region leads to an increase in the level of transcription of the regulated genes (de Graaff et al., 1994). The promoter region of the *xlnA* gene from *A. tubigensis* has also been studied with respect to xylan induction and carbon catabolite repression (de Graaff et al., 1994; Orejas et al., 1999) and contains four potential CreAp target sites. de Graaff et al. (1994) suggest that

Table 2. Proteins known to regulate cell wall degrading enzymes.

Regulatory factor	Organism	Comments	Reference
AnRP	*Chaetomium gracile*	Binds to a xylanase gene (*cgxA*) promoter and represses its expression.	Mimura et al., 1999
XlnRp	*A. niger*	Transcriptional activator of xylanase genes.	van Peij et al., 1998
CreAp	*A. niger* *B. cinerea* *E. nidulans* *G. fujikuroi* *N. crassa* *S. sclerotiorum* *T. reesei*	Carbon catabolite repressor which binds to the promoter region of enzymes and down-regulates gene expression.	de Graaff et al., 1994; Orejas et al., 1999; Zeilinger et al., 1996; Mach et al., 1996; Reymond-Cotton et al., 1996 Drysdale et al., 1993 Dowzer and Kelly, 1991 Shroff et al., 1996
ccSnf1p	*C. carbonum*	Relieves CreAp inhibition, thereby promoting expression of catabolite-repressible genes including CWDEs.	Tonukari et al., 2000

carbon catabolite repression of the *xlnA* gene is controlled at two levels, directly by repression of *xlnA* gene transcription and indirectly by repression of the transcriptional activator. *T. reesei xyn1* and *Sclerotinia sclerotiorum* polygalacturonase *pg1* genes are also regulated by CreAp (Mach et al., 1996; Reymond-Cotton et al., 1996; Zeilinger et al., 1996).

Because the biosynthesis of most CWDEs is substrate-inducible, it may appear that these enzymes are made upon encountering plant cell walls. In contrast, most CWDE genes are repressed by glucose. Repression by the CreA complex is relieved by Snf1p, which was first isolated in *S. cerevisiae* (Celenza and Carlson, 1984). Snf1p is a serine/threonine protein kinase (encoded by *SNF1*) and functions as an activator of gene expression by inactivating Mig1p in yeast, and presumably, the Mig1p homolog CreAp in fungi (Östling and Ronne, 1998; Treitel et al., 1998). The yeast *SUC2* gene which encodes invertase is repressed by Mig1p, and yeast carrying a *snf1* mutation cannot grow on sucrose because they are unable to express invertase. *Candida glabrata snf1* mutants also cannot use trehalose as carbon source (Petter and Kwon-Chung, 1996). Snf1p is not active in the presence of glucose, and preliminary evidence suggests that it is in a dephosphorylated state under these conditions (Hardie, 1999; Sugden et al., 1999). Snf1p also activates transcription in response to glucose limitation by modulating transcriptional activators such as Sip4p (Lesage et al., 1996; Vincent and Carlson, 1998). The *SNF1* gene is highly conserved among eukaryotes, and has been isolated from mammals, plants, and the nematode, *Caenorhabditis elegans* (Hardie et al., 1998). The mammalian homolog of Snf1p, AMP-activated protein kinase (AMPK), phosphorylates and regulates hydroxymethylglutaryl-CoA reductase and acetyl-CoA carboxylase, both of which are

key regulatory enzymes of sterol synthesis and fatty acid synthesis, respectively (Hardie, 1999). AMPK is activated by high AMP and low ATP levels via a complex mechanism, which involves allosteric regulation, promotion of phosphorylation by an upstream protein kinase (AMPK kinase), and inhibition of dephosphorylation. This protein-kinase cascade is a sensitive system which is activated by cellular stresses that deplete ATP and thus acts like a cellular fuel gauge. When AMPK detects a 'low-fuel' situation, it protects the cell by switching off ATP-consuming pathways (e.g. fatty acid synthesis and sterol synthesis) and switching on alternative pathways for ATP generation (e.g. fatty acid oxidation) (Hardie and Carling, 1997). Like Snf1p, AMPK can also regulate gene expression (Foretz et al., 1998). A tobacco Snf1p homolg, NPK5p, has a predicted kinase domain whose amino acid sequence is 65% identical to that of the *S. cerevisiae SNF1* product. Expression of *NPK5* in yeast cells allows *snf1* mutant cells to utilize sucrose for growth and causes constitutive expression of *SUC2* in wild-type cells. *NPK5*-related genes are expressed in the roots, leaves, and stems of tobacco plants (Muranaka et al., 1994).

Therefore, a gene disruption that abolishes Snf1p activity should down-regulate CWDE expression. Mutation of the *SNF1* gene in *C. carbonum* led to a decreased expression and activities of several CWDEs including β-1,3-glucanases, pectinases and xylanases (Tonukari et al., 2000; and in this thesis). The *C. carbonum snf1* mutant also shows reduced virulence on maize which is consistent with the hypothesis that CWDEs are virulence factors.

In *S. cerevisiae*, Snf1p is regulated by protein phosphatase 1, which is made up of the regulatory subunit Reg1p and the catalytic subunit Glc7p (Sanz et al., 2000).

Expression of the glucose transporter gene (*HXT*) in *S. cerevisiae* also depends on the Rgt1p transcriptional repressor and two glucose sensors in the membrane, Snf3p and Rgt2p. these sensors bind glucose and generate the intercellular signal to which Rgt1p responds (Ozcan and Johnston, 1999). Morever, mutation of two other *S. cerevisiae* genes, *SSN3* and *SSN8*, suppresses the effect of a *SNF1* mutation. *SSN3* encodes a cyclin-dependent protein kinase (cdk) homolog, and *SSN8* encodes a cyclin homolog, and both are likely to function as a cdk-cyclin pair (Kuchin et al., 1995). A spinach homolog of Snf1p, protein kinase III, is regulated by glucose-6-phosphate, and this suggests that the inhibition of Snf1p may be through glucose-6-phosphate (Huang et al., 1997; Toroser et al., 2000).

RESPONSE OF THE PLANT TO CWDEs

CWDEs as inducers of Plant Defense

Although plants are exposed to many microorganisms, some of which are potential pathogens, they are naturally resistant to the vast majority. Only a few fungi are pathogenic though most are endowed with CWDEs that could serve as virulence factors. The key to pathogenicity may depend to a large extent on the degree of resistance or susceptibility of the host. Saprophytes with their wide array of digestive enzymes are unable to consume living plants suggesting that all plants may have constitutive mechanisms that resist these enzymes. In addition to the cell wall barrier, other pathways that activate a battery of defense mechanisms against potential pathogens may also exist.

It has been specifically shown that a 21-kDa xylanase from *T. viride* elicits defense responses in tobacco plants, including tissue necrosis and increased production of pathogenesis-related proteins (Avni et al., 1994). In tomato, a cytoplasmic protein T-SUMO (tomato small ubiquitin-related modifier) is involved in mediating the signal generated by the ethylene-inducing xylanase from *T. viride* that leads to induction of plant defense responses (Hanania et al., 1999). In general, resistance could involve small molecules or proteins that inhibit or promote the digestion of the fungal secreted enzymes thereby denying access to the host cell. For a pathogen to be successful, it must be able to circumvent or overcome these antifungal defenses.

Protease Inhibitors

It now common knowledge that plants produce protease inhibitors that can inactivate fungal proteins, possibly including CWDEs. A proteinase-inhibitor protein (MPI) is induced in response to fungal infection in germinating maize embryos (Cordero et al., 1994). Increase in the proteinase inhibitor mRNA is a consequence of the wound produced by the penetration and colonization of the host tissues by the pathogen. Similarly, an antifungal cysteine protease inhibitor has been identified in pearl millet (Joshi et al., 1998), while a 14-kDa trypsin inhibitor from maize inhibits both conidial germination and hyphal growth of nine plant pathogenic fungi, including *Aspergillus parasiticus* and *Fusarium moniliforme* (Chen et al., 1999). Whether protease inhibitors specifically interact with CWDEs, however, is not known at present.

CWDE Inhibitors

The identification of CWDE inhibitors in plants provides indirect evidence that CWDEs are virulence factors. Polygalacturonase-inhibiting proteins (PGIP) are typically effective against fungal endopolygalacturonases (Cervone et al., 1989, 1990). The PGIPs are predominantly bound to plant cell walls, and their levels increase in bean hypocotyls when primary leaves begin to develop (Salvi et al, 1990). PGIP forms specific and reversible high affinity complexes with fungal polygalacturonases, thereby regulating the activity of the fungal enzymes (Cervone et al, 1989). Rapid accumulation of *pgip* mRNA correlates with the appearance of the hypersensitive response in incompatible interactions between *C. lindemuthianum* and *P. vulgaris*, and the inhibitor transcripts accumulate to higher levels in epidermal cells proximal to the site of infection (Devoto et al., 1997).

Recently, a glycosylated, basic protein that inhibits *A. niger* and *Trichoderma viride* endo-1,4-β-xylanases was identified in wheat (McLauchlan et al., 1999). This inhibitor, which is heat and protease sensitive, is a glycosylated protein with a basic isoelectric point similar to the PGIP from tomato (Stotz et al., 1994). An inhibitor of pectin methylesterase from kiwi fruit is also a glycoprotein, but has an acidic isoelectric point (Giovane et al., 1995). High-level expression of pear PGIPs in transgenic tomato fruits leads to increased resistance to *B. cinerea* (Powell et al., 1994). The PGIP inhibition of *B. cinerea* polygalacturonases slows the expansion of disease lesions and the associated tissue maceration in transgenic tomato plants (Powell et al., 2000). It is envisaged that more CWDE inhibitor proteins will be identified in the near future.

CONCLUSION

Virtually all fungi produce a great abundance and variety of CWDEs that may be needed for softening up of the plant cell walls for penetration by fungal hyphae, as well as provision of nutrients for growth. The application of molecular technologies to the study of fungal-plant interactions offers a new and more definitive approach for examining the role played by CWDEs in disease development. Resistance to CWDEs may be part of the constitutive and/or acquired resistance that plants possesses against various pathogens. Any factor that will inhibit the production of CWDEs in fungi could lead to reduced virulence. Though it has not yet been possible to demonstrate that individual CWDEs are required for *C. carbonum* pathogenicity (Apel et al., 1993; Apel-Birkhold and Walton, 1996; Gorlach et al., 1998; Murphy and Walton, 1996; Scott-Craig et al., 1990; Scott-Craig et al., 1998; Wegener et al., 1999), the focus of this study was to determine whether CWDEs are globally necessary for *C. carbonum* pathogenicity.

REFRENCES

Abbas, H.K., Tanaka, T., and Shier, W.T. (1995). Biological activities of synthetic analogues of *Alternaria alternata* toxin (AAL-toxin) and fumonisin in plant and mammalian cell cultures. Phytochemistry **40**, 1681-1689.

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

Annis, S.L., and Goodwin, P.H. (1997). Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. Eur. J. Plant Pathol. **103**, 1-14.

Apel, P.C., Panaccione, D.G., Holden, F.R., and Walton, J.D. (1993). Cloning and targeted gene disruption of *XYL1*, a β-1,4-xylanase gene from the maize pathogen *Cochliobolus carbonum*. Mol. Plant-Microbe Interact. **6**, 467-473.

Apel-Birkhold, P.C., and Walton, J.D. (1996). Cloning, disruption, and expression of two endo-β-1, 4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. Appl. Environ. Microbiol. **62**, 4129-4135.

Avni, A., Bailey, B. A., Mattoo, A. K., and Anderson, J. D. (1994). Induction of ethylene biosynthesis in *Nicotiana tabacum* by a *Trichoderma viride* xylanase is correlated to the accumulation of 1-aminocyclopropane-1-carboxylic acid (*ACC*) synthase and *ACC* oxidase transcripts. Plant. Physiol. **106**, 1049-1055.

Beffa, R. S., Neuhaus, J. M., and Meins, F. Jr. (1993). Physiological compensation in antisense transformants: specific induction of an "ersatz" glucan endo-1,3-beta-glucosidase in plants infected with necrotizing viruses. Proc. Natl. Acad. Sci. USA **90**, 8792-8796.

Caprari, C., Bergmann, C., Migheli, Q., Salvi, G., Albersheim, P., Darville, A., Cervone, F., and De Lorenzo, G. (1993). *Fusarium moniliforme* secretes four endopolygalacturonases derived from a single gene product. Physiol. Mol. Plant Pathol. **43**, 453-462.

Celenza, J.L., and Carlson, M. (1984). Cloning and genetic mapping of *SNF1*, a gene required for expression of glucose-repressible genes in *Saccharomyces cerevisiae*. Mol. Cell Biol. **4**, 49-53.

Cervone, F., De Lorenzo, G., Pressey, R., Daville, A.G., and Albershim, P. (1990). Can Phaseolus PGIP inhibit pectic lyases from microbes and plants? Phytochemistry **29**, 447-449.

Cervone, F., Hahn, M.G., De Lorenzo, G., Daville, A.G., and Albershim, P. (1989). Host-pathogen interactions. XXXIII. A plant protein converts a fungal

pathogenesis factor into an elicitor of plant defense responses. Plant Physiol. **90**, 542-548.

Chen, Z.Y., Brown, R.L., Lax, A.R., Cleveland, T.E., and Russin, J.S. (1999). Inhibition of plant-pathogenic fungi by a corn trypsin inhibitor overexpressed in *Escherichia coli*. Appl. Environ. Microbiol. **65**, 1320-1324.

Cooper, R.M., Longman, D., Campbell, A., Henry, M., and Lees, P.E. (1988). Enzymatic adaptation of cereal pathogens to the monocotyledonous primary wall. Physiol. Mol. Plant Pathol. **32**, 33-47.

Cordero, M.J., Raventos, D., and San Segundo, B. (1994). Expression of a maize proteinase inhibitor gene is induced in response to wounding and fungal infection: systemic wound-response of a monocot gene. Plant J. **6**, 141-150.

Crotti, L.B., Terenzi, H.F., Jorge, J.A., de Lourdes, M., and Polizeli, M,L. (1998). Regulation of pectic enzymes from the exo-1 mutant strain of *Neurospora crassa*: effects of glucose, galactose, and galacturonic acid. J. Basic Microbiol. **38**, 181-188.

de Graaff, L.H., van den Broeck, H.C., van Ooijen, A.J., and Visser, J. (1994). Regulation of the xylanase-encoding *xlnA* gene of *Aspergillus tubigensis*. Mol. Microbiol. **12**, 479-490.

Devoto, A., Clark, A.J., Nuss, L., Cervone, F., and De Lorenzo, G. (1997). Developmental and pathogen-induced accumulation of transcripts of polygalacturonase-inhibiting protein in *Phaseolus vulgaris* L. Planta **202**, 284-292.

Dowzer, C.E., and Kelly, J.M. (1991). Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. Mol. Cell. Biol. **11**, 5701-5709.

Drysdale, M.R., Kolze, S.E., and Kelly, J.M. (1993). The *Aspergillus niger* carbon catabolite repressor encoding gene, *creA*. Gene **130**, 241-245.

Foretz, M., Carling, D., Guichard, C., Ferre, P., and Foufelle, F. (1998). AMP-activated protein kinase inhibits the glucose-activated expression of fatty acid synthase gene in rat hepatocytes. J. Biol. Chem. **273**, 14767-14771.

Giovane, A., Balestrieri, C., Quagliuolo, L., Castaldo, D., and Servillo, L. (1995). A glycoprotein inhibitor of pectin methylesterase in kiwi fruit. Purification by affinity chromatography and evidence of a ripening-related precursor. Eur. J. Biochem. **233**,926-929.

Gorlach, J.M., Van Der Knaap, E., and Walton, J.D. (1998). Cloning and targeted disruption of *MLG1*, a gene encoding two of three extracellular mixed-linked glucanases of *Cochliobolus carbonum*. Appl. Environ. Microbiol. **64**, 385-391.

Hanania, U., Furman-Matarasso, N., Ron, M., and Avni, A. (1999). Isolation of a novel SUMO protein from tomato that suppresses EIX-induced cell death. Plant J. **19**, 533-541.

Hardie, D.G. (1999). Roles of the AMP-activated/SNF1 protein kinase family in the response to cellular stress. Biochem. Soc. Symp.**64**,13-27.

Hardie, D.G., and Carling, D. (1997). The AMP-activated protein kinase--fuel gauge of the mammalian cell? Eur. J. Biochem. **246**, 259-273.

Hardie, D.G., Carling, D., and Carlson, M. (1998). The AMP-activated/Snf1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu. Rev. Biochem. **67**, 821-855.

Howard, R.J., Ferrari, M.A., Roach, D.H., and Money, N.P. (1991). Penetration of hard substrates by a fungus employing enormous turgor pressures. Proc. Natl. Acad. Sci. USA **88**, 11281-11284.

Huang, D., Wilson, W.A., and Roach, P.J. (1997). Glucose-6-P control of glycogen synthase phosphorylation in yeast. J. Biol. Chem. **272**, 22495-22501.

Ilmen, M., Saloheimo, A., Onnela, M.L., and Penttila, M.E. (1997). Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. Appl. Environ. Microbiol. **63**, 1298-1306.

Joshi, B.N., Sainani, M.N., Bastawade, K.B., Gupta, V.S., and Ranjekar, P.K. (1998). Cysteine protease inhibitor from pearl millet: a new class of antifungal protein. Biochem. Biophys. Res. Commun. **246**, 382-387.

Kelemu, S., and Colemer, A. (1993). *Erwinia chrysanthemi* EC16 produces a second set of plant-inducible pectate lyase isozymes. Appl. Environ. Microbiol. **59**, 1756-1761.

Kolattukudy, P.E., Rogers, L.M., Li, D., Hwang, C.S., and Flaishman, M.A. (1995). Surface signaling in pathogenesis. Proc. Natl. Acad. Sci. USA **92**, 4080-4087.

Kuchin, S., Yeghiayan, P., and Carlson, M. (1995). Cyclin-dependent protein kinase and cyclin homologs SSN3 and SSN8 contribute to transcriptional control in yeast. Proc. Natl. Acad. Sci. USA **92**, 4006-4010.

Lesage, P., Yang, X., and Carlson, M. (1996). Yeast SNF1 protein kinase interacts with SIP4, a C_6 zinc cluster transcriptional activator: a new role for SNF1 in the glucose response. Mol. Cell. Biol. **16**, 1921-1928.

Mach, R.L., Strauss, J., Zeilinger, S., Schindler, M., and Kubicek, C.P. (1996). Carbon catabolite repression of xylanase I (*xyn1*) gene expression in *Trichoderma reesei*. Mol. Microbiol. **21**, 1273-1281.

McLauchlan, W.R., Garcia-Conesa, M.T., Williamson, G., Roza, M., Ravestein, P., and Maat, J. (1999). A novel class of protein from wheat which inhibits xylanases. Biochem. J. 338, 441-446.

Mendgen, K., Hahn, M. and Deising, H., (1996). Morphogenesis and mechanisms of penetration by plant pathogenic fungi. Annu. Rev. Phytopathol. **34**, 367-386.

Mimura, S., Rao, U., Yoshino, S., Kato, M., and Tsukagoshi, N. (1999). Depression of the xylanase-encoding *cgxA* gene of *Chaetomium gracile* in *Aspergillus nidulans*. Microbiol. Res. **153**, 369-376.

Mirocha, C.J., Xie, W., Xu, Y., Wilcoxson, R.D., Woodward, R.P., Etebarian, R.H., and Behele, G. (1994). Production of trichothecene mycotoxins by *Fusarium graminearum* and *Fusarium culmorum* on barley and wheat. Mycopathologia **128**, 19-23.

Money, N.P. (1997). Mechanism linking cellular pigmentation and pathogenicity in rice blast disease. Fungal Genet. Biol. **22**, 151-152.

Muranaka, T., Banno, H., and Machida, Y. (1994). Characterization of tobacco protein kinase NPK5, a homolog of *Saccharomyces cerevisiae SNF1* that constitutively activates expression of the glucose-repressible *SUC2* gene for a secreted invertase of *S. cerevisiae*. Mol. Cell. Biol. **14**, 2958-2965.

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

Neer, E.J., Schmidt, C.J., Nambudripad, R., and Smith, T.F. (1994). The ancient regulatory-protein family of WD-repeat proteins. Nature **371**, 297-300.

Nikolskaya, A.N., Pitkin, J.W., Schaeffer, H.J., Ahn, J.H., and Walton, J.D. (1998). EXG1p, a novel exo-β-1,3-glucanase from the fungus *Cochliobolus carbonum*, contains a repeated motif present in other proteins that interact with polysaccharides. Biochim. Biophys. Acta **1425**, 632-636.

O'Donnell, K., Kistler, H.C., Tacke, B.K., and Casper, H.H. (2000). Gene genealogies reveal global phylogeographic structure and reproductive isolation among

lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proc. Natl. Acad. Sci. USA **97**, 7905-7910.

Orejas, M., MacCabe, A.P., Perez, Gonzalez, J.A., Kumar, S., and Ramon, D. (1999). Carbon catabolite repression of the *Aspergillus nidulans xlnA* gene. Mol. Microbiol. **31**, 177-184.

Östling, J., and Ronne, H. (1998). Negative control of the Mig1p repressor by Snf1p-dependent phosphorylation in the absence of glucose. Eur. J. Biochem. **252,** 162-168.

Ozcan, S., and Johnston, M. (1999). Function and regulation of yeast hexose transporters. Microbiol. Mol. Biol. Rev. **63**, 554-569.

Petter, R, and Kwon-Chung, K.J. (1996). Disruption of the *SNF1* gene abolishes trehalose utilization in the pathogenic yeast *Candida glabrata*. Infect Immun 64, 5269-5273.

Powell, A.L.T., D'hallewin, G., Hall, B.D., Stotz, H., Labavitch, J.M., and Bennett, A.B. (1994). Glycoprotein inhibitors of fungal polygalacturonases: expression of pear PGIP improves resistance in transgenic tomatoes. Plant Physiol. **105**, 159.

Powell, A.L., van Kan, J., ten Have, A., Visser, J., Greve, L.C., Bennett, A.B., and Labavitch, J.M. (2000). Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant Microbe Interact. **13**, 942-950.

Ransom, R.F., and Walton, J.D. (1997). Purification and characterization of extracellular β-xylosidase and α-arabinosidase from the plant pathogenic fungus *Cochliobolus carbonum*. Carbohydr. Res. **297**, 357-364.

Reymond-Cotton, P., Fraissinet-Tachet, L., and Fevre, M. (1996). Expression of the *Sclerotinia sclerotiorum* polygalacturonase *pg1* gene: possible involvement of *CREA* in glucose catabolite repression. Curr. Genet. **30**, 240-245.

Ronne, H. (1995). Glucose repression in fungi. Trends genet. **11**, 12-17.

Ruiz-Roldan, M.C., Di Pietro, A., Huertas-Gonzalez, M.D., and Roncero, M.I. (1999). Two xylanase genes of the vascular wilt pathogen *Fusarium oxysporum* are differentially expressed during infection of tomato plants. Mol. Gen. Genet. **261**, 530-536.

Salvi, G., Giarrizzo, F., De Lorenzo, G., and Cervone, F. (1990). A polygalacturonase-inhibiting protein in the flowers of *Phaseolus vulgaris* L. J. Plant Physiol. **136**, 513-518.

Sanz, P., Alms, G.R., Haystead, T.A., and Carlson, M. (2000). Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase. Mol. Cell Biol. **20**, 1321-1328.

Schaeffer, H.J., Leykam, J., and Walton, J.D. (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.

Scott-Craig, J.S., Cheng, Y.Q., Cervone, F., De Lorenzo, G., Pitkin, J.W., and Walton, J.D. (1998). Targeted mutants of *Cochliobolus carbonum* lacking the two major extracellular polygalacturonases. Appl. Environ. Microbiol. **64**, 1497-1503.

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

Shieh, M.T., Brown, R.L., Whitehead, M.P., Cary, J.W., Cotty, P.J., Cleveland, T.E., and Dean, R.A. (1997). Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl. Env. Microbiol. **63**, 3548-3552.

Shroff, R.A., Lockington, R.A., and Kelly, J.M. (1996). Analysis of mutations in the *creA* gene involved in carbon catabolite repression in *Aspergillus nidulans*. Can. J. Microbiol. **42**, 950-959.

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

St Leger, R.J., Joshi, L., and Roberts, D.W. (1997). Adaptation of proteases and carbohydrates of saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches. Microbiology **143**, 1983-1992.

Stotz, H.U., Contos, J.J., Powell, A.L., Bennett, A.B., and Labavitch, J.M. (1994). Structure and expression of an inhibitor of fungal polygalacturonases from tomato. Plant Mol. Biol. **25**, 607-617.

Sugden, C., Crawford, R.M., Halford, N.G., and Hardie, D.G. (1999). Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5'-AMP. Plant J. **19**, 433-439.

Tanaka, A., Shiotani, H., Yamamoto, M., and Tsuge, T. (1999). Insertional mutagenesis and cloning of the genes required for biosynthesis of the host-

specific AK-toxin in the Japanese pear pathotype of *Alternaria alternata*. Mol. Plant Microbe Interact. **12**, 691-702.

ten Have, A., Mulder, W., Visser, J., and van Kan, J.A.L. (1998). The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol. Plant-Microbe Interact. **11**, 1009-1016.

Tonukari, N.J., Scott-Craig, J.S. and Walton, J.D. (2000) The *Cochliobolus carbonum SNF1* gene is required for cell wall-degrading enzyme expression and virulence on maize. Plant Cell **12**, 237-248.

Toroser, D., Plaut, Z., and Huber, S.C. (2000). Regulation of a plant SNF1-related protein kinase by glucose-6-phosphate. Plant Physiol. **123**, 403-412.

Treitel, M.A., Kuchin, S., and Carlson, M. (1998). Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **18**, 6273-6280.

van Peij, N.N., Visser, J., and de Graaff, L.H. (1998). Isolation and analysis of *xlnR*, encoding a transcriptional activator co-ordinating xylanolytic expression in *Aspergillus niger*. Mol. Microbiol. **27**, 131-142.

Vincent, O., and Carlson, M. (1998). Sip4, a Snf1 kinase-dependent transcriptional activator, binds to the carbon source-responsive element of gluconeogenic genes. EMBO J. **17**, 7002-7008.

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

Wegener, S., Ransom R.F., and Walton, J.D. (1999). A unique eukaryotic β-xylosidase gene from the phytopathogenic fungus *Cochliobolus carbonum*. Microbiology **145**, 1089-1095.

Wu, C., Ham, K.-S., Darvill, A.G., and Albersheim, P. (1997). Deletion of two endo-□-1,4-xylanase genes reveals additional isozymes secreted by the rice blast fungus. Mol. Plant-Microbe Interact. **10**, 700-708.

Yague, E., Mehak-Zunic, M., Morgan, L., Wood, D.A., and Thurston, C.F. (1997). Expression of CEL2 and CEL4, two proteins from *Agaricus bisporus* with similarity to fungal cellobiohydrolase I and beta-mannanase, respectively, is regulated by the carbon source. Microbiology **143**, 239-244.

Yao, C., and Koller, W. (1995). Diversity of cutinases from plant pathogenic fungi: different cutinases are expressed during saprophytic and pathogenic stages of *Alternaria brassicicola*. Mol. Plant Microbe Interact. **8**, 122-130.

Zeilinger, S., Mach, R.L., Schindler, M., Herzog, P., and Kubicek, C.P. (1996). Different inducibility of expression of the two xylanase genes *xyn1* and *xyn2* in *Trichoderma reesei*. J. Biol. Chem. **271**, 25624-25629.

Chapter Two

The *Cochliobolus carbonum SNF1* gene is required for cell wall-degrading enzyme expression and virulence on maize

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ABSTRACT

The production of cell wall-degrading enzymes (wall depolymerases) by plant pathogenic fungi is under catabolite (glucose) repression. In *Saccharomyces cerevisiae*, the *SNF1* gene is required for expression of catabolite-repressed genes when glucose is limiting. An ortholog of *SNF1*, *ccSNF1*, was isolated from the maize pathogen *Cochliobolus carbonum* and *ccsnf1* mutants of HC toxin-producing ($Tox2^+$) and HC toxin nonproducing ($Tox2^-$) strains created by targeted gene replacement. Growth in vitro of the *ccsnf1* mutants was reduced by 50 to 95% on complex carbon sources, such as xylan, pectin, or purified maize cell walls. Growth on simple sugars was affected depending on the sugar. Whereas growth on sucrose or glucose, fructose, or sucrose was normal, growth on galactose, galacturonic acid, maltose, or xylose was somewhat reduced, and growth on arabinose was strongly reduced. HC toxin production was normal in the $Tox2^+$ *ccsnf1* mutant, as were conidiation, conidial morphology, conidial germination, and in vitro appressorium formation. Activities of secreted β-1,3-glucanase, pectinase, and xylanase in culture filtrates of the $Tox2^+$ *ccsnf1* mutant were reduced by 53, 24 and 65%, respectively. mRNA expression under inducing conditions of *XYL1*, *XYL2*, *XYL3*, *XYL4*, *XYP1*, *ARF1*, *MLG1*, *EXG1*, *PGN1*, and *PGX1*, which encode secreted wall-degrading enzymes, was downregulated. The $Tox2^+$ *ccsnf1* mutant was strongly reduced in virulence on susceptible maize, forming fewer spreading lesions, which, however, had normal morphology. The $Tox2^-$ *ccsnf1* mutant also formed fewer nonspreading lesions that also had normal morphology. The results indicate that *ccSNF1* is required for biochemical processes that are important for pathogenesis by *C. carbonum* and suggest

penetration as the single most important step at which *ccSNF1* is required. The specific biochemical processes controlled by *ccSNF1* probably include, but are not necessarily restricted to, an ability to degrade polymers of the plant cell wall and to take up and metabolize the sugars that are produced.

INTRODUCTION

The plant cell wall is a major barrier to the penetration and spread of potential pathogenic organisms, and all of the major groups of cellular plant pathogens are known to make extracellular enzymes that can degrade cell wall polymers. Although the involvement of wall-degrading enzymes and their genes in penetration, ramification, plant defense induction, and symptom expression has been extensively studied, conclusive evidence for or against a role for any particular enzyme activity in any aspect of pathogenesis has been difficult to obtain (Walton, 1994).

The major obstacle to addressing the function of wall-degrading enzymes has been redundancy: all pathogens that have been studied in detail have multiple genes for any particular enzyme activity. Thus, most fungal strains mutated in wall-degrading enzyme genes – either by conventional (e.g., Cooper, 1987) or molecular (e.g., Scott-Craig et al., 1990) methods – retain at least some residual enzyme activity. For example, the pea pathogen *Nectria haematococca* (*Fusarium solani* f sp *pisi*) has four functional pectate lyase genes, the maize pathogen *Cochliobolus carbonum* and the rice pathogen *Magnaporthe grisea* each have at least four xylanase genes, and the cosmopolitan pathogen *Botrytis cinerea* has up to five endo-polygalacturonase genes (Apel-Birkhold and Walton, 1996; Guo et al., 1996; Wu et al., 1997; ten Have et al., 1998). Even strains of fungi with multiple mutations still retain residual enzyme activity and are still pathogenic (Apel-Birkhold and Walton, 1996; Scott-Craig et al., 1998; J. S. Scott-Craig and J. D. Walton, unpublished results). Despite redundancy, single genes of a particular class have been shown, in two cases, to contribute to the virulence of pathogenic fungi.

Particular constitutive pectinases are virulence factors for *Aspergillus flavus* on cotton bolls and for *B. cinerea* on tomato (Shieh et al., 1997; ten Have et al., 1998).

The gamut of extracellular wall-degrading enzymes produced by the ascomycete *C. carbonum* includes pectinases, xylanases, cellulases, mixed-linked (β-1,3-β-1,4)-glucanases, β-1,3-glucanases, proteases, xylosidases, arabinosidase, and undoubtedly others. None of the strains generated to date with single mutations in any of the genes encoding these enzymes have reduced virulence. Furthermore, with only a few exceptions, the mutants still grow as well as wild type on the appropriate substrate in vitro (Scott-Craig et al., 1990; Apel et al., 1993; Schaeffer et al., 1994; Sposato et al., 1995; Apel-Birkhold and Walton, 1996; Murphy and Walton, 1996; Nikolskaya et al., 1998; Görlach et al., 1998; Scott-Craig et al., 1998; Wegener et al., 1999).

An alternative approach to the individual isolation and disruption of each wall-degrading enzyme gene would be to identify genetic regulatory elements whose mutation results in the simultaneous loss or downregulation of multiple enzymes. If a mutant globally impaired in its ability to make wall-degrading enzymes were still pathogenic, it would bring into serious doubt a significant role for such enzymes in pathogenesis (Walton, 1994).

In culture, the expression of most wall-degrading enzymes by most fungi, including plant pathogens, is inhibited by glucose or other simple sugars, which is a well-studied metabolic process known as catabolite or glucose repression (Ruijter and Visser, 1997). Most and perhaps all of the examined extracellular enzyme activities of *C. carbonum* are subject to catabolite repression (Walton and Cervone, 1990; van Hoof et al., 1991; Holden and Walton, 1992; Ransom and Walton, 1997). In yeast, release from

catabolite repression requires a protein kinase called Snf1p. Snf1p is required for the expression of glucose-repressed genes such as invertase (*SUC2*) when glucose is limiting. That is, glucose-repressed genes remained repressed in a *snf1* mutant even in the absence of glucose (Celenza and Carlson, 1984; Hardie et al., 1998; Östling and Ronne, 1998; Treitel et al., 1998). A major function of Snf1p is to phosphorylate Mig1p, a DNA-binding transcriptional repressor. The ortholog of *MIG1* in filamentous fungi is called *creA* (Ronne, 1995). Phosphorylation of Mig1p inhibits its binding to the promoters of the genes that it represses and promotes movement of Mig1p out of the nucleus into the cytoplasm (DeVit et al., 1997). Snf1p controls the response to glucose through additional mechanisms, because it also activates via phosphorylation the transcriptional activators Sip4p and Cat8p (Lesage et al., 1996; Vincent and Carlson, 1998).

Orthologs of *SNF1* are present in many other organisms, including mammals and plants. Its counterpart in mammals is AMP-dependent protein kinase (Hardie et al., 1998). A *SNF1* ortholog is involved in the response of *Arabidopsis* to glucose (Bhalerao et al., 1999). To the best of our knowledge, the biology of *SNF1* genes has not been studied previously in filamentous fungi.

Because *SNF1* is required for derepression of catabolite-repressed genes in yeast, mutation of the orthologous gene in *C. carbonum* might cause irreversible downregulation of catabolite-repressed wall-degrading enzymes. *snf1* mutants might therefore be useful for testing whether wall-degrading enzymes are virulence factors in pathogenic fungi.

METHODS

Fungal Cultures, Media, and Growth Conditions

The wild-type HC toxin-producing ($Tox2^+$) isolate of *Cochliobolus carbonum*, designated 367-2A, was derived from isolate SB111 (ATCC 90305) and maintained on V8 juice agar plates (Apel et al., 1993). The wild-type HC toxin nonproducing ($Tox2^-$) isolate was 164R1, which is a progeny of SB111 (Walton, 1987). The fungus was grown in liquid media or agar plates containing mineral salts, 0.2% yeast extract, and trace elements (Van Hoof et al., 1991). Carbon sources were 2% (w/v) glucose, sucrose, oat spelt xylan (Fluka), citrus pectin (Sigma P-9135), or maize cell walls (Sposato et al., 1995). For quantitation of growth on agar plates, 5 μL of a conidial suspension (10^4 conidia per mL) in 0.1% Tween 20 was pipetted onto the center of the plate. Plates were incubated under fluorescent lights at 21°C.

Nucleic Acid Manipulations

The *C. carbonum* genomic and cDNA libraries have been previously described (Scott-Craig et al., 1990). DNA and total RNA were extracted from lyophilized mats (Apel et al., 1993; Pitkin et al., 1996). The methods used for DNA and RNA electrophoresis, gel blotting, probe labeling, and hybridization have also been described elsewhere (Apel-Birkhold and Walton, 1996). For RNA blots, the *Cochliobolus heterostrophus GPD1*

gene, encoding glyceraldehyde-3-phosphate dehydrogenase, was used as a loading control (Van Wert and Yoder, 1992).

Polymerase chain reaction (PCR) was performed in a thermocycler (MJ Research, Callahan, CA) using *Taq* DNA polymerase (GIBCO-BRL) and two degenerate oligonucleotide primers based on the conserved regions of *SNF1* genes (sense, 5'-CAYCCNCAYATHATHAA-3'; antisense, 5'-TCNGGNGCNGCRTARTT-3', where Y is T or C; R is G or A; H is T, C or A; N is A, T, G, or C). Touchdown PCR (Don et al., 1991) with these primers and *C. carbonum* genomic DNA as template was performed under the following conditions: initial denaturation at 94°C for 3 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing for 1 min, and polymerization at 72°C for 2 min. The annealing temperature ranged from 60 to 45°C with a decrease of 1°C every three cycles. This was followed by 10 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and polymerization at 72°C for 2 min. The PCR product was cloned into the *Eco*RV site of pBluescript II KS+ (Stratagene, La Jolla, CA).

The transcriptional start site of *ccSNF1* was determined using the 5' rapid amplification of cDNA ends (RACE) system, version 2.0, following the instructions of the manufacturer (GIBCO-BRL) (Frohman et al., 1988). An oligonucleotide of sequence 5'-GCCCTCGCCCAGGGTGC-3' was used to prime first-strand cDNA synthesis, which was then amplified by PCR using a nested primer of the sequence 5'-GCCGAGACGCTGGCTCG-3'.

Automated fluorescence DNA sequencing was done at the Department of Energy-Plant Research Laboratory Sequencing Facility at Michigan State University. Sequence data were analyzed with Lasergene software (DNASTAR, Inc., Madison, Wisconsin).

Functional Complementation of Yeast *snf1*

Saccharomyces cerevisiae was grown at 30°C on plates containing YPD (1% yeast extract, 2% [w/v] peptone, 2% [w/v] glucose, and 2% [w/v] agar). The *ura* reference strain was MG106 (MATa *ade2-1 can1-100 his3-1115 leu2-3112 trp1-1 ura3-1*). The *ccSNF1* cDNA was excised from plasmid pSnf1c-3 with SacI and XbaI and cloned into pVT100-U, a yeast expression vector with the alcohol dehydrogenase *ADH1* promoter and terminator and the *URA3* selectable marker. The resulting construct, pSnf1c-4, or the empty vector pVT100-U as a control, were used to transform yeast MCY1846 (MATa *snf1Δ10 lys2-801 ura3-52)* cells as described by Gietz and Woods (1994). Transformants were first selected on synthetic dextrose-uracil (SD-URA) medium, which contains 0.67% yeast nitrogen base without amino acids (DIFCO Laboratories, Detroit, MI), 0.062 % -Leu/-Trp/-Ura DO (dropout) supplement (Clonetech Laboratories, Palo Alto, CA), 0.01% Leu, 0.002% tryptophan, 2% (w/v) glucose, and 2% (w/v) agar at 30°C. The transformed cells were grown in liquid YPD medium overnight. The cultures were then streaked on YPS agar plates, which contain 1% yeast extract, 2% peptone, 2 % (w/v) sucrose and 2 % (w/v) agar. The plates were photographed after 2 days at 30°C.

Disruption of *ccSNF1*

The gene replacement vector was derived from a 3.3-kb *Sac*I-*Eco*RV genomic fragment containing *ccSNF1* (Figures 1A and 1B) cloned into pBluescriptII KS+. An internal 2.1-kb *Pst*I-*Sal*I fragment was deleted and replaced with a 1.4-kb *Pst*I-*Sal*I fragment

containing the *hph* gene conferring hygromycin resistance. To create convenient restriction sites, the *Hpa*I fragment from plasmid CB1003 (Carroll et al., 1994) was cloned into the *Eco*RV site of pBluescriptII KS+.

The replacement vector was linearized at the *Bss*HII-*Sac*I sites prior to transformation of *C. carbonum* 367-2A ($Tox2^+$) or 164R1 ($Tox2^-$) protoplasts (Pitkin et al., 1996; Scott-Craig et al., 1990). Mycelium for protoplasts for transformation was obtained from germinating conidia (Apel et al., 1993). Transformants were purified by two rounds of single-spore isolation.

Enzyme Assays

Total xylanase, pectinase, and β-1,3-glucanase activities were assayed by release of reducing sugars from the indicated substrate (Lever, 1972). Thirty microliters of culture supernatant was assayed in a 300-μL reaction volume containing 1.0% oat spelt xylan, polygalacturonic acid, or β-1,3-glucan (laminarin), and 50 mM sodium acetate, pH 5.0, at 37°C for 30 min. A 25- or 100-μL aliquot of the reaction mixture was mixed with 1.5 mL of a working solution of p-hydroxybenzoic acid hydrazide, the mixture heated at 100°C for 10 min, and the absorbance measured at 410 nm (Lever, 1972). Enzyme activities are based on the amount of monomers (glucose, galacturonic acid and xylose for β-1,3-Glucanase, pectinase and xylanase activities, respectively) released per μL of reaction volume.

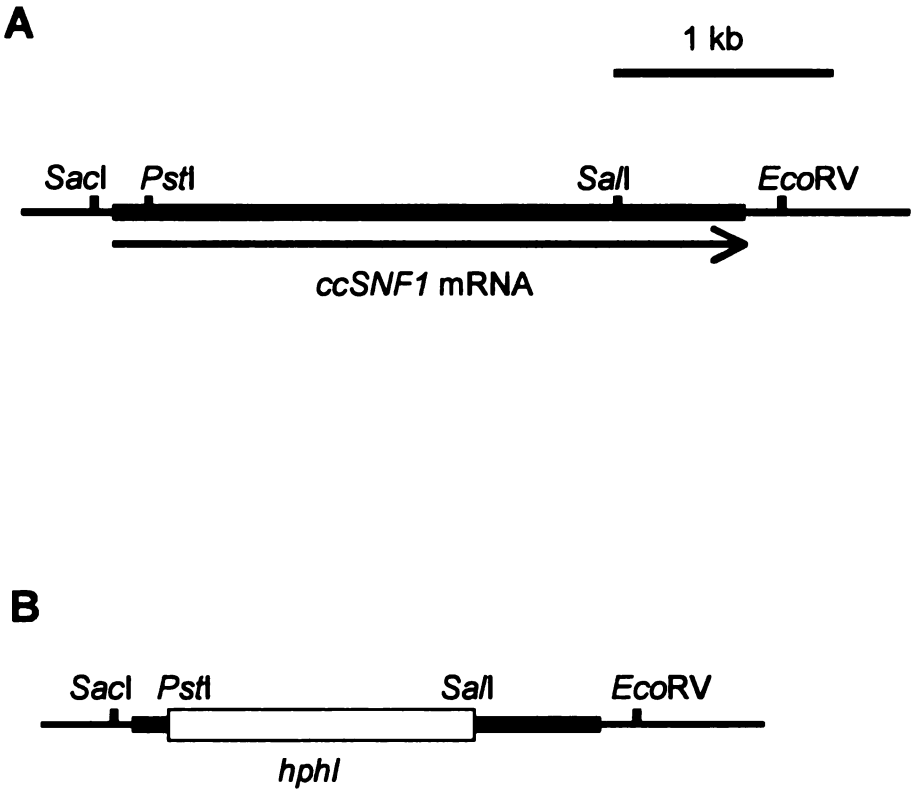

Figure 1. Construction of *ccsnf1* Disruption Transformants.

(A) Map of the *ccSNF1* gene.
(B) An internal 2.1-kb *Pst*I-*Sal*I fragment of *ccSNF1* was replaced with *hphI*.

HC toxin Analysis

HC toxin was extracted with chloroform from culture filtrates of *C. carbonum* grown for 14 days, fractionated by thin-layer chromatography on silica using a solvent system of dichloromethane:acetone (1:1 by volume), and detected using an epoxide-specific colorimetric reagent (Meeley and Walton, 1991).

Pathogenicity Assay

Pathogenicity was tested by spray-inoculating 18-day-old susceptible inbred maize line Pr (genotype *hm1/hm1*) with a suspension of conidia (10^4 per mL) in 0.1% Tween 20. The plants were inoculated in the afternoon and covered with plastic bags overnight. The plants were grown in a greenhouse and monitored daily for eight days or until death.

RESULTS

Isolation of *C. carbonum SNF1* (*ccSNF1*)

Two degenerate oligonucleotide primers were synthesized based on conserved regions of Snf1p-related sequences of *Saccharomyces cerevisiae*, *Candida glabrata*, and tobacco *NPK5* (Celenza and Carlson, 1984; Muranaka et al., 1994; Petter and Kwon-Chung, 1996). Amplification by polymerase chain reaction (PCR) with *C. carbonum* genomic

DNA as template yielded a product of the predicted size (400 bp). The PCR product was used to isolate the *SNF1* gene and cDNA copies from *C. carbonum* genomic and cDNA libraries, respectively. The *C. carbonum* cDNA clone of *ccsnf1* was cloned into pGEM7 to create plasmid pSnf1c-3. The *C. carbonum* gene (designated *ccSNF1*) has two introns of 60 and 51 bp. The 3'-untranslated region is 159 bp and the 5'-untranslated region is 120 bp in length (Figure 2).

The open reading frame of the product of *ccSNF1*, ccSnf1p, is 880 amino acids and has a molecular mass of 98 kD. ccSnf1p has ~40 % overall identity with yeast Snf1p. As shown in Figure 2, the similarity is very strong at the N terminus, a region of the protein that includes the 'activation segment,' which is conserved in all known related protein kinases (Johnson et al., 1996; Hardie et al., 1998). This block of amino acids is 100% conserved between Snf1p and ccSnf1p. The similarity between Snf1p and ccSnf1p is weak to nonexistent at the C terminus, with the possible exception of a few blocks of conserved amino acids that are apparent only with the introduction of many large gaps (Figures 3 and 4).

ccSnf1p is the largest (98 kD) of the known Snf1p-related proteins. Those of various yeasts are ~70 kD, those of plants are ~56 kD, and those of *Caenorhabditis elegans*, Drosophila, and mammals are ~63 kD. ccSnf1p also differs from other proteins related to Snf1p in that the PSORT program (Nakai and Kanehisa, 1992) strongly predicts localization in the nucleus (Table 3). The computed probability of ccSnf1p having a nuclear localization is 78%, whereas yeast Snf1p is predicted to be in the nucleus with only a 48% probability. The difference is due to the presence of three regions of basic amino acids in ccSnf1p that are absent in yeast Snf1p (PTKKPRA

```
CAGGCGGCCGCGAATTCACTAGTGATTCTGCACACAATTGTGCAACTTCGAGCCCTGTAC     60

CTGTGCTTCTGCACACTCCCACGGGCCGCCAACAAGCTCTCTTCCTCTCCCTCTTTCTCT   120
CCCTCTCGTTGCCTCTCCCCACCGACGCGCTCCTCTTCGCATGCTTCCGCCCTGCGTCGG   180
CACACCTGCTAACCGGATGTTACCTGTATAGTCCCCGCAGACGCAAACCGCCCTCCAGCC   240
            ^
CTACGTATCCCCGCAAAAGCTTCTGGCCAGCGCCTCCAAGCACGACCGGCGCCACCATAC   300
CACTCGCCATACAATGTCGGCCGCAATCGATAATGAGGACCTGGAGGAGCTCTCCATCTC   360
              M  S  A  A  I  D  N  E  D  L  E  E  L  S  I  S     16
CATGCCCTCGCAGCGGCGGGGCGCCGCCCAAACCAGCACAACAAAGGCCCAGGACCCCGC   420
  M  P  S  Q  R  R  G  A  A  Q  T  S  T  T  K  A  Q  D  P  A     36
CCCGCCGCCGCCTACTGCGCTCGGAACTGCGGTGCACGAAACCAAGAGCAAGGATACAAA   480
  P  P  P  P  T  A  L  G  T  A  V  H  E  T  K  S  K  D  T  K     56
GGCGAGCCAGCGTCTCGGCCAGTACACCATTGTCCGCACCCTGGGCGAGGGCTCCTTCGG   540
  A  S  Q  R  L  G  Q  Y  T  I  V  R  T  L  G  E  G  S  F  G     76
CAAGGTCAAGCTGGCCACCCACCAGGTTAGCGGCCAAAAGGTCGCCCTCAAGATCATCAA   600
  K  V  K  L  A  T  H  Q  V  S  G  Q  K  V  A  L  K  I  I  N     96
TCGCAAGAGGCTCGTCACCAGAGATATGGCAGGCAGGATCGAGCGTGAGATTCAGTATCT   660
  R  K  R  L  V  T  R  D  M  A  G  R  I  E  R  E  I  Q  Y  L    116
GCAGCTGCTGCGCCATCCGCATATCATCAAGCTgtacgttgtttgcctgtagccgcgctt   720
  Q  L  L  R  H  P  H  I  I  K  L                               127
gcctcattttcatgctcacttttgctccctagCTATACCGTCATAACAACGCCGACCGA   780
                                 Y  T  V  I  T  T  P  T  E    136
AATCATCATGGTCCTCGAATACGCAGGCGGGGAATTGTTCGACTACATCGTCAACCACGG   840
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TAAACTGCAAGAGGCACAGGCTCGAAAGTTCTTCCAGCAAATTGTATGCGCTGTCGAATA   900
  K  L  Q  E  A  Q  A  R  K  F  F  Q  Q  I  V  C  A  V  E  Y    176
CTGCCATCGACACAAGATTGTCCACCGAGATCTGAAGCCCGAGAACCTCCTCCTCGACCA   960
  C  H  R  H  K  I  V  H  R  D  L  K  P  E  N  L  L  L  D  H    196
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TCTCAAGACAAGCTGTGGCAGCCCCAACTATGCTGCGCCCGAGGTCATTTCTGGCAAGTT  1080
  L  K  T  S  C  G  S  P  N  Y  A  A  P  E  V  I  S  G  K  L    236
GTACGCTGGTCCCGAAGTCGACGTCTGGAGCTGTGGTGTCATACTATACGTTTTGTTAGT  1140
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CGGCCGGCTACCCTTCGACGACGAATATATCCCGACCCTCTTTAAGAAAATTGCCGCGGG  1200
  G  R  L  P  F  D  D  E  Y  I  P  T  L  F  K  K  I  A  A  G    276
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TGTGCAAGATGCCTTGGCACGCGATGAGCCGAGTGCCATTAAAGATGCTTACCTCATTGT  1560
  V  Q  D  A  L  A  R  D  E  P  S  A  I  K  D  A  Y  L  I  V    396
```

Sequence continued next page.

Figure 2. Nucleotide (Genbank accession number: AF159253) and deduced amino acid sequence (accession number: AAD43341) of *C. carbonum SNF1* gene.
The open reading frame encodes a protein of 880 amino acids; ^ transcription start site; * stop codon; # polyadenylation site. The two introns, 60 and 51 bp, are typed in *italic lowercase*.

Sequence continued from previous page.

```
CCGAGAGAATGAGATGATGCGGGAGAACCgtaggcatgactactcttatggtattgcctg  1620
  R  E  N  E  M  M  R  E  N                                     405
tagctgactgtttattctagCTTTGTTAACCAACCAAGATGGTGTTCCGGTGTGGAATCA  1680
                     P  L  L  T  N  Q  D  G  V  P  V  W  N  H  419
CCAGTCGCCGCCTGCGCACGACAGTTATATGGAAAAGTTTAGACCACAATCATTGAATGC  1740
  Q  S  P  P  A  H  D  S  Y  M  E  K  F  R  P  Q  S  L  N  A  439
TGTATCACGTCCGCAATTCATTCCCCCGGCGCCTTCAGACCATGAAAGAGCACGCCAAGG  1800
  V  S  R  P  Q  F  I  P  P  A  P  S  D  H  E  R  A  R  Q  G  459
ATCCAACGCCAGCAGTCAGCTTGCAAGCATTCGCAGCCCGGTCAGCACCATAGCAATTCT  1860
  S  N  A  S  S  Q  L  A  S  I  R  S  P  V  S  T  I  A  I  L  479
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CAAGATTTCGGAAAGCGAGGCTCTCCCACCAACACCTGAACAGACGGAAGAACAACGGCA  1980
  K  I  S  E  S  E  A  L  P  P  T  P  E  Q  T  E  E  Q  R  Q  519
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  E  *                                                          880
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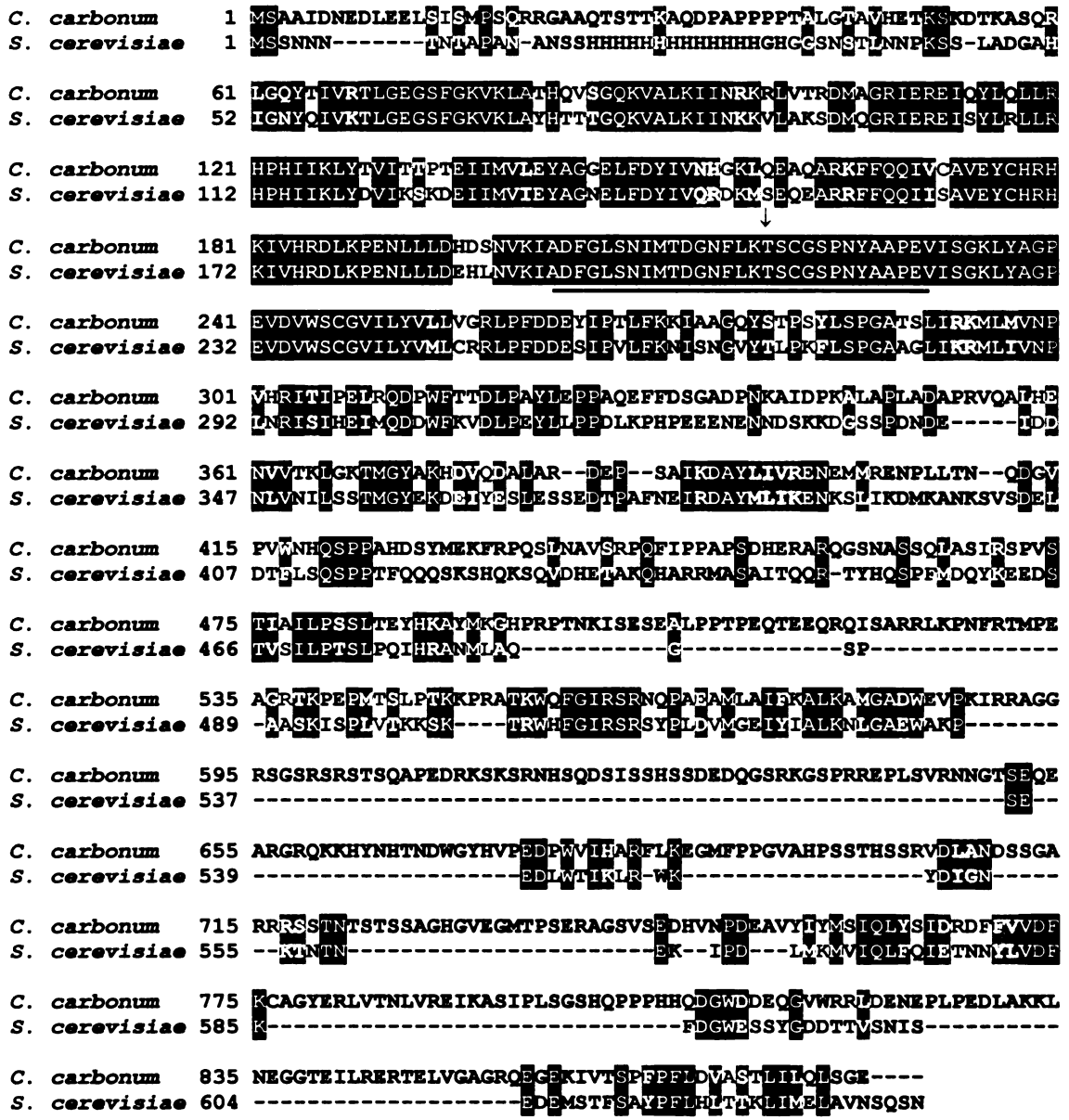

Figure 3. Comparison of Deduced Amino Acid Sequences of *C. carbonum ccSNF1* and yeast *SNF1*.

Residues that are identical in both sequences are indicated by black shading while conserved substitutions are in gray shading. The protein kinase activation segment situated between Asp-Phe-Gly and Ala-Pro-Glu is underlined (Johnson et al., 1996; Hardie et al., 1998). An arrow indicates the phosphorylated threonine in Snf1p.

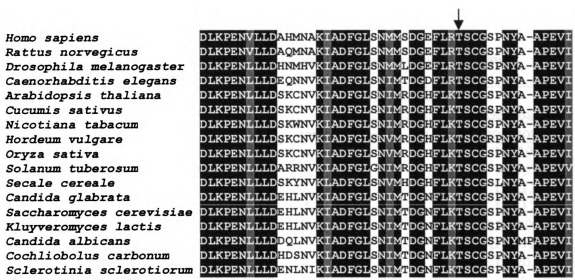

Figure 4. Alignment of the N-terminal region of Snf1p homologs from several eukaryotic organisms.

Residues that are identical in both sequences are indicated by black shading while conserved substitutions are in gray shading. The protein kinase activation segment situated between Asp-Phe-Gly (DFG) and Ala-Pro-Glu (APE) is underlined (Johnson et al., 1996; Hardie et al., 1998). An arrow indicates the phosphorylated threonine in Snf1p.

Table 3. Properties of Snf1 protein kinases from several eukaryotic organisms.

Source	Mwt	# of aa	Isoelectric point	Predicted Localization (%) Nucleus	Cytoplasm	Genebank accession #
Saccharomyces cerevisiae	72.0	633	6.46	48	26	M13971
Kluyveromyces lactis	68.6	602	6.47	52	26	X87975
Candida glabrata	70.0	611	6.59	56	26	L78130
Candida albicans	70.0	620	7.85	52	30	L78129
Sclerotinia sclerotiorum	80.5	722	6.62	61	30	AJ238009
Cochliobolus carbonum	97.9	880	8.35	78	4	AF159253
Oryza sativa	57.6	505	7.92	9	74	D82039
Secale cereale	57.7	502	8.43	22	39	M74113
Nicotiana tabacum	58.3	511	8.36	13	26	D26602
Hordeum vulgare	55.3	484	8.78	4	83	X82548
Arabidopsis thaliana	58.4	512	7.90	13	26	M93023
Cucumis sativus	57.8	504	8.65	13	26	Y10036
Solanum tuberosum	57.9	504	8.20	17	78	X95996
Caenorhabditis elegans	63.1	562	7.07	30	52	U58726
Drosophila melanogaster	64.6	582	8.46	9	65	AF020309
Rattus norvegicus	62.2	552	7.47	21	65	U12149
Homo sapiens	63.3	552	7.68	21	65	U06454

starting at amino acid 547, PKIRRAG at amino acid 587, and PEDRKSK at amino acid 607) (Figure 3). Of 14 additional Snf1p-like proteins analyzed by using PSORT, most were predicted to be in the cytoplasm, with nuclear localization probabilities ranging from 9% (*Drosophila* and rice) to 56% (*C. glabrata*).

Hybridization of a *ccSNF1* probe to a blot of *C. carbonum* genomic DNA digested with different restriction enzymes (*Cla*I, *Eco*RV, *Nco*I, or *Sac*I) resulted in a single band in each case (Figure 5), indicating the existence of a single copy of the gene in *C. carbonum*.

Complementation of a Yeast *snf1* Mutant by *ccSNF1*

Yeast *snf1* mutants cannot grow on a medium containing sucrose as the sole source of carbon, because the Snf1 protein kinase is essential for derepression of invertase (Celenza and Carlson, 1984). To determine whether *ccSNF1* gene can complement the *snf1* mutation, the 2.94-kb *ccsnf1* cDNA of pSnf1c-3 was subcloned into the yeast vector pVT100-U under the control of the constitutive alcohol dehydrogenase *ADH1* promoter. Both transformed and untransformed cells, including the wild-type reference strain MG106, grew on glucose (YPD medium; see Methods), whereas only yeast transformed with either pSnf1c-4 or pVT100-U grew on SD-URA (synthetic dextrose minus uracil) medium, which contains glucose but lacks uracil. Only the transformant expressing *ccSNF1* also grew on YPS medium (see Methods), which contains sucrose as carbon source (Figure 6). This result indicates that despite their differences in size and in C terminus sequence, ccSnf1p can functionally replace the yeast Snf1p.

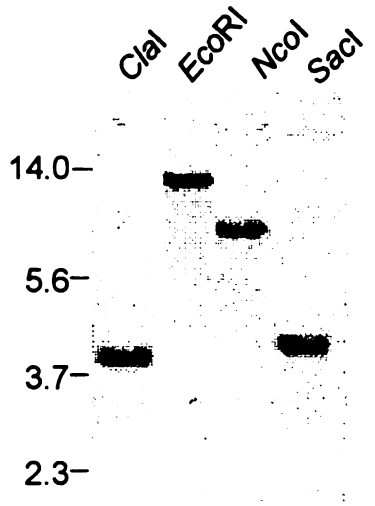

Figure 5. Copy number of *SNF1* in *C. carbonum*.

Southern blot analysis of total genomic DNA from *C. carbonum* digested with restriction enzymes *Cla*I, *Eco*RV, *Nco*I, or *Sac*I

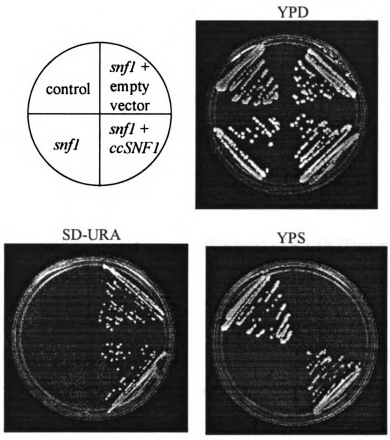

Figure 6. Complementation of Yeast *snf1* by *ccSNF1*.

The control reference strain was MG106. *snf1* denotes the yeast *snf1* mutant MCY1846. *snf1* + empty vector is MCY1846 transformed with vector pVT100-U. *snf1* + *ccSNF1* is MCY1846 transformed with vector pSnf1c-4 (pVT100-U containing *ccSNF1*). YPD is YP medium plus glucose as carbon source; SD-URA is glucose medium lacking uracil (see Methods); YPS is YP medium plus sucrose as carbon source.

Targeted Disruption of *ccSNF1*

A ccsnf1 mutant in an HC toxin-producing ($Tox2^+$) background was generated by DNA-mediated transformation. Two hygromycin-resistant transformants from two separate transformation experiments were isolated and purified. Both transformants (T688 and T669) were determined by DNA gel blot analysis to have undergone gene replacement by homologous integration at the *ccSNF1* locus. Genomic DNA from both transformants and the wild type was digested with *Sac*I, blotted, and probed with the 2.1-kb *Pst*I-*Sal*I internal *ccSNF1* fragment. Neither transformant showed hybridization whereas the wild type exhibited the expected hybridization with a band of 4.3 kb (Figure 7A). A similar blot was probed with the *Escherichia coli hph* gene encoding hygromycin phosphotransferase. As predicted, the *hph* gene hybridized with a band of 3.7 kb in the two mutant strains and did not hybridize with the DNA from the wild-type strain. A control probe consisting of a genomic fragment of *XYL3* hybridized with the wild type and both transformants (Figure 7A). There are also HC toxin nonproducing ($Tox2^-$) isolates of *C. carbonum* that can successfully penetrate resistant maize leaves (genotype *Hm1*/-) but cause only small necrotic lesions that do not expand (Panaccione et al., 1992; Ahn and Walton, 1997). A *ccsnf1* mutant of a wild-type $Tox2^-$ isolate was made in the same way as the $Tox2^+$/*ccsnf1* mutant. Disruption was confirmed by DNA gel blot analysis (Figure 7B).

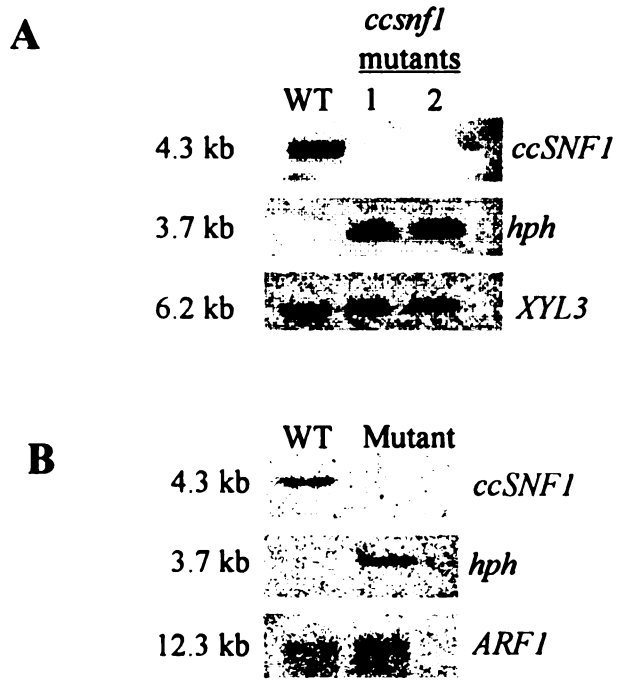

Figure 7. Screening of *ccsnf1* Disruption Transformants.

(A) DNA blotting of Tox2$^+$ wild type (WT) and two *ccsnf1* disruptant transformants (lane 1 is T688 and lane 2 is T669). DNA was digested with *Sac*I. Similar blots were probed with *ccSNF1*, *hph*, or *XYL3* as a control. **(B)** DNA blotting of the Tox2$^-$ wild type (WT) and a *ccsnf1* disruptant transformants (T707). DNA was digested with *Sac*I. Similar blots were probed with *ccSNF1*, *hph*, or *ARF1* as a control.

Expression of CWDE Activities and mRNAs in the *ccsnf1* Mutant

In preliminary experiments, both *ccsnf1* mutants (T688 and T689) displayed similar phenotypes with regard to growth and enzymatic activities; therefore, *ccsnf1* mutant T688 was used for all further experiments. Strains 367-2A (wild type) and T688 (*ccsnf1*) were grown in liquid still culture with purified maize cell walls as the sole carbon source. After 7 days of growth, β-1,3-glucanase, α-1,4-polygalacturonase, and β-1,4-xylanase activities in the culture filtrates were measured. All three of these activities are due to multiple enzymes encoded by different genes (Schaeffer et al., 1994; Scott-Craig et al., 1998; Apel-Birkhold and Walton, 1996). Enzyme activities were reduced by 53 ± 4, 24 ± 6, and 65 ± 7 % (means of three independent replicates, ± one standard deviation), respectively, in the mutant compared to the wild type (Figure 8). In the Tox2$^-$/*ccsnf1* mutant, reduction in the same enzyme activities was 78 ± 5, 41 ± 8, and 83 ± 12 %, respectively (Figure 9).

The expression of particular structural genes encoding wall-degrading enzymes was further analyzed by RNA gel blotting. Expression of *XYL3* (encoding endo-β-1,4-xylanase 3) and *ARF1* (encoding α-arabinosidase; Y. Cheng, S. Wegener, and J.D. Walton, unpublished results) was undetectable in the *ccsnf1* mutant under conditions that normally induce them (Figure 4). Expression of *XYL1*, *XYL2*, *XYL4*, *XYP1*, *PGX1*, *PGN1*, *MLG1*, and *EXG1* (encoding endo-β-1,4-xylanases 1, 2, and 4; β-xylosidase; exo-α-1,4-polygalacturonase; endo-α-1,4-polygalacturonase; mixed-linked (β-1,3-β-1,4-) glucanase; and exo-β-1,3-glucanase, respectively) was moderately to strongly reduced in the *ccsnf1* mutant (Figure 4). Expression of *ccRPD3* (encoding a histone deacetylase

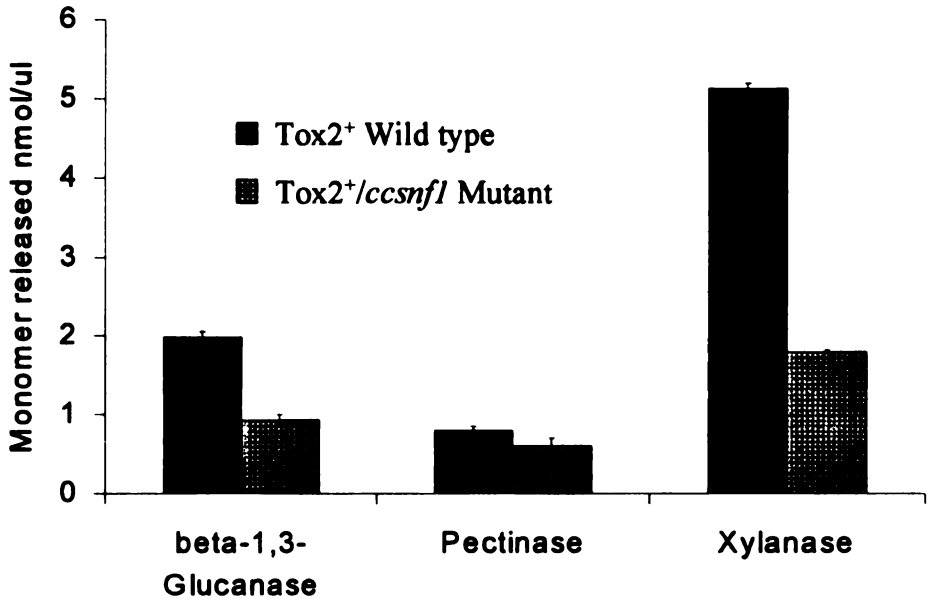

Figure 8. Effect of *SNF1* mutation on extracellular enzyme activities.

β-1,3-Glucanase, pectinase and xylanase activities of *C. carbonum* Tox2$^+$ wild type and the *ccsnf1* mutant after growth for 7 days in liquid cultures containing 2 % corn cell walls. Monomers were glucose, galacturonic acid and xylose for β-1,3-Glucanase, pectinase and xylanase activities, respectively.

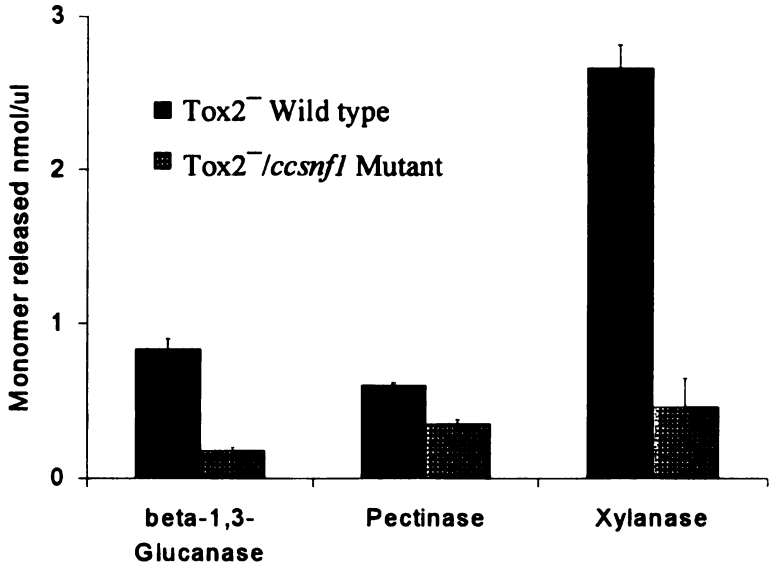

Figure 9. Effect of *SNF1* mutation on extracellular enzyme activities in Tox2$^-$ *C. carbonum* strains.

β-1,3-Glucanase, pectinase and xylanase activities of *C. carbonum* Tox2$^-$ wild type and the Tox2$^-$ *ccsnf1* mutant after growth for 7 days in liquid cultures containing 2 % corn cell walls. Monomers were glucose, galacturonic acid and xylose for β-1,3-Glucanase, pectinase and xylanase activities, respectively.

related to yeast Rpd3p; S. Wegener and J. D. Walton, unpublished results) and *GPD1* (encoding glyceraldehyde-3-phosphate dehydrogenase) was not affected by the *ccsnf1* mutation (Figure 10).

Growth of the *ccsnf1* Mutant on Complex and Simple Carbon Sources

T688 and the wild type strain 367-2A grew at the same rate on glucose and almost the same rate on sucrose (Figure 11). This is in contrast to yeast *snf1* mutants, which grow on glucose but not sucrose (Celenza and Carlson, 1984; Figure 11). This apparent contradiction can be explained by the fact that expression of secreted invertase is subject to glucose repression in yeast but not in *C. carbonum*. When tested, invertase activity in the wild type was the same when grown on glucose or sucrose, and invertase activity in the *ccsnf1* mutant was not significantly different from that of the wild type (reduced by only about 19 %). When grown on glucose, conidia of the *ccsnf1* mutant were normal in numbers and morphology. Conidia of the *ccsnf1* mutant germinated and formed appressoria at the same rate as did the wild type on glass slides (Horwitz et al., 1999).

The *ccsnf1* mutant was moderately to severely impaired in its ability to grow on pectin, xylan, or corn cell walls compared to the wild type (Figure 11). Growth on corn cell walls by *C. carbonum snf1* mutant was particularly impaired. Reduced growth could be due to decreased ability to degrade the substrates or to an inability to take up and metabolize the released sugars. To test the latter possibility, growth of the *ccsnf1* mutant was compared to the wild type on various

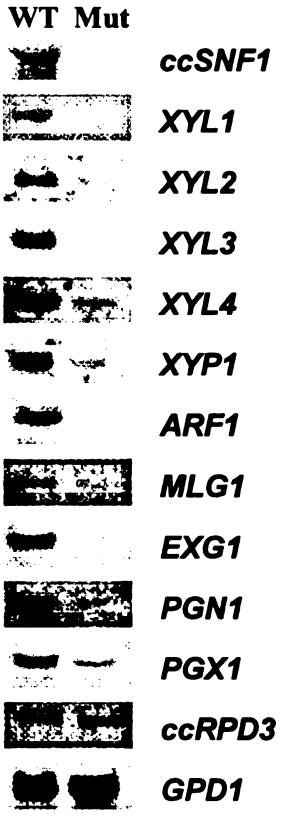

Figure 10. RNA expression of Wall-Degrading Enzyme Genes in the Wild Type (WT) and in a *ccsnf1* Mutant (Mut) of *C. carbonum*.

Fungi (367-2A and T688) were grown for 7 days in liquid still culture containing, as sole carbon source, 2 % xylan (*XYL1, XYL2, XYL3, XYL4, XYP1*, and *ARF1*), 2% pectin (*PGN1* and *PGX1*), or 2% maize cell walls (*ccSNF1, MLG1, EXG1, ccRPD3,* and *GPD1*).

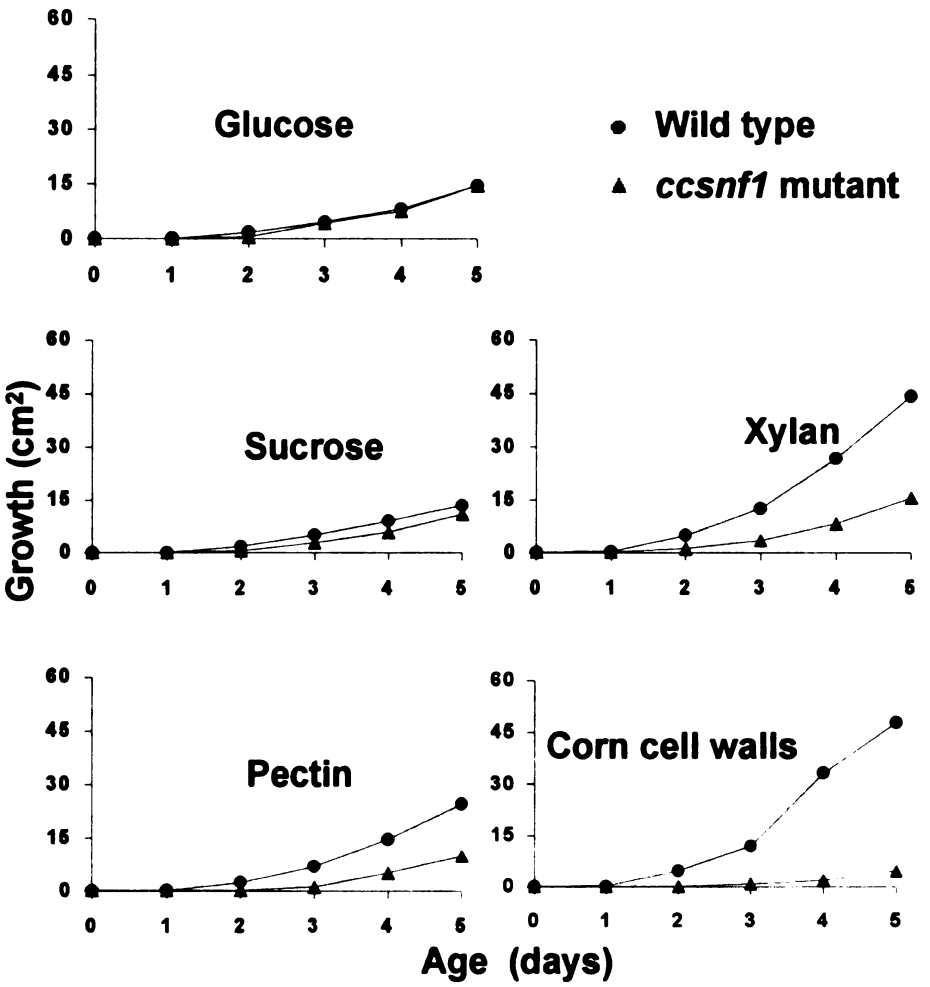

Figure 11. Growth of *C. carbonum* Wild Type and a *ccsnf1* Mutant on Glucose, Sucrose, or Complex Carbon Sources.

Fungi (367-2A and T688) were grown on agar in 15-cm Petri plates. Basal salts medium was supplemented with glucose, sucrose, xylan, pectin, or maize cell walls at 2% (w/v) as sole carbon source.

simple sugars as the sole carbon source. The wild type grew best on xylose and reasonably well on, in descending order, L-arabinose, fructose, maltose, galactose, and glucose. Growth on galacturonic acid or D-arabinose was relatively poor (Figure 12). The *ccsnf1* mutant was able to grow almost as well as wild type on fructose, but showed significantly reduced growth on galactose, galacturonic acid, xylose, or maltose. Growth of the mutant was most strikingly reduced on either L- or D-arabinose (Figure 12). Reduction in growth of the *ccsnf1* mutant was most pronounced on L-arabinose of the substrates on which the wild type grew well. L-arabinose is one of the most abundant sugars in maize cell walls (Kato and Nevins, 1984). These results indicate that *ccSNF1* is required not just for the expression of extracellular depolymerases but also for uptake and/or metabolism of the products of those enzymes.

Effect of the *ccsnf1* Mutation on Pathogenicity

The Tox2$^+$/*ccsnf1* mutant was tested for pathogenicity by spray-inoculation of maize plants of genotype ***hm1/hm1*** (sensitive to HC toxin). The Tox2$^+$/*ccsnf1* mutant caused fewer lesions (<15%) than did the wild type. Most of the lesions caused by the mutant were similar to those caused by the wild type in appearance and rate of expansion, but did not develop as uniformly (Figure 13). Furthermore, whereas the wild type eventually killed the entire seedling, the Tox2$^+$/*ccsnf1* mutant never did so (Figure 14). Like the Tox2$^+$/*ccsnf1* mutant, the Tox2$^-$/*ccsnf1* mutant caused many fewer lesions than did the wild-type Tox2$^-$/*ccSNF1* strain (Figure 13).

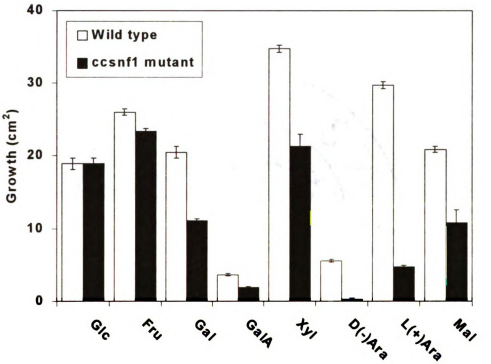

Figure 12. Growth of *C. carbonum* Wild Type and *ccsnf1* Mutant on Simple Sugars.

Fungi (367-2A and T688) were grown on agar in 15-cm Petri plates. Basal salts medium was supplemented with glucose (Glc), fructose (Fru), galactose (Gal), galacturonic acid (GalA), xylose (Xyl), D-arabinose (D-Ara), L-arabinose (L-Ara), or maltose (Mal) at 2% (w/v) as sole carbon source. Growth was measured after 6 days.

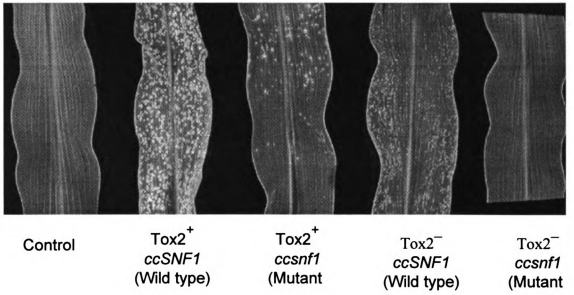

Figure 13. Pathogenicity Assays of *ccsnf1* Mutants.

Maize plants of genotype *hm1/hm1* (inbred Pr) were inoculated with wild types and *ccsnf1* mutants. $Tox2^{+}$ indicates a strain that produces HC-toxin; $Tox2^{-}$ indicates a strain that does not produce toxin.

Figure 14. Pathogenicity Assays of Tox2$^+$/*ccsnf1* Mutant.

Maize plants of genotype *hm1*/*hm1* (inbred Pr) were inoculated with wild type and *ccsnf1* mutant. Plants were photographed after 8 days.

To determine whether the reduced virulence of the *ccsnf1* mutant was due to reduced HC toxin production, culture filtrates of the wild type and *ccsnf1* mutant were analyzed. Toxin production was normal in the *ccsnf1* mutant (Figure 15), indicating that HC toxin synthesis does not require *ccSNF1* and that the reduced virulence of the mutant is not due to a defect in toxin biosynthesis or secretion.

When examined microscopically, it was observed that spores of the mutant adhered, germinated, and formed appressoria at the same rate as did the wild type. Whereas the efficiency of penetration (as manifested by the appearance of either compatible or incompatible macroscopic lesions) by adhered spores of the wild type was quite high (ranging from 50 to 80% in different experiments), infection efficiency by both the Tox2^{+}/*ccsnf1* and Tox2^{-}/*ccsnf1* mutants was much lower (Figures 16A to 16D).

Yeast *snf1* mutants have reduced thermotolerance (Thompson-Jaeger et al., 1991). Because the temperature in the greenhouse where the pathogenicity tests were done sometimes exceeded 27°C, the tests were repeated when the temperature stayed consistently below 24°C, and an identical reduction in virulence was obtained. Growth on agar plates of the wild type and *ccsnf1* mutant were also compared in the laboratory. Both isolates grew equally well at 26°C, both were equally reduced in growth at 30°C, and neither grew at 37°C (Figure 17). Therefore, the reduced growth and virulence of the *ccsnf1* mutant was not due to decreased thermotolerance.

Figure 15. HC-toxin Production by Tox2$^+$ Wild Type (3672A) and Tox2$^+$/*ccsnf1* (T688) Mutant.

The uppermost (and darkest) spot in both lanes is HC-toxin I, the major form of HC-toxin.

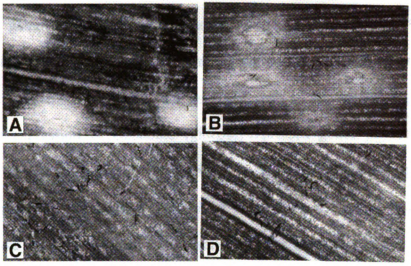

Figure 16. Infection by Wild Type and *ccsnf1* Mutants.

Plants were of genotype *hm1*/*hm1*. Photographs were taken at 30x magnification 3 days after inoculation.
(A) Wild type (Tox2^{+}/*ccSNF1*). Three lesions, each with a spore in its center, are shown. Although not clearly visible, the fungus has already spread well outside the necrotic zones, and the entire leaf will be dead in another 48 hr.
(B) Wild type (Tox2^{-}/*ccSNF1*). The lesions are typical of an incompatible reaction. Penetration and necrotic lesions have formed, but the fungus fails to spread further. The leaf will remain alive.
(C) and (D) Tox2^{+}/*ccsnf1* and Tox2^{-}/*ccsnf1* mutants, respectively. Whereas penetration efficiency by *ccSNF1* wild-type isolates is high (cf. [A] and [B]), penetration by *ccsnf1* mutants is much reduced. Most of the spores shown in (C) and (D) have germinated, but none have penetrated the leaf to give either a compatible (cf. [A]) or incompatible (cf. [B]) reaction.

DISCUSSION

The *ccSNF1* gene of *C. carbonum* is structurally and functionally related to *SNF1* of yeast. Its overall amino acid similarity is strong within the region conserved among all known Snf1p proteins, and *SNF1* and its counterpart in *C. carbonum*, *ccSNF1*, are required for expression of catabolite (glucose)-repressed genes in both organisms. *ccSNF1* can complement growth on sucrose of a yeast *snf1* mutant. The genes that are actually regulated by *SNF1* and *ccSNF1* differ in yeast and *C. carbonum* because yeast does not make xylanase or many other wall-degrading enzymes, and invertase is not subject to *SNF1*-mediated catabolite repression in *C. carbonum*. HC toxin synthesis does not require *ccSNF1*, indicating that HC toxin, although necessary, is not sufficient for virulence of *C. carbonum*.

The *ccsnf1* mutant of *C. carbonum* grew at the same rate as did the wild type on glucose and was also normal in other respects, such as colony morphology, conidiation, and appressorium formation. This argues that *ccSNF1* is not required for essential housekeeping functions in *C. carbonum*. The *ccsnf1* mutant was impaired in growth on complex polysaccharide substrates as well as on certain simple sugars and, most importantly, had decreased virulence on maize. Microscope analysis of infection by $Tox2^{+}$/*ccsnf1* and $Tox2^{-}$/*csnf1* mutants indicated that *ccSNF1* has a role specifically in penetration.

As with any regulatory gene, it is possible that the reduced virulence of the *ccsnf1* mutant is due to a defect in some process unrelated to expression of extracellular depolymerases or an ability to utilize their breakdown products. *SNF1* in yeast and

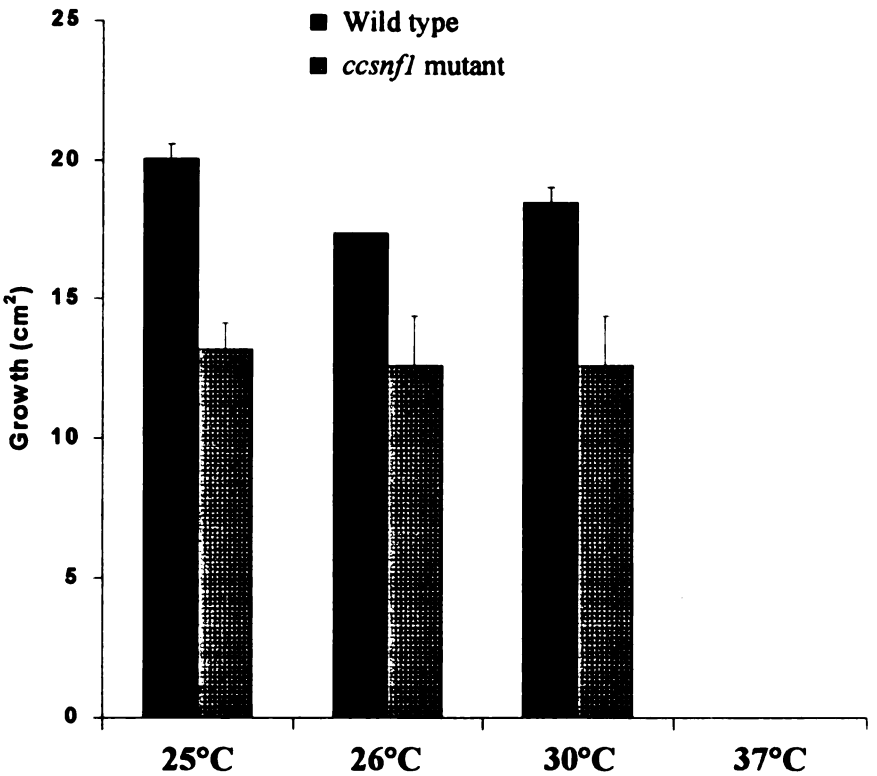

Figure 17. Thermotolerance of *C. carbonum* wild type and *snf1* mutant.

The Tox2$^+$ wild type and *snf1* mutant were grown on V8 juice agar plates at room temperature (25°C) for 2 days, and then transferred to 26, 30 or 37 °C. The difference in growth was measured after 2.5 days.

related genes in other organisms also regulate other cellular processes associated with carbon metabolism, such as glycogen, sterol, and fatty acid biosynthesis, and fatty acid β-oxidation (Hardie et al., 1998). However, unlike wall-degrading enzymes, these processes have not previously been implicated by experimental data in the process of fungal pathogenesis. It seems more likely that the *ccsnf1* mutant has reduced virulence because of its reduced ability to express wall-degrading enzymes and/or to utilize the products of those enzymes.

These results complement earlier studies in which structural enzyme genes were directly mutated. A striking difference is the much more drastic growth and pathogenicity phenotype of the *ccsnf1* mutant compared to any combination of mutated structural genes. That is, the reduction in growth of the *ccsnf1* mutant was stronger than would be predicted by the degree to which measurable enzyme activities were decreased, when compared to the results obtained by mutations in the structural genes for the enzymes themselves. For example, although Xyl1p accounts for ~80% of the total extracellular endo-β1,4-xylanase activity of *C. carbonum* grown in vitro, mutation of *XYL1* does not reduce growth on xylan or maize cell walls (Apel et al., 1993; Apel-Birkhold and Walton, 1996). In contrast, *XYL1*, *XYL2*, and *XYL4* are only partially downregulated, and total xylanase activity is decreased by only 65% in the *ccsnf1* mutant, yet growth on xylan is decreased by >60%. Similarly, disruption of *PGN1* and *PGX1* together causes a >95% loss of extracellular polygalacturonase activity in *C. carbonum*, yet disruption of both genes results in only a 40-60% decrease in growth on pectin (Scott-Craig et al., 1998; J.S. Scott-Craig and J.D. Walton, unpublished results). In contrast, disruption of *ccSNF1* only

partially down-regulates *PGN1* and *PGX1* expression and decreases total pectinase activity by only 24%, yet growth of the mutant is decreased by >50%.

These comparative results are consistent with the hypothesis that *ccSNF1* is also necessary for expression of enzymes needed for utilization of xylan or pectin, such as enzymes need for the transport and intracellular catabolism of xylose or galacturonic acid. This hypothesis is supported by the data shown here that indicates that *ccsnf1* mutants have reduced ability to grow on simple sugars, such as xylose or galacturonic acid. It is also supported by studies in other systems. Catabolism of the products of pectinase in *Erwinia chrysanthemi* requires at least two transport proteins and seven cytoplasmic enzymes, which are coregulated with the extracellular pectinases (Hugouvieux-Cotte-Pattat et al., 1996). Glucose, acting at least partially through *SNF1*, regulates the transcription of hexose transporters in yeast (Carlson, 1998).

ccSNF1 is required for the expression of wall-degrading enzymes and for growth on simple sugars, but our results do not indicate which is more important for virulence of *C. carbonum*. Wall-degrading enzymes might be important for the actual process of penetration. Species of *Cochliobolus* do not require either melanization or appressoria to cause disease and, therefore, have been presumed, by default, to penetrate enzymatically and not by mechanical force (Horwitz et al., 1999). A decreased ability to produce wall-degrading enzymes would therefore be predicted to result in decreased penetration, which is what was observed for the *ccsnf1* mutant. Alternatively, or in addition, *C. carbonum* might require free sugars as a source of nutrition to sustain the metabolic activity necessary for penetration. These sugars could conceivably come from the plant leaf surface or epidermis, although the concentrations of free sugars (other than glucose or

sucrose, which are utilized equally well by the *ccsnf1* mutant and the wild type) are probably quite low in these environments. A more likely source of sugars, such as arabinose or xylose, is from the action of extracellular depolymerases on the maize cell wall. Therefore, even if the proximal cause of the decreased virulence of the *ccsnf1* mutant is a defect in the uptake and/or metabolism of simple sugars, the ultimate cause would still be its reduced expression of wall-degrading enzymes.

Insofar as wall-degrading enzymes are important for virulence of *C. carbonum*, it is of interest to determine which enzymes in particular are important. Reduced virulence of the *ccsnf1* mutant could be due to downregulation of one enzyme, all enzymes of a particular class, or many enzymes partially. It is not possible to distinguish between these alternatives from our results, because all of the ones studied were downregulated. Previous studies have excluded many individual structural genes from making a major contribution to virulence. Of those that have not yet been directly tested, *ARF1* is intriguing as a possible candidate for a solo virulence gene because (1) its expression is completely dependent on *ccSNF1*, (2) the *ccsnf1* mutant has strongly impaired growth on arabinose, (3) arabinose is a major component of maize cell walls, and (4) α-arabinosidase has been implicated as a virulence factor in two other diseases (Howell, 1975; Rehnstrom et al., 1994).

REFERENCES

Ahn, J.-H., and Walton, J.D. (1997). A fatty acid synthase gene required for production of the cyclic tetrapeptide HC-toxin, cyclo(D-prolyl-L-alanyl-D-alanyl-L-2-amino-9,10-epoxi-8-oxodecanoyl). Mol. Plant-Microbe Interact. **10**, 207-214.

Apel-Birkhold, P.C., and Walton, J.D. (1996). Cloning, disruption, and expression of two endo-β-1,4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. Appl. Environ. Microbiol. **62**, 4129-4135.

Apel, P.C., Panaccione, D.G., Holden, F.R., and Walton, J.D. (1993). Cloning and targeted gene disruption of *XYL1*, a β-1,4-xylanase gene from the maize pathogen *Cochliobolus carbonum*. Mol. Plant-Microbe Interact. **6**, 467-473.

Bhalerao, R.P., Salchert, K., Bakó, L., Okrész, L., Szabados, L., Muranaka, T., Machida, Y., Schell, J., and Koncz, C. (1999). Regulatory interaction of PRL1 WD protein with *Arabidopsis* SNF1-like protein kinases. Proc. Natl. Acad. Sci. USA **96**, 5322-5327.

Carlson, M. (1998). Regulation of glucose utilization in yeast. Curr. Opin. Genet. Devel. **8**, 560-564.

Carroll, A.M., Sweigard, J.A., and Valent, B. (1994). Improved vectors for selecting resistance to hygromycin. Fung. Genet. Newslett. **41**, 22.

Celenza, J.L., and Carlson, M. (1984). Cloning and genetic mapping of *SNF1*, a gene required for expression of glucose-repressible genes in *Saccharomyces cerevisiae*. Mol. Cell Biol. **4**, 49-53.

Cooper, R.M. (1987). The use of mutants in exploring depolymerases as determinants of pathogenicity. In Genetics and Plant Pathogenesis, P. R. Day and G. J. Jellis, eds (Oxford: Blackwell), pp. 261-281.

DeVit, M.J., Waddle, J.A., and Johnston, M. (1997). Regulated nuclear translocation of the Mig1 glucose repressor. Mol. Biol. Cell **8**, 1603-1618.

Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K., and Mattick, J.S. (1991). 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. **19**, 4008.

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

Gietz, R.D., and Woods, R.A. (1994). High efficiency transformation in yeast. In Molecular Genetics of Yeast:Practical Approaches, J.A. Johnston, ed (Oxford: Oxford University Press), pp. 121-134.

Görlach, J.M., Van Der Knaap, E., and Walton, J.D. (1998). Cloning and targeted disruption of *MLG1*, a gene encoding two of three extracellular mixed-linked glucanases of *Cochliobolus carbonum*. Appl. Environ. Microbiol. **64,** 385-391.

Guo, W.J., Gonzalez-Candelas, L., and Kolattukudy, P.E. (1996). Identification of a novel *pelD* gene expressed uniquely in planta by *Fusarium solani* f sp *pisi* (*Nectria haematococca*, mating type VI) and characterization of its protein product as an endo-pectate lyase. Arch. Biochem. Biophys. **332**, 305-312.

Hardie, D.G., Carling, D., and Carlson, M. (1998). The AMP-activated/Snf1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu. Rev. Biochem. **67,** 821-855.

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

Horwitz, B.A., Sharon, A., Lu, S.-W., Ritter, V., Sandrock, T.M., Yoder, O.C., and Turgeon, B.G. (1999). A G protein alpha subunit from *Cochliobolus heterostrophus* involved in mating and appressorium formation. Fung. Genet. Biol. **26**, 19-32.

Howell, H.E. (1975). Correlation of virulence with secretion in vitro of three wall-degrading enzymes in isolates of *Sclerotinia fructigena* obtained after mutagen treatment. J. Gen. Microbiol. **90**, 32-40.

Hugouvieux-Cotte-Pattat, N., Reverchon, S., Nasser, W., Condemine, G.T., and Robert-Baudouy, J. (1996). Regulation of pectinase biosynthesis in *Erwinia chrysanthemi*. In Pectins and Pectinases, J. Visser and A.G.J. Voragen, eds (Amsterdam: Elsevier), pp. 311-330.

Johnson, L.N., Noble, M.E., and Owen, D.J. (1996). Active and inactive protein kinases: structural basis for regulation. Cell **85**, 149-158.

Kato, Y., and Nevins, D. J. (1984). Enzymic dissociation of *Zea* shoot cell wall polysaccharides. Plant Physiol. **75**, 740-744.

Lesage, P., Yang, X., and Carlson, M. (1996). Yeast SNF1 protein kinase interacts with SIP4, a C_6 zinc cluster transcriptional activator: a new role for SNF1 in the glucose response. Mol. Cell. Biol. **16**, 1921-1928.

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

Meeley, R.B., and Walton, J.D. (1991). Enzymatic detoxification by maize of the host-selective toxin HC-toxin. Plant Physiol. **97,** 1080-1086.

Muranaka, T., Banno, H., and Machida, Y. (1994). Characterization of tobacco protein kinase NPK5, a homolog of *Saccharomyces cerevisiae SNF1* that constitutively activates expression of the glucose-repressible *SUC2* gene for a secreted invertase of *S. cerevisiae*. Mol. Cell. Biol. **14,** 2958-2965.

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

Nakai, K., and Kanehisa, M. (1992). A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics **14,** 897-911.

Nikolskaya, A.N., Pitkin, J.W., Schaeffer, H.J., Ahn, J.H., and Walton, J.D. (1998). EXG1p, a novel exo-β-1,3-glucanase from the fungus *Cochliobolus carbonum*, contains a repeated motif present in other proteins that interact with polysaccharides. Biochim. Biophys. Acta **1425,** 632-636.

Östling, J., and Ronne, H. (1998). Negative control of the Mig1p repressor by Snf1p-dependent phosphorylation in the absence of glucose. Eur. J. Biochem. **252,** 162-168.

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

Petter, R., and Kwon-Chung, K. J. (1996). Disruption of the *SNF1* gene abolishes trehalose utilization in the pathogenic yeast *Candida glabrata*. Infect. Immun. **64,** 5269-5273.

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

Ransom, R.F., and Walton, J.D. (1997). Purification and characterization of extracellular β-xylosidase and α-arabinosidase from the plant pathogenic fungus *Cochliobolus carbonum*. Carbohydr. Res. **297,** 357-364.

Rehnstrom, A.L., Free, S.J., and Pratt, R.G. (1994). Isolation, characterization and pathogenicity of *Sclerotinia trifoliorum* arabinofuranosidase-deficient mutants. Physiol. Mol. Plant Pathol. **44,** 199-206.

Ronne, H. (1995). Glucose repression in fungi. Trends Genet. **11,** 12-17.

Ruijter, G.J.G., and Visser, J. (1997). Carbon repression in *Aspergilli*. FEMS Microbiol. Lett. **151**, 103-114.

Schaeffer, H.J., Leykam, J., and Walton, J.D. (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.

Scott-Craig, J.S., Cheng, Y.Q., Cervone, F., De Lorenzo, G., Pitkin, J.W., and Walton, J.D. (1998). Targeted mutants of *Cochliobolus carbonum* lacking the two major extracellular polygalacturonases. Appl. Environ. Microbiol. **64,** 1497-1503.

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

Sposato, J.P., Ahn, J.-H., and Walton, J.D. (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.

Shieh, M.T., Brown, R.L., Whitehead, M.P., Cary, J.W., Cotty, P.J., Cleveland, T.E., and Dean, R.A. (1997). Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl. Env. Microbiol. **63**, 3548-3552.

ten Have, A., Mulder, W., Visser, J., and van Kan, J.A.L. (1998). The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol. Plant-Microbe Interact. **11**, 1009-1016.

Thompson-Jaeger, S., Francois, J., Gaughran, J.P., and Tatchell, K. (1991). Deletion of *SNF1* affects the nutrient response of yeast and resembles mutations which activate the adenylate cyclase pathway. Genetics **129**, 697-706.

Treitel, M.A., Kuchin, S., and Carlson, M. (1998). Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **18,** 6273-6280.

Van Hoof, A., Leykam, J., Schaeffer, H.J., and Walton, J.D. (1991). A single β-1,3-glucanase secreted by the maize pathogen *Cochliobolus carbonum* acts by an exolytic mechanism. Physiol. Mol. Plant Pathol. **39,** 259-267.

Van Wert, S.L., and Yoder, O.C. (1992). Structure of the *Cochliobolus heterostrophus* glyceraldehyde-3-phosphate dehydrogenase gene. Curr. Genet. **22,** 29-35.

Vincent, O., and Carlson, M. (1998). Sip4, a Snf1 kinase-dependent transcriptional activator, binds to the carbon source-responsive element of gluconeogenic genes. EMBO J. **17**, 7002-7008.

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

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

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

Wegener, S., Ransom, R.F., and Walton, J.D. (1999). A unique eukaryotic β-xylosidase from the phytopathogenic fungus *Cochliobolus carbonum*. Microbiology **145**, 1089-1095.

Wu, C., Ham, K.-S., Darvill, A.G., and Albersheim, P. (1997). Deletion of two endo-β-1,4-xylanase genes reveals additional isozymes secreted by the rice blast fungus. Mol. Plant-Microbe Interact. **10**, 700-708.

Chapter Three

Expression of cell wall degrading enzymes and characterization of the *CREA* gene in *Cochliobolus carbonum*

ABSTRACT

The expression of four endo-1,4-β-xylanase genes (*XYL1*, *XYL2*, *XYL3*, *XYL4*), an exo-1,4-β-xylosidase gene (*XYP1*) and an α-arabinosidase (*ARF1*) was studied in the presence and absence of glucose. Repression of all six genes was observed with glucose as the sole carbon source, whereas expression was seen when the culture medium contained xylan. The xylanase genes are substrate-induced, but differentially expressed. The *CREA* gene has been implicated in glucose repression in several fungi, and a homolog was isolated and characterized in *Cochliobolus carbonum*. The deduced amino acid sequences of the *C. carbonum ccCREA* gene was very similar to the CreA proteins of *Aspergillus niger* (55% identity), *Gibberella fujikuroi* (48% identity), *Sclerotinia sclerotiorum* (46% identity), and *Trichoderma reesei* (46% identity). It also had 22% identity with the *Saccharomyces cerevisiae* Mig1p. The zinc finger region of the *C. carbonum* ccCreAp protein had between 92 and 100% identity with the corresponding zinc finger regions of the other fungal proteins. The CreAp pathway may play an important role in the regulatory process that leads to cell wall degrading enzyme expression and therefore virulence in pathogenic fungi.

INTRODUCTION

The full expression of cell wall degrading enzyme (CWDE) genes by fungi depends on mechanisms that are associated with the glucose regulation pathway (Tonukari et al., 2000). In many organisms, glucose represses genes whose products are used to metabolize other carbon sources. Work in yeast and filamentous fungi has revealed a mechanism for glucose repression in eukaryotes that is different from that found in bacteria (Ronne, 1995). In yeast, a Cys_2His_2 zinc-finger-containing protein, Mig1p, binds GC-boxes (GCGGGG) in promoters of several genes (Bu and Schmidt, 1998; Lundin et al., 1994) and plays a key role in mediating glucose repression. In the presence of glucose, Mig1p represses the transcription of genes required for the utilization of alternative sugars (Lutfiyya and Johnston, 1996). Carbon catabolite repression is mediated in *Aspergillus nidulans* by a repressor, CreAp, which is a homolog of the yeast Mig1p (Dowzer and Kelly, 1991). CreAp also binds to promoters (Ruijter and Visser, 1997) and prevents transcription of several genes in the presence of glucose. The *CREA* gene has also been isolated from other fungi including *Sclerotinia sclerotiorum* (Vautard et al., 1999), *Trichoderma reesei*, and *T. harzianum* (Ilmen et al., 1996). Most CreA proteins have the signature zinc finger domain of the Cys_2His_2 type (C-x(2,4)-C-x(3)-[LIVMFYWC]-x(8)-H-x(3,5)-H) (Berg, 1988; Miller et al., 1985; Rosenfeld and Margalit, 1993). The role of the CreAp in glucose repression has been summarized by Ronne (1995) and Ruijter and Visser (1997).

Deletion of *CREA* in *A. nidulans* has an extremely severe effect on growth under both carbon catabolite-repressing and nonrepressing conditions (Dowzer and Kelly, 1991;

Shroff et al., 1997). Other authors have shown that an *A. nidulans crea* mutant has two- and threefold increased expression of hexokinase and fructose-6-phosphate reductase activities, respectively, while phosphofructokinase and pyruvate kinase activities decrease (van der Veen et al., 1995).

Repression by CreAp is mainly relieved by the protein kinase, Snf1p, which is highly conserved in fungi, plants and mammals (Celenza and Carlson, 1984; Hardie et al., 1998). We have previously reported that in the pathogenic fungus, *Cochliobolus carbonum*, a homolog of *SNF1* (the gene which encodes Snf1p) is required for the expression of many cell wall degrading enzymes (Tonukari et al., 2000). Mutation of *ccSNF1* in the fungus leads to varying levels of repression of cell wall degrading enzyme genes, reduced growth on complex polymers such as xylan and pectin, and also reduced virulence on its host, maize. As part of an effort to determine whether the phenomenon of glucose repression in *C. carbonum* depends on the CreA-Snf1 pathway studied in yeast, we isolated a homolog of the *CREA* gene from *C. carbonum* and studied the relatedness of its deduced protein to other known CreA proteins. We also studied the expression of CWDE genes following the growth of the fungus in minimal medium containing different carbon sources.

METHODS

Fungal cultures, media and growth conditions

The wild type race 1 strain of *C. carbonum* (367-2A) used in this study was derived from strain SB111 (ATCC 90305) and was maintained on V8 plates (Apel et al., 1993). The fungus was grown in liquid media containing mineral salts, 0.2 % yeast extract, and trace elements (Van Hoof et al., 1991) with 2 % glucose, sucrose, xylose, oat spelt xylan (Fluka, Switzerland), pectin, cellulose, corn cell walls (Sposato et al., 1995), lyophilized corn leaves, or 5% of fresh corn leaf material as the carbon source.

Nucleic acid manipulations

DNA and total RNA were extracted from lyophilized mats (Pitkin et al., 1996; Apel et al., 1993). The methods used for DNA and RNA electrophoresis, blotting, probe labeling and hybridization have also been described elsewhere (Apel-Birkhold et al., 1996). The *Cochliobolus heterostophus GPD1* gene, encoding glyceraldehyde-3-phosphate dehydrogenase (Van Wert and Yoder, 1992), was used as a reference.

PCR was performed in a thermo cycler (MJ Research, Watertown, MA) using *Taq* DNA polymerase (Life Technologies, Gaithersburg, MD) and two degenerate primers based on conserved amino acid sequences of CreA homologs from *Aspergillus niger*, *Neurospora crassa*, *Saccharomyces cerevisiae*, *Sclerotinia sclerotiorum*, and

Trichoderma reesei. Forward primer 5' GARAARCCNCAYGCNTG 3' is based on the amino acid sequence "EKPHAC," and reverse primer 5' GTRTGRTCNGGNGTNGG 3' is based on the amino acid sequence "PTPDHT" where Y = T or C; R = G or A; I = inosine; N = A, T, G or C). The degenerate primers were used in Touchdown PCR (Don et al., 1991) to amplify a 631-bp fragment from *C. carbonum* genomic DNA. Touchdown PCR was performed under the following conditions: initial denaturation at 94 °C for 3 min, 45 cycles of denaturation, 94 °C for 1 min; annealing for 1 min; and polymerization at 72 °C for 2 min. The annealing temperature ranged from 60 °C to 45 °C with a decrease of 1 °C every three cycles. This was followed by 10 cycles of denaturation at 94 °C for 1 min; annealing at 45 °C for at for 1 min; and polymerization at 72 °C for 2 min. The PCR product was cloned into pGEM T-easy vector (Promega).

A specific primer based on the amplified *CREA* sequence from the touchdown PCR (above), 5' CTCCTTCTCCAACTACTCTCCTG 3', was made and used with a vector-specific primer, 5' CGTGAATGTAAGCGTGACAT 3', to isolate a *CREA* cDNA from a *C. carbonum* cDNA library in the yeast vector, pMYR (GenBank Accession number of pMYR is AF102577; CytoTrap XR Library Construction Kit, Stratagene). The *C. carbonum* cDNA library was originally constructed for the CytoTrap yeast two-hybrid screen (Stratagene), following the instructions of the manufacturer, using total mRNA from *C. carbonum* grown on corn cell walls. The PCR reaction was as follows: initial denaturation at 94 °C for 3 min, 35 cycles of denaturation; 94 °C for 1 min; annealing at 50 °C for 1 min; and polymerization at 72 °C for 2 min. The *CREA* cDNA was then cloned into the pGEM T-easy vector.

The transcriptional start site of the *CREA* cDNA was determined using the 5' RACE (Rapid Amplification of cDNA Ends) system, version 2.0, following the instructions of the manufacturer (GIBCO-BRL) (Frohman et al., 1988). A 21-bp oligonucleotide (5'- TCATGTGTGCAAGGTTAGATC -3') was used to prime first-strand cDNA synthesis, which was then amplified by PCR using a nested primer of the sequence 5'- GCTCCATGAGACCCGCCTGTA -3'.

DNA nucleotide sequences were determined by automated fluorescence sequencing at the DOE-Plant Research Laboratory Plant Biochemistry Facility, Michigan State University. Sequence data were analyzed with the Lasergene software (DNASTAR Inc., Madison, WI).

RESULTS

Repression of *C. carbonum* CWDE genes in glucose substrate

To illustrate the glucose repression mechanism in *C. carbonum*, the expression of cell wall degrading enzymes was analyzed by RNA gel blotting. Four endo-1,4-β-xylanase genes (*XYL1*, *XYL2*, *XYL3*, *XYL4*) (Apel et al., 1993; Apel-Birkhold et al., 1996; Scott-Craig et al., unpublished), an exo-1,4-β-xylosidase gene (*XYP1*) (Ransom and Walton, 1997; Wegener et al., 1999) and an α-L-arabinofuranosidase gene (*ARF1*) (in this thesis) has been cloned from *C. carbonum*. The expression of these genes was studied following growth of the fungus in minimal medium containing glucose or xylan, and their

expression was detected only when the culture medium contained xylan (Figure 18). All of these genes were repressed when the culture medium contained only glucose (Figure 18).

Expression of *C. carbonum* CWDE genes in different growth media

The expression of *XYL1*, *XYL2*, *XYL3*, *XYL4*, and *XYP1* was also studied during growth of the fungus in minimal media containing sucrose, xylose, xylan, pectin, cellulose, purified corn cell walls, lyophilized corn leaves (ground or whole), corn leaf extracts or whole corn leaves (fresh) as the sole carbon source (Figures 19). As analyzed by RNA blotting, *XYL1* was expressed only when the culture medium contained xylan, cellulose, purified corn cell walls, or the various corn leaf materials. *XYL2* was induced under these same conditions but only weakly by the corn leaves. *XYL3* was expressed only when xylose or xylan was in the medium, and *XYL4* was transcribed in the presence of xylose, xylan or corn leaf materials. *XYP1* expression was induced by xylose, xylan, pectin and cellulose. The presence of sucrose in the medium suppressed the expression of all five genes.

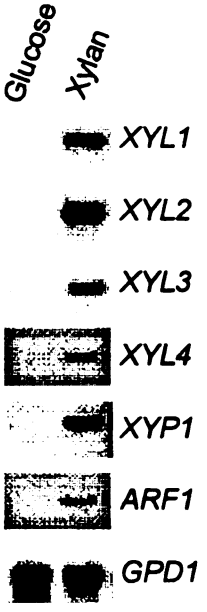

Figure 18. Expression of *C. carbonum* Arabinoxylan-Degrading Enzyme Genes in Glucose and Xylan media.

Fungal strains were grown for 7 d in liquid still culture containing, as sole carbon source, 2 % xylan, or 2 % glucose. *GPD1* encodes the glyceraldehyde-3-phosphate dehydrogenase gene.

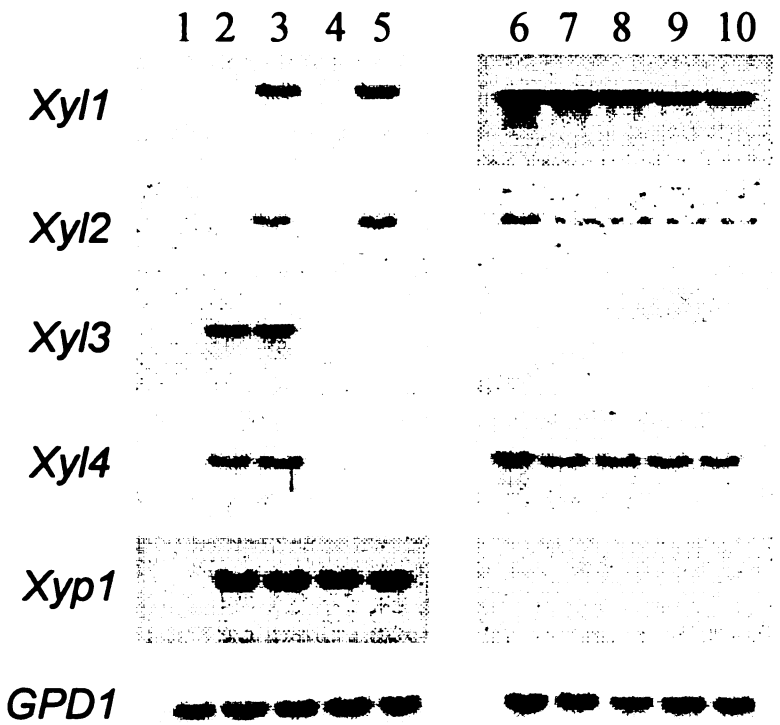

Figure 19. Effect of carbon source on xylanase gene expression..

Fungal strains were grown for 7 d in liquid still culture containing 2% sucrose (1), 2% xylose (2), 2% xylan (3), 2% pectin (4), 2% cellulose (5), 1% corn cell walls (6), 1% lyophilized corn leaves (whole) (7), 1% lyophilized corn leaves (ground) (8), fresh corn leaf extracted with water, 1:10 (w/v) (9), or 5% fresh corn leaves (10) as sole carbon source. *GPD1* encodes glyceraldehyde-3-phosphate dehydrogenase gene.

Cloning of *C. carbonum CREA*

Two degenerate oligonucleotide primers were designed based on conserved regions of CreAp sequences of *A. niger* (Drysdale et al., 1993), *S. cerevisiae* (Lutfiyya and Johnston, 1996), *S. sclerotiorum* (Vautard et al., 1999), and *T. reesei* (Ilmen et al., 1996). Amplification by PCR, with *C. carbonum* genomic DNA as template, yielded a single 631-bp DNA fragment. A gene-specific primer and a vector-specific primer were used to amplify a corresponding cDNA. The full length cDNA contains an open reading frame encoding a predicted protein product of 430 amino acids with a molecular mass of 46.3 kD and a pI of 9.38. The 3'-untranslated region is 20 bp and the 5'-untranslated region is 212 bp in length (Figure 20). The deduced amino acid sequence of the *C. carbonum CREA* (called *ccCREA*) has 55, 48, 46, and 46 % identity with the CreA proteins of *A. niger*, *Gibberella fujikuroi*, *S. sclerotiorum* and *T. reesei*, respectively (Figure 21). It also show 22% identity with Mig1p of *S. cerevisiae*. Two putative DNA-binding protein zinc fingers of the Cys_2His_2 type situated between amino acids 70-90 and 98-120 are predicted by the PROSITE program (Hofmann et al., 1999). The ccCreAp zinc finger region is 92-100% identical to that of other fungal CreA proteins. The similarity between ccCreAp and its fungal homologs can be seen across the entire protein including both the N and C termini (Figures 21).

Although smaller than *S. cerevisiae* Mig1 (504 aa, 55.5 kD), ccCreAp is similar in size (430 aa, 46.3 kD) to the *A. niger* (402 aa, 43.7 kD), *G fujikuroi* (420 aa, 45.9kD), *S. sclerotiorum* (429 aa, 47 kD) and *T. reesei* (402 aa, 43.7 kD) CreA proteins. Like other

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AAACTTTCCCCTTCGGCCACATGCAATCCAACTCTGCCTCGACAGGTTTCGCCAACCTGC     60
                      M  Q  S  N  S  A  S  T  G  F  A  N  L      13
TCAACCCCGAAACAGCCTCCCAGAACCAACAGCAACAACAACAACAACCCACCTCTACAC    120
L  N  P  E  T  A  S  Q  N  Q  Q  Q  Q  Q  Q  Q  P  T  S  T      33
CAACCGCCTCCATGGCCGCTGCCACCGTCAGCCTCATGGCGCCTCTTCTCCAGAACGCCC    180
P  T  A  S  M  A  A  A  T  V  S  L  M  A  P  L  L  Q  N  A      53
CGCAACAGACAGAGGAGCCTCGACAGGATCTTCCCAGGCCTTACAAGTGCCCTCTCTGCG    240
P  Q  Q  T  E  E  P  R  Q  D  L  P  R  P  Y  K  C  P  L  C      73
ACAAGGCCTTCCACCGTCTGGAGCACCAGACTCGCCACATCCGAACCCACACTGGAGAGA    300
D  K  A  F  H  R  L  E  H  Q  T  R  H  I  R  T  H  T  G  E      93
AGCCACACGCCTGCACTTTCCCTGGATGCACAAAGAGATTTTCCCGCTCTGACGAACTGA    360
K  P  H  A  C  T  F  P  G  C  T  K  R  F  S  R  S  D  E  L     113
CTCGACACTCGAGGATACATAACAACCCAAACTCGCGGCGAGGCAAGGGCCAGCAACATG    420
T  R  H  S  R  I  H  N  N  P  N  S  R  R  G  K  G  Q  Q  H     133
CTGCTGCCACGCAAGCTGCCGTTGCCGCTGTACAGGCGGGTCTCATGGAGCCTGGATCTA    480
A  A  A  T  Q  A  A  V  A  A  V  Q  A  G  L  M  E  P  G  S     153
ACCTTGCACACATGATGCCTCCCCCATCAAAGCCcATTTCTCGCAGCGCCCCGGGTTCTC    540
N  L  A  H  M  M  P  P  P  S  K  P  I  S  R  S  A  P  G  S     173
AACTAGGCTCACCCAACGTCTCGCCACCTCACTCCTTCTCCAACTACTCTCCTGGCATGA    600
Q  L  G  S  P  N  V  S  P  P  H  S  F  S  N  Y  S  P  G  M     193
GCAACGACCTGGCCGCATACCACCAGGGCGGCTTGAGCAACAGCAGCAGCCCCAGCGGTC    660
S  N  D  L  A  A  Y  H  Q  G  G  L  S  N  S  S  S  P  S  G     213
TTGCCCGCCCCATGGATCTCCTTGCTGATGCTGCGTCAAGACTGGAGCAACGTCCTGGAC    720
L  A  R  P  M  D  L  L  A  D  A  A  S  R  L  E  Q  R  P  G     233
ACATTTCCCACTCGAGTAGACATCACCTTACGAGCGGGTACCACCCTTACGCCAACCGAC    780
H  I  S  H  S  S  R  H  H  L  T  S  G  Y  H  P  Y  A  N  R     353
TGCCAGGCCTCTCTCAATACGCCTACTCGTCGCAGCCCATGTCAAGATCGCACTCACACG    840
L  P  G  L  S  Q  Y  A  Y  S  S  Q  P  M  S  R  S  H  S  H     273
AGGACGACGACCCGTACTCGCACAGGATGACGAAGAAGTCGAGGCCAGGCTCACCGTCGT    900
E  D  D  D  P  Y  S  H  R  M  T  K  K  S  R  P  G  S  P  S     293
CGACTGCCCCGCCATCTCCAACATTTTCGCACGATTCCTGCAGTCCGACGCCAGACCACA    960
S  T  A  P  P  S  P  T  F  S  H  D  S  C  S  P  T  P  D  H     313
CGCCTCTGGCAACCCCTGCACACTCGCCAAGATTACGGCCCCACGGCTTCAGTGATCTCC   1020
T  P  L  A  T  P  A  H  S  P  R  L  R  P  H  G  F  S  D  L     333
AGCTACCACATCTACGTCATCTCAGCCTCAACCAAAACTTTGTGCCAGCACTTGCGCCCA   1080
Q  L  P  H  L  R  H  L  S  L  N  Q  N  F  V  P  A  L  A  P     353
TGGAGCCGTCGACGGAGCGTGAGCAACCGTACGTGCCAAGCCAGTCGTCGGGACTTCGTA   1140
M  E  P  S  T  E  R  E  Q  P  Y  V  P  S  Q  S  S  G  L  R     373
TTGGGGACATTATTTCCAAGCCCGAAGGTGCGCAGCGCAAATTGCCGGTTCCGCAAGTAC   1200
I  G  D  I  I  S  K  P  E  G  A  Q  R  K  L  P  V  P  Q  V     393
CCAAGGTTGCGGTGCAAGATCTGCTCAATGGCCCGAGCAACAGCGGCTTCTCTTCCGGTA   1260
P  K  V  A  V  Q  D  L  L  N  G  P  S  N  S  G  F  S  S  G     413
ATAACTCCGCAACGGCGTCATTAGCCGGCGAAGACCTCTCGAATCGCAACTAAACCCACT   1320
N  N  S  A  T  A  S  L  A  G  E  D  L  S  N  R  N  .          430
CTCGACTCCAACTAGCATGGCATCGGCGTTTTCGGTCTGCATCCTCTGCTTTTTTTTACT   1380
TTTTCACCTCTCTTGCATTTCGTCATTTTTTTCACGGTCAGCAAGCGATGAGATTAGACA   1440
AGGGCACACACACACACACAAAGACCTTTTTTTGTAGGGTGGAGCCTGTGGCATATTACG   1500
ATTTCCCTAATTGAAACCTGAAAGCAAAAAAAAAAAAAAA                       1540
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Figure 20. Nucleotide and deduced amino acid sequence of *C. carbonum CREA* cDNA (*ccCREA*).

The open reading frame encodes a protein of 430 amino acids; '.' stop codon

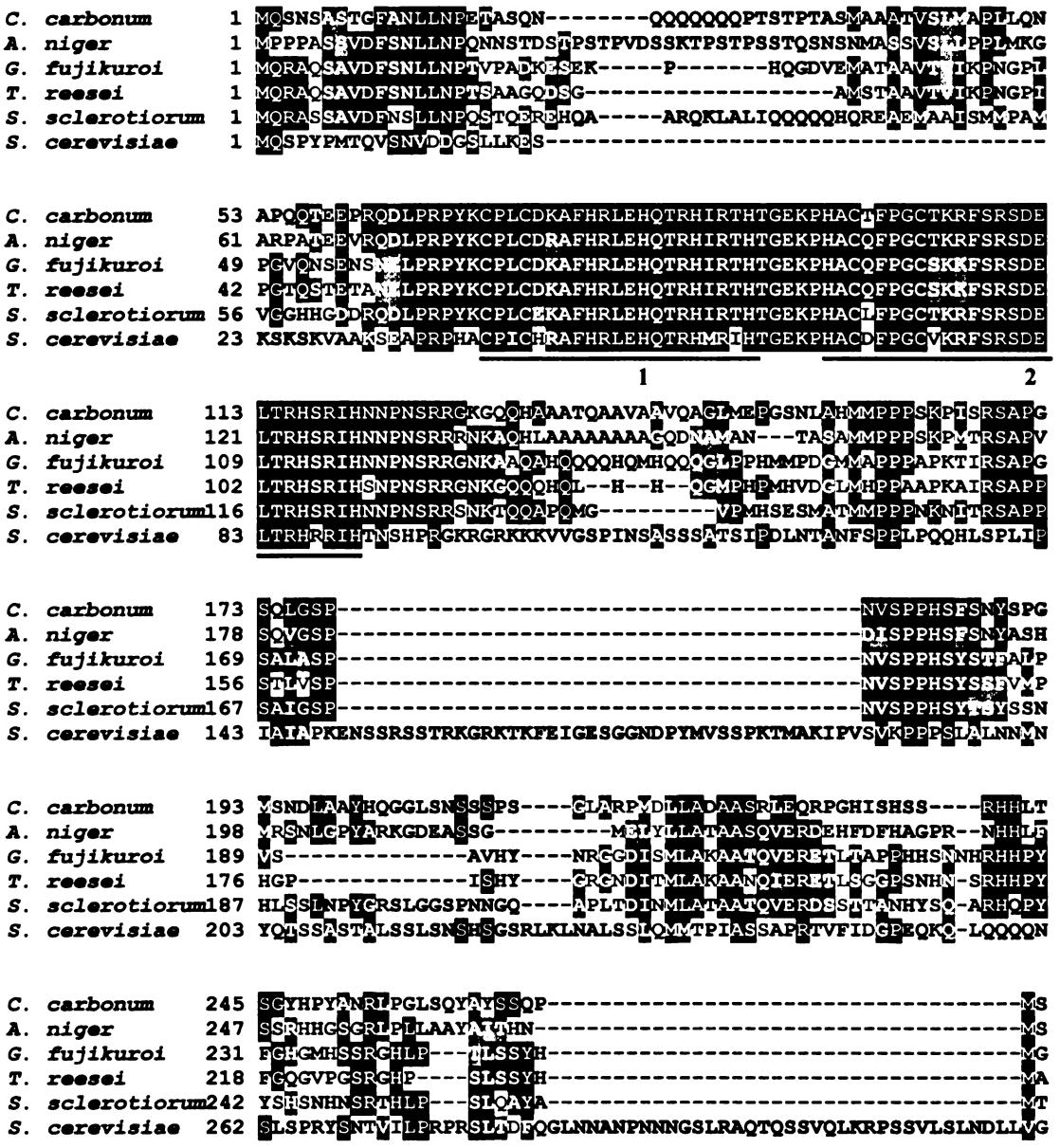

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Figure 21. Comparison of Deduced Amino Acid Sequences of *ccCREA*, other fungal CreAps, and *S. cerevisiae* Mig1p.

Residues that are identical in at least four sequences are indicated by black shading while conserved substitutions are in gray shading. The two Cys_2His_2 zinc-finger region (Berg, 1988; Miller et al., 1985; Rosenfeld and Margalit, 1993) are underlined (1,2).

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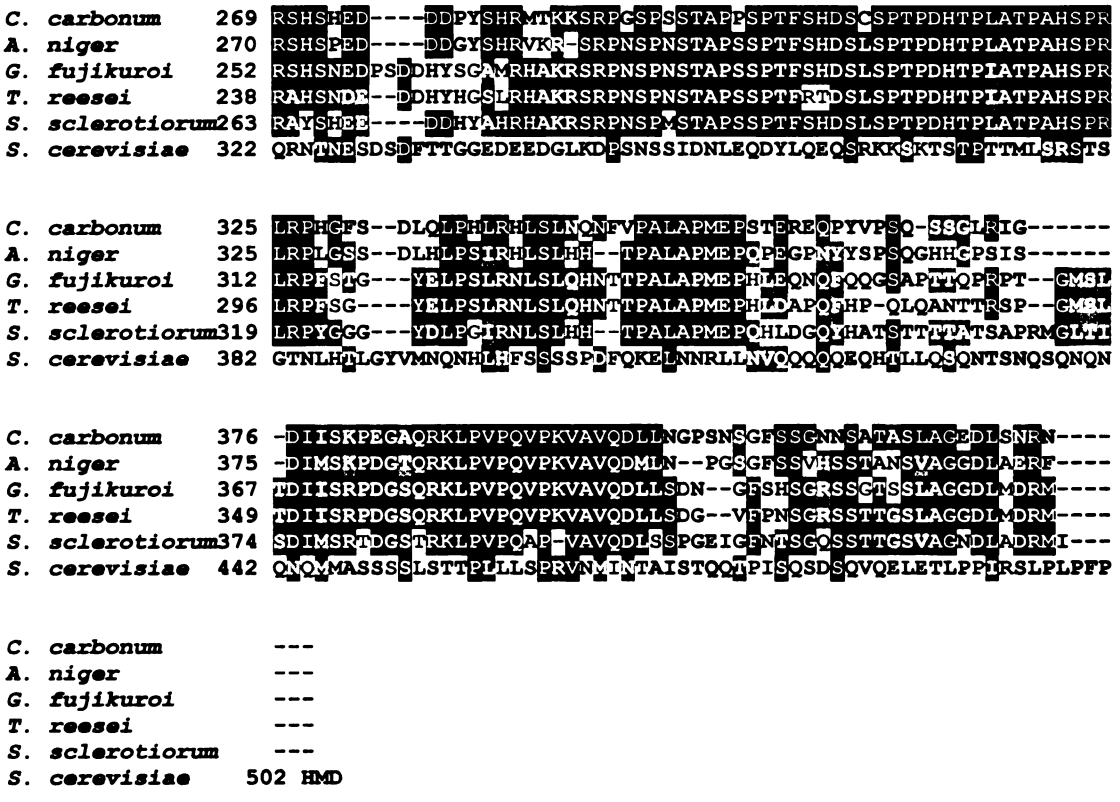


CreA proteins, the deduced ccCreAp also contains a nuclear localization signal (Hicks and Raikhel, 1995), PNSRRGK, at residues 123-129.

Hybridization of a *ccCREA* probe to *C. carbonum* genomic DNA digested with *Pst*I, *Sac*I, *Sal*I, or *Spe*I resulted in a single band in each case (Figure 22), indicating the existence of a single copy of the *ccCREA* gene in *C. carbonum*.

DISCUSSION

Most CWDE genes are activated only in the appropriate environment and therefore are only expressed when required (Hensel and Holden, 1996). The biosynthesis of most xylanases are induced by xylan. Monomers such as xylose, a product of xylan digestion, can also induce expression of some CWDEs. This differential expression of multiple xylanases may serve to ensure that enzymes capable of degrading xylan are present under the various conditions encountered by the fungus during growth in its host plant. The repression of CWDEs by preferred carbon sources such as glucose (Asymeric et al., 1988) is an efficient energy-conserving mechanism because the activity of enzymes that degrade xylan and other carbon sources are not required in the presence of glucose. One of the mechanisms to achieve this is repression at the transcriptional level of genes that encode enzymes involved in the catabolism of the alternative carbon sources. The involvement of a carbon catabolite repressor (CreAp) in the regulation of CWDEs has been reported in several fungi (de Graaff et al., 1994; Orejas et al., 1999; Mach et al.,

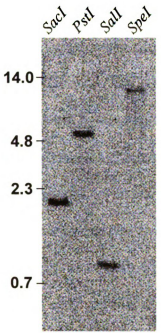


Figure 22. Copy number of *CREA* gene in *C. carbonum*.

Southern blot analysis of total genomic DNA from *C. carbonum* digested with restriction enzymes *Pst*I, *Sac*I, *Sal*I, or *Spe*I.

1996; Reymond-Cotton et al., 1996; Zeilinger et al., 1996). CreAp, which is the homolog of yeast Mig1p, binds to the promoter region of target genes in the presence of glucose and inhibits their expression (Dowzer and Kelly, 1991; Ronne, 1995). ccCreAp, like Mig1p and other CreA proteins, has two Cys_2His_2 zinc fingers that are well conserved among all known CreA proteins. Cys_2His_2 zinc finger-bearing proteins are a large superfamily of nucleic acid binding proteins that constitute a major subset of eukaryotic transcription factors (Berg, 1988; Miller et al., 1985; Rosenfeld and Margalit, 1993).

CreAp inhibits gene transcription by binding to specific sequences (5'-SYGGRG-3') in the promoters of these genes (Cubero and Scazzocchio, 1994; Espeso and Penalva, 1994). This consensus sequence is present in several *C. carbonum* CWDE genes (Table 4). In *S. cerevisiae*, Mig1p, binds similar promoter sequences (GCGGGG) and recruits two other corepressor proteins, Tup1p and Ssn6p which inhibit the expression of genes encoding enzymes that catabolize other carbon sources (Treitel and Carlson, 1995). Ssn6p-Tup1p is a general repressor of transcription in yeast (Keleher et al., 1992; Smith and Johnson, 2000). It has been shown that *CREA* transcript levels are independent of the carbon source in *G. fujikuroi* and *Botrytis cinerea* (Tudzynski et al., 2000). De Vit et al. (1997) have also found that the subcellular localization of the *S. cerevisiae* Mig1p is regulated by glucose. Mig1p is imported into the nucleus within minutes after the addition of glucose and is just as rapidly transported back to the cytoplasm when glucose is removed.

Although our knowledge of catabolite repression is still very incomplete, it is possible in certain cases to propose a partial model of the way in which the different elements involved in catabolite repression may be integrated. It appears that the Snf1p

Table 4. Sequence in 5' region of *C. carbonum* CWDE genes that corresponds to the consensus sequence (5'-SYGGRG-3') that CreAp binds ((Cubero and Scazzocchio, 1994; Espeso and Penalva, 1994). The consensus sequence is in either '+' or '-' strand, and numbering is from the ATG encoding the first amino acid.

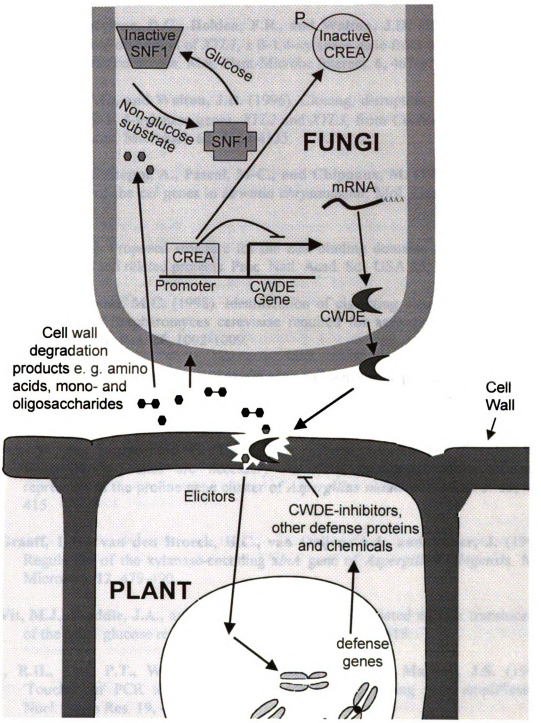
Gene	Putative CreAp binding site
ARF1	-111 CTGGAG -106
XYP1	-163 GTGGGG -168
XYL3	-453 GTGGGG -448
XYL4	-118 CTGGGG -123

in the presence of non-glucose substrate phosphorylates Mig1p and probably CreAp (Östling and Ronne, 1998; Treitel et al., 1998). The phosphorylated CreAp is unable to bind to DNA and repress CWDE gene expression, and therefore they are transcribed. Translation of the messages produces CWDE proteins that are processed and secreted to the external surface of the fungal hyphae (Figure 23). Upon encountering plant cell walls, the CWDEs degrade the constituent polymers generating simple compounds including amino acids and mono- and oligosaccharides that are assimilated by the fungus for growth. As long as most of the absorbed molecules are not glucose, Snf1p is active, CreAp is inhibited, and CWDEs are made. The Snf1/CreA regulatory pathway is the first link of a possible signaling pathway from the cell wall of the host plant to the global expression of enzymes as probable fungal virulence factors that advance the disease condition.

(See figure next page)

Figure 23. Schematic representation of the Snf1/CreA possible pathway of cell wall degrading enzyme regulation.

Snf1 protein kinase in the presence of non-glucose substrate phosphorylates CreAp. The phosphorylated CreA is unable to bind DNA and repress CWDE gene expression. Translation of the message produces CWDE proteins that are processed and secreted to the external surface of the fungal hyphae. Upon encountering it, the CWDEs degrade the plant cell wall generating simple compounds including amino acid, and mono- and oligosaccharides that are assimilated by the fungus for growth. As long as most of the absorbed molecules are not glucose, Snf1p is active and CreAp is inhibited and CWDEs are made.


P
Inactive SNF1
Glucose
Inactive CREA
Non-glucose substrate
SNF1
FUNGI
mRNA
AAAA
CREA
Promoter
CWDE Gene
CWDE
Cell wall degradation products e. g. amino acids, mono- and oligosaccharides
Cell Wall
Elicitors
CWDE-inhibitors, other defense proteins and chemicals
PLANT
defense genes

REFERENCES

Apel, P.C., Panaccione, D.G., Holden, F.R., and Walton, J.D. (1993). Cloning and targeted gene disruption of *XYL1*, a β-1,4-xylanase gene from the maize pathogen *Cochliobolus carbonum*. Mol. Plant-Microbe Interact. **6,** 467-473.

Apel-Birkhold, P.C., and Walton, J.D. (1996). Cloning, disruption, and expression of two endo-β-1, 4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. Appl. Environ. Microbiol. **62**, 4129-4135.

Asymeric, J-L., Guiseppi, A., Pascal, M-C., and Chippaux, M. (1988). Mapping and regulation of the *cel* genes in *Erwinia chrysanthemi*. Mol. Gen. Genet. **211,** 95-101.

Berg, J.M. (1988). Proposed structure for the zinc-binding domains from transcription factor IIIA and related proteins. Proc. Natl. Acad. Sci. USA **85**, 99-102.

Bu, Y., and Schmidt, M.C. (1998). Identification of cis-acting elements in the SUC2 promoter of Saccharomyces cerevisiae required for activation of transcription. Nucleic Acids Res. **26**, 1002-1009.

Celenza, J.L., and Carlson, M. (1984). Cloning and genetic mapping of *SNF1*, a gene required for expression of glucose-repressible genes in *Saccharomyces cerevisiae*. Mol. Cell Biol. **4,** 49-53.

Cubero, B., and Scazzocchio, C. (1994). Two different, adjacent and divergent zinc finger binding sites are necessary for CREA-mediated carbon catabolite repression in the proline gene cluster of *Aspergillus nidulans*. EMBO J. **13**, 407-415.

de Graaff, L.H., van den Broeck, H.C., van Ooijen, A.J., and Visser, J. (1994). Regulation of the xylanase-encoding *xlnA* gene of *Aspergillus tubigensis*. Mol. Microbiol. **12**, 479-490.

De Vit, M.J., Waddle, J.A., and Johnston, M. (1997). Regulated nuclear translocation of the Mig1 glucose repressor. Mol. Biol. Cell **8**, 1603-1618.

Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K., and Mattick, J.S. (1991). 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucl. Acids Res. **19,** 4008.

Dowzer, C.E., and Kelly, J.M. (1991). Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. Mol. Cell. Biol. **11**, 5701-5709.

Drysdale, M.R., Kolze, S.E., and Kelly, J.M. (1993). The *Aspergillus niger* carbon catabolite repressor encoding gene, *creA*. Gene **130**, 241-245.

Espeso, E.A., and Penalva, M.A. (1994). In vitro binding of the two-finger repressor CreA to several consensus and non-consensus sites at the *ipnA* upstream region is context dependent. FEBS Lett. **342**, 43-48.

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

Hensel, M., and Holden, D. W. (1996). Molecular genetic approaches for the study of virulence in both pathogenic bacteria and fungi. Microbiology **142**, 1049-1058.

Hicks, G.R., and Raikhel, N.V. (1995). Protein import into the nucleus: an integrated view. Annu. Rev. Cell Dev. Biol. **11**, 155-88.

Hofmann K., Bucher P., Falquet L., and Bairoch A. (1999). The PROSITE database, its status in 1999. Nucleic Acids Res. **27**, 215-219.

Ilmen, M., Thrane, C., and Penttila, M. (1996). The glucose repressor gene *cre1* of *Trichoderma*: isolation and expression of a full-length and a truncated mutant form. Mol. Gen. Genet. **251**, 451-460.

Keleher, C.A., Redd, M.J., Schultz, J., Carlson, M., and Johnson, A.D. (1992). Ssn6-Tup1 is a general repressor of transcription in yeast. Cell **68**, 709-719.

Lundin, M., Nehlin, J.O., and Ronne, H. (1994). Importance of a flanking AT-rich region in target site recognition by the GC box-binding zinc finger protein MIG1. Mol. Cell Biol. **14**, 1979-1985.

Lutfiyya, L.L., and Johnston, M. (1996). Two zinc-finger-containing repressors are responsible for glucose repression of *SUC2* expression. Mol. Cell Biol. **16**, 4790-4797.

Mach, R.L., Strauss, J., Zeilinger, S., Schindler, M., and Kubicek, C.P. (1996). Carbon catabolite repression of xylanase I (*xyn1*) gene expression in *Trichoderma reesei*. Mol. Microbiol. **21**, 1273-1281.

Miller, J., McLachlan, A.D., and Klug, A. (1985). Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. EMBO J. **4**, 1609-1614.

Orejas, M., MacCabe, A.P., Perez, Gonzalez, J.A., Kumar, S., and Ramon, D. (1999). Carbon catabolite repression of the *Aspergillus nidulans xlnA* gene. Mol. Microbiol. **31**, 177-184.

Östling, J., and Ronne, H. (1998). Negative control of the Mig1p repressor by Snf1p-dependent phosphorylation in the absence of glucose. Eur. J. Biochem. **252,** 162-168.

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

Ransom, R.F., and Walton, J.D. (1997). Purification and characterization of extracellular β-xylosidase and α-arabinosidase from the plant pathogenic fungus *Cochliobolus carbonum*. Carbohydr. Res. **297,** 357-364.

Reymond-Cotton, P., Fraissinet-Tachet, L., and Fevre, M. (1996). Expression of the *Sclerotinia sclerotiorum* polygalacturonase *pg1* gene: possible involvement of *CREA* in glucose catabolite repression. Curr. Genet. **30**, 240-245.

Ronne, H. (1995). Glucose repression in fungi. Trends genet. **11**, 12-17.

Rosenfeld, R., and Margalit, H. (1993). Zinc fingers: conserved properties that can distinguish between spurious and actual DNA-binding motifs. J. Biomol. Struct. Dyn. **11**, 557-70.

Ruijter, G.J.G., and Visser, J. (1997). Carbon repression in *Aspergilli*. FEMS Microbiol. Lett. **151**, 103-114.

Shroff, R.A., O'Connor, S.M., Hynes, M.J., Lockington, R.A., and Kelly, J.M. (1997). Null alleles of *creA*, the regulator of carbon catabolite repression in *Aspergillus nidulans*. Fungal Genet. Biol. **22**, 28-38.

Smith, R.L., and Johnson, A.D. (2000). Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. Trends Biochem. Sci. **25**, 325-330.

Sposato, J P., Ahn, J-H., and Walton, J.D. (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.

Tonukari, N.J., Scott-Craig, J.S., and Walton, J.D. (2000) The *Cochliobolus carbonum SNF1* gene is required for cell wall-degrading enzyme expression and virulence on maize. Plant Cell **12**, 237-248.

Treitel M.A., and Carlson, M. (1995). Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. Proc. Natl. Acad. Sci. USA **92**, 3132-3136.

Treitel, M.A., Kuchin, S., and Carlson, M. (1998). Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **18,** 6273-6280.

Tudzynski, B., Liu, S., and Kelly, J.M. (2000). Carbon catabolite repression in plant pathogenic fungi: isolation and characterization of the *Gibberella fujikuroi* and *Botrytis cinerea CreA* genes. FEMS Microbiol. Lett. **184**, 9-15.

van der Veen, P., Ruijter, G.J., and Visser, J. (1995). An extreme *creA* mutation in *Aspergillus nidulans* has severe effects on D-glucose utilization. Microbiology 141, 2301-2306.

Van Hoof, A., Leykam, J., Schaeffer, H.J., and Walton, J.D. (1991). A single β-1,3-glucanase secreted by the maize pathogen *Cochliobolus carbonum* acts by an exolytic mechanism. Physiol. Mol. Plant Pathol. **39,** 259-267.

Van Wert, S.L., and Yoder, O.C. (1992). Structure of the *Cochliobolus heterostrophus* glyceraldehyde-3-phosphate dehydrogenase gene. Curr. Genet. **22,** 29-35.

Vautard, G., Cotton, P., and Fevre, M. (1999). The glucose repressor CRE1 from *Sclerotinia sclerotiorum* is functionally related to CREA from *Aspergillus nidulans* but not to the Mig proteins from *Saccharomyces cerevisiae*. FEBS Lett. **453**, 54-58.

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

Wegener, S., Ransom R.F., and Walton, J.D. (1999). A unique eukaryotic β-xylosidase gene from the phytopathogenic fungus *Cochliobolus carbonum*. Microbiology **145**, 1089-1095.

Zeilinger, S., Mach, R.L., Schindler, M., Herzog, P., and Kubicek, C.P. (1996). Different inducibility of expression of the two xylanase genes *xyn1* and *xyn2* in *Trichoderma reesei*. J. Biol. Chem. **271**, 25624-25629.

Chapter Four

Cloning and targeted mutation of the *Cochliobolus carbonum* α-L-arabinofuranosidase genes *ARF1* and *ARF2*

ABSTRACT

The filamentous fungus *Cochliobolus carbonum* produces many CWDEs that can depolymerize plant cell walls. A strain of *C. carbonum* carrying a *snf1* mutation has reduced virulence and does not express *ARF1*, a gene encoding α-L-arabinofuranosidase. The *ARF1* gene was isolated using degenerate PCR primers based on *Aspergillus niger*, *Aspergillus sojae*, *Streptomyces coelicolor*, and *Streptomyces lividans* α-L-arabinofuranosidases. Its encoded protein of 325 amino acid residues and 35 kD shares 64 to 66% identity with the above named arabinofuranosidase proteins. An internal fragment of *ARF1* was used to create *arf1* mutants by transformation-mediated gene deletion. Mutant strains were analyzed for growth, arabinofuranosidase activity and virulence. Residual arabinofuranosidase activity in the *arf1* mutant remained high, but one of the peaks of arabinofuranosidase activity, separated by cation-exchange HPLC, disappeared. A second arabinofuranosidase gene, *ARF2*, encoding a protein of 503 amino acid residues was cloned from *C. carbonum*. It has 66 to 70% amino acid identity with a similar arabinofuranosidase present in *Aspergillus niger*, *Emericella nidulans*, *Trichoderma reesei*. The disruption of *ARF2* in the *arf1* mutant, making an *arf1/arf2* double mutant led to the disappearance of the two major arabinofuranosidase activity peaks in culture filtrate fractionated by HPLC. The remaining activity was due to a bifunctional β-xylosidase/α-L-arabinofuranosidase (Ransom and Walton, 1997; Wegener et al., 1999). Growth of the *arf1* mutant was normal on xylan but somewhat reduced on xylose, arabinose or corn cell walls. Growth of the *arf1/arf2* double mutant was reduced

in the above sugars, but more strongly on arabinose. Both the *arf1* mutant and *arf1/arf2* double mutant had similar virulence on maize as the wild type *C. carbonum*.

INTRODUCTION

The role of fungal-secreted extracellular enzymes in diseases caused by plant fungal pathogens has been postulated to include degradation of the plant cell walls to enable penetration into inter- and intracellular regions, release of nutrients that can be assimilated for growth, and triggering of plant defense responses (Walton, 1994). A number of such enzymes and their encoding genes have been isolated from plant pathogenic microorganisms and studied for their role in the disease process. *Cochliobolus carbonum*, an ascomycetous pathogen of maize, penetrates into and ramifies through intact leaves and in the process obtains nutrients for growth from the plant cell cytoplasm and walls. The disease caused by this organism, Northern leaf spot of corn, is characterized by extensive necrotization of susceptible maize tissues. The fungus synthesizes numerous extracellular enzymes that can degrade the polysaccharides of the maize cell wall (Walton, 1994). Several *C. carbonum* cell wall degrading enzyme genes have been cloned and characterized. *C. carbonum* mutants made by disruption or deletion of specific genes including polygalacturonase genes (Scott-Craig et al., 1990, 1998) and endo-1,4-β-xylanase genes (Apel et al., 1993; Apel-Birkhold et al., 1996) were unaltered in virulence on maize. A possible reason for the lack of a role of individual enzymes in the disease process is the redundancy of these enzymes, that is, the presence of multiple CWDEs with overlapping function.

In graminaceous crops and grasses, arabinoxylan can account for up to 60 % of total cell wall carbohydrate (Carpita and Gibeaut 1993). It is composed of a β-1,4-linked D-xylopyranosyl backbone that is highly substituted, at the O-2 and/or O-3 positions by various mono- or oligosacchaide side chains. The side chains consist largely of

arabinosyl, xylosyl, and/or glucuronic acid residues (Carpita and Gibeaut 1993). Arabinoxylan-degrading enzymes includes β-xylosidases, α-L-arabinofuranosidases and endo-β-1,4-D-xylanases (Sunna and Antranikian, 1997). The mRNA expression of *XYL3* (an endo-1,4-β-xylanase gene) and *ARF1* (an α-L-arabinofuranosidase gene) cannot be detected in a *C. carbonum ccsnf1* mutant (Tonukari et al., 2000). Mutation of *ccSNF1* also leads to down-regulation of other *C. carbonum* CWDE genes resulting in a decreased amount of enzyme production, poor growth on corn cell walls, and reduced virulence (Tonukari et al., 2000). Growth of the *ccsnf1* mutant is strongly impaired when D or L-arabinose is the sole carbon source (Tonukari et al., 2000). In order to determine whether *XYL3* and *ARF1* are required for virulence, it would be necessary to create a strain of the fungus that completely lacked these activities. However, Apel-Birkhold and Walton (1996) have previously shown by gene disruption that the *XYL1*, *XYL2*, and *XYL3* genes are not required for pathogenicity in *C. carbonum*. The *C. carbonum xyl1*, *xyl2*, and *xyl3* mutants exhibits similar growth in culture and virulence on maize as the wild-type fungus. In addition to *XYL1*, *XYL2*, and *XYL3*, *C. carbonum* has at least one other endo-1,4-β-xylanase encoding gene, *XYL4* (Apel et al., 1993; Apel-Birkhold and Walton, 1996; Scott-Craig et al., unpublished).

Unlike xylanases, α-L-arabinofuranosidase has been implicated as a virulence factor in diseases caused by *Sclerotinia fructigena* (Howell, 1975) and *Sclerotinia trifoliorum* (Rehnstrom et al., 1994). Arabinofuranosidases are a class of enzymes that specifically release arabinose residues from xylan side chains (Sunna and Antranikian, 1997). These enzymes in combination with exo- and endoxylanases are important for the complete degradation of arabinoxylan to xylose and L-arabinose. Ransom and Walton

(1997) have described the isolation of a non-glycosylated α-L-arabinofuranosidase from *C. carbonum* with a molecular weight of 63 kD. In this research, we describe the isolation of the ccSnf1p-regulated *C. carbonum ARF1*, and a second arabinofuranosidase gene, *ARF2*. We also analyze the effects of their targeted mutation on growth, total arabinofuranosidase activity, and virulence on maize.

METHODS

Fungal cultures, media and growth conditions

The wild-type race 1 strain of *C. carbonum* (367-2A) was derived from strain SB111 (ATCC 90305) and maintained on V8 plates (Apel et al. 1993). The fungus was grown in liquid media on 1 % agar plates containing minimal salts, 0.2 % yeast extract, and trace elements (Van Hoof et al., 1991) with 1% D-xylose, D-arabinose, L-arabinose, oat spelt xylan (Fluka, Switzerland), or corn cell walls (Sposato et al., 1995) as the sole carbon source. For liquid culture, four fungal plugs (each 5 mm^2) were inoculated into a 1 L Erlenmeyer flask containing 125 ml of medium and grown in still culture for 7 to 14 days at room temperature. For growth on the agar plates, 5 µl of a suspension of conidia (10^4/ml) in 0.1 % Tween 20 was pipetted onto the center of the plate. Mycelium for protoplasts preparation was obtained from germinating conidia (Apel et al., 1993). For routine enzyme production, cultures were grown on 0.8% corn cell walls plus 0.2% sucrose.

Nucleic acid manipulations

The *C. carbonum* genomic library has been previously described (Scott-Craig et al., 1990). DNA and total RNA were extracted from lyophilized mats (Pitkin et al., 1996; Apel et al., 1993). The methods used for DNA and RNA electrophoresis, blotting, probe labeling and hybridization have also been described elsewhere (Apel-Birkhold et al., 1996). The *Cochliobolus heterostophus GPD1* gene, encoding glyceraldehyde-3-phosphate dehydrogenase (Van Wert and Yoder, 1992), was used as a loading control.

To isolate *ARF1*, PCR was performed in a thermocycler (MJ Research, Watertown, MA) using *taq* DNA polymerase (Life Technologies, Gaithersburg, MD) and two degenerate primers based on conserved amino acid sequence of α-L-arabinofuranosidase proteins from *Aspergillus niger*, *Streptomyces coelicolor*, and *Streptomyces lividans*. Forward primer 5' GGNGAYAAYGGIAARATITAYMG 3' is based on the amino acid sequence "GDNGKIYR," and reverse primer 5' ATRTCRTYI GTCCANGTIGCNCC 3' is based on the amino acid sequence "GATWTN/DDI" (where Y = T or C; R = G or A; I = inosine; N = A, T, G or C). The degenerate primers were used to amplify a 310-bp genomic fragment from *C. carbonum* DNA using the following PCR: initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 45 sec; annealing at 50 °C for 30 sec; and polymerization at 72 °C for 1 min. This was followed by polymerization at 72 °C for 7 min. The PCR product was cloned into the pGEM T-easy vector (Promega). For *ARF2* isolation, degenerate primers were also made based on conserved amino acid sequence of arabinofuranosidases from *Aspergillus niger*,

Emericella nidulans, and *Trichoderma reesei*. Forward primer 5' ARAARGCNTAYG GNGTNTT 3' is based on the amino acid sequence "Q\KKAYGVF," and reverse primer 5' ADRTCNRCCATDATCCA 3' is based on the amino acid sequence "WIMA/VDM/L" (where D = A, G or T). The degenerate primers were used to amplify a 264-bp genomic fragment from the *C. carbonum* cDNA library originally constructed for the CytoTrap yeast two-hybrid screen. A touchdown PCR (Don et al., 1991) with the *C. carbonum* cDNA library as template and the above primers was performed under the following conditions: initial denaturation at 94 °C for 3 min, 45 cycles of denaturation, 94 °C for 1 min; annealing for 1 min; and polymerization at 72 °C for 2 min. The annealing temperature ranged from 60 °C to 45 °C with a decrease of 1 °C every three cycles. This was followed by 10 cycles of denaturation at 94 °C for 1 min; annealing at 45 °C for at for 1 min; and polymerization at 72 °C for 2 min. The PCR product was cloned into pGEM T-easy vector (Promega).

An *ARF1* cDNA was amplified by PCR from a CytoTrap yeast two-hybrid cDNA library made from *C. carbonum* mRNAs. An *ARF1* specific primer, 5' TTCTTCGCC GGTGACAACGGC 3' and the MYR 3' primer, 5'CGTGAATGTAAGCGTGACAT, which is specific to the pMYR vector into which the cDNA library was made (GenBank Accession number: AF102577; CytoTrap XR Library Construction Kit, Stratagene) were used in a PCR reaction (initial denaturation at 94 °C for 3 min, 35 cycles of denaturation; 94 °C for 1 min; annealing at 50 °C for 1 min; and polymerization at 72 °C for 2 min) to amplify an 800-bp *ARF1* cDNA which was then cloned into pGEM T-easy vector. *ARF2* cDNAs were amplified from the CytoTrap yeast two-hybrid cDNA library. A 1.1-kb *ARF2* cDNA was amplified using an *ARF2* specific primer, 5' GGAACCGGGTACCGC

AATAAC 3' and the vector pMYR primer. The PCR reaction conditions were initial denaturation at 94 °C for 3 min, 35 cycles of denaturation; 94 °C for 1 min; annealing at 50 °C for 1 min; and polymerization at 72 °C for 2 min. The *ARF2* cDNA was cloned into pGEM T-easy vector.

The transcriptional start sites of *ARF1* and *ARF2* were determined using the 5' RACE (Rapid Amplification of cDNA Ends) system, version 2.0, following the instructions of the manufacturer (GIBCO-BRL) (Frohman et al. 1988). An oligonucleotide of sequence 5' TAGCACCAGTGCTGCTGCCGG 3' was used to prime first-strand cDNA synthesis, which was then amplified by PCR using a nested primer of the sequence 5'-TGGACA CCACCCCAGCTGTTG-3'. The initial primer for the *ARF2* 5' RACE reaction was GATGGCTCCCATGTGGCCGTT and PCR amplification primer was 5' GTTGTTATTGCG GTACCCGGT 3'.

DNA Nucleotide sequence was determined by automated fluorescence sequencing at the DOE-Plant Research Laboratory facility, Michigan State University. Sequence data were analyzed with the Lasergene software (DNASTAR Inc., Madison, WI).

Construction of gene replacement vector and transformation

A gene replacement vector for *ARF1* was constructed using a 3.8-kb *Xho*I genomic fragment cloned into pBluescript SK+/- (Stratagene). An internal 1.0-kb *Hin*dIII-*Eco*RI fragment was deleted, and replaced with a 1.4-kb *Hpa*I fragment containing the *hph1* gene that confers hygromycin resistance (Sweigard et al., 1992) (Figure 23A and 23B). A major portion of the coding region was thus replaced by *hph1* such that 0.8 to 1.5 kb of

colinear DNA remained on each side. The gene replacement fragment was released from vector sequences by using two restriction endonucleases, *Sma*I and *Apa*I to prevent recircularization prior to transformation of *C. carbonum* 367-2A protoplasts (Scott-Craig et al., 1990). Transformants were selected for their ability to grow in the presence of hygromycin B (Calbiochem, La Jolla, Calif.).

For *ARF2* gene disruption, a 2.8-kb genomic fragment containing the *ARF2* gene was cloned into pBluescript KS+/-, and a 1.35-kb *Sal*I-*Hin*dIII region was deleted and replaced with a 3.0-kb *Stu*I-*Hin*dIII *sh-ble* gene conferring phleomycin resistance (Figure 25A and 25B). A major portion of the coding region was thus replaced by the *sh-ble* gene such that 650-bp of *ARF2* genomic DNA remained on one side. The *sh-ble* fragment was obtained from pUT 720 (CAYLA, Toulouse, France). The *ARF2* disruption vector was linearized at the *Kpn*I/*Spe*I site and used to transform protoplasts of *C. carbonum arf1* mutant (T708). Transformants were selected for their ability to grow in the presence of phleomycin.

HPLC

Seven-day old culture filtrates of *C. carbonum* grown in 1 L flasks containing 125 mL of liquid medium with 0.2 % sucrose and 0.8 % corn cell walls were filtered through through four layers of cheesecloth and one layer of Whatman #1 filter paper. Five

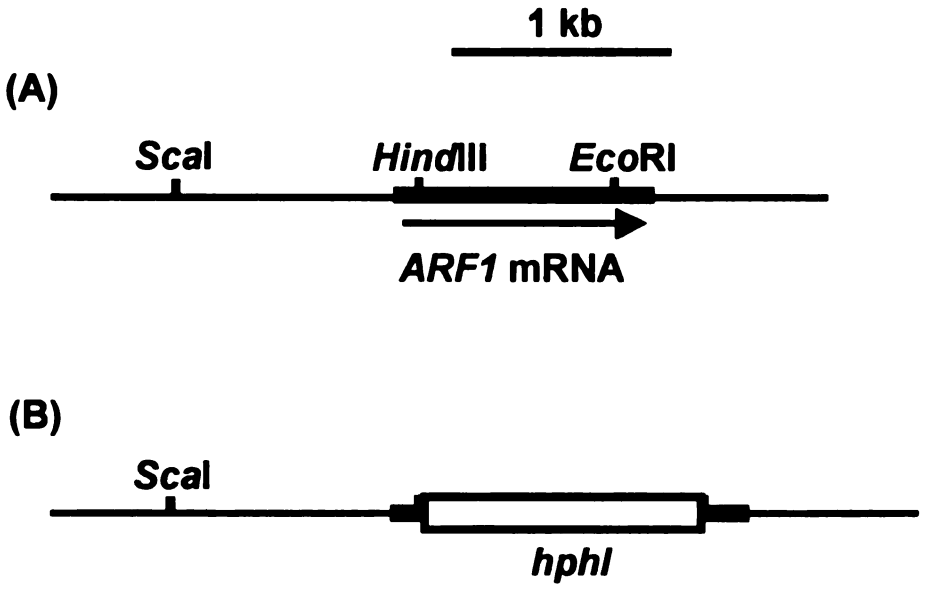


Figure 24. Construction of *arf1* Disruption Transformants.

(A) Map of the *ARF1* gene.
(B) An internal 1.0-kb *Hind*III-*Eco*RI fragment of *ARF1* was replaced with *hphI*.

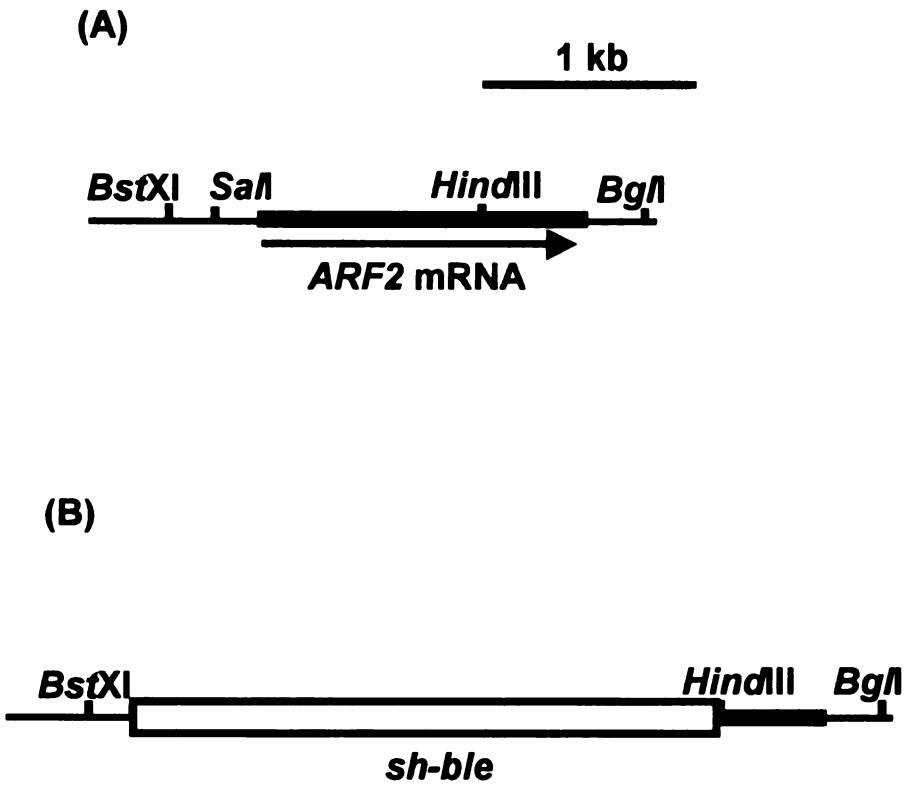


Figure 25. Construction of *arf2* Disruption Transformants.

(A) Map of the *ARF2* gene.

(B) A 1.35-kb *Sal*I-*Hin*dIII region was replaced with *sh-ble*.

hundred milliliters of culture filtrate was concentrated by rotary evaporation to about 50 mL and then dialyzed against 4 L of 25 mM sodium acetate, pH 5.0, for 24 h with two changes of buffer. The dialysate was then applied to a column of DEAE-cellulose to remove strongly anionic species and pigments. Material not binding to the DEAE-cellulose column was pooled and concentrated by ultrafiltration through an Amicon YM30 membrane to about 10 mL. This concentrated and clarified culture filtrate was then fractionated by cation-exchange high-performance liquid chromatography (HPLC) on a sulfoethylaspartamide cation-exchange HPLC column (The Nest Group, Southboro, Mass.) with a linear gradient of 25 mM sodium acetate (pH 5) to 25 mM sodium acetate (pH 5) plus 0.4 M KCl over 30 min. The flow rate was 1 ml/min, and 1-ml fractions were collected and assayed for α-L-arabinofuranosidase activity.

Enzyme assays

α-L-arabinofuranosidase, β-glucosidase and β-xylosidase activities were determined by production of *p*-nitrophenol from *p*-nitrophenol-α-L-arabinofuranoside, *p*-nitrophenol-β-D-glucopyranoside and *p*-nitrophenol-β-D-xylopyranoside (Sigma), respectively. A mixture of 100 µl of enzyme, 200 µl of 50 mM sodium acetate, pH 5, and 100 µl 10 mM substrate was incubated for 30 min at 37°C. Sodium carbonate (600 µl of 1 M) was added and the absorbance measured at 400 nm (Ransom and Walton, 1997).

Pathogenicity test

Eighteen days old plants of susceptible inbred maize line Pr (genotype *hm/hm*) were inoculated with a suspension of conidia (10^4/ml) in 0.1% Tween 20. After inoculation in the afternoon, the plants were covered in plastic bags overnight (approximately 12-16h) and then grown in a greenhouse. The plants were observed daily for about seven days or until they died.

RESULTS

Cloning of *ARF1* and *ARF2*

Two degenerate oligonucleotides were synthesized based on the conserved regions of α-L-arabinofuranosidases from other fungi (Gielkens et al., 1997; Redenbach et al., 1996; Vincent et al., 1997) and a 310-bp fragment of *ARF1* was amplified by PCR, using *C. carbonum* genomic DNA as template. The PCR product was used as a probe to obtain the genomic copy of the encoding gene, *ARF1*, which was sequenced on both strands (Figure 26). The *ARF1* cDNA was amplified by PCR from a cDNA library. The transcriptional start site of *ARF1* was determined by 5'-RACE. The *ARF1* transcript has a 64-bp 5' untranslated region, and 161-bp 3' untranslated region. The DNA sequence of *ARF1* contains an open reading frame of 978 bases predicting a protein of 325 amino acid residues with a molecular mass of 34.6 kDa. Comparison of the genomic and cDNA

```
CCGCCCGTAGGATTTCCTTCTTTATCTCTTAGGAGCTGCTCTCTTAGTGTTCGGGGGGAA    60
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CGATGTTCAAAAGCCGACGTGGAGAAAAACCCCGGATAGAGGAGAATCCACCTGCACCAC   180
CCCACCCACCCAACCTCGCAGGAACGGGCATTGGCGGGCGAACTCCGGAAGATTTGTAAG   240
TAGTGTTCTATCGGTGTAATAGTGGAGATGTGCCAAGCGGTGTGGGAAAAAGGTTCAAAG   300
GTTGAATATTTGATGGATTGTTCAGAGATTGATATGGGCACTTTTTTTGAACCAGGGGCG   360
CAATCGGCAAAAGTTACTTTTTTACGTACTGTTTAAATGTTGGTTGGGTGGTAAACAATT   420
TGCTCCTTGGGAGACGAAAGCCGGTGTGCAAAGTAGCATTTTTTGAGCATCTTTTGTCCA   480
GCATGCTCCTTACTCTAGACATGGGCTTGGTAGGTGGGCCGAGTTACATTGGCAACGCTC   540
ACAACCGCCTTGGCAAGGCTAAGTACTGCGTACAATCCAAGCGCTGTGGTTACCGCTACT   600
CTTTAATCTGTGCGGCCGAACAAATTCCGCGAGTCGATCTAGAAGCTGACCCTCCTCCCA   660
CGTGTTTGCTGGCCACTGACAGTGAATGTTGTTTAGTGGAGTTGAGAGTGCTTAGTGGCA   720
AAAAAGTGCCCTTGCTTATGGCCGGAGAGTGTTGCGTCAGTTATAGTTTTCATAACTGCA   780
GATGCGCGTCCTTTGGGCGGCTCCTTACAGCGGCTCCCTGCTGTAGCACGATAATTGTGG   840
TTTTGCAGTTTTGCGTGATTAAGGCGCAGAGCACAGTTGACGTCGGTGTAGGGACAATTT   900
CTCGCGCCCTAGCTGTCCATGCGGCATCAATCAAGCAATGCCAAACCTCAAACCCGCTAA   960
GTCCAAGTCTTGACTTGTGGGCGCAGCGGAGGCAGCCCTTCGACTGGTGGCACTTTTGCG  1020
CCACTGGCACCCATGCTTCTAGATTCCGGCCACCGGGTGTTTCATCGCGTGTTGACGTTT  1080
CGCTCTAGTGGAGAGACTAGCATCGGATAGGAACACTCGCGGATTCTAGTAAACACCCGG  1140
TGGGACTTTTTGGAGCAATACCGCCGAGATTGGGGCCTGGCAATCAGCGCAGCAGGCCAT  1200
GTTTCTTTGTTCGGCGCAAGAGACGCCAACAGGACTGCTTGTTAGCGCAGTGTTGGTACG  1260
TAGTCGGCCGGTCATACATCAGACGAATTGACGTCGACGTCAAAACATAGTCGCCAAGCT  1320
AGCGGGTTAGGCGAATGGGGTGCAGCACATACCTGCATTAGGATTTAAGCATGCATATAG  1380
TAGTAGATTAAGGAATTGCCCAGCCTCACTTGTTTCTGCAGTCCTACGTGCCCTTTGCCG  1440
TGCGTTCCCCGCATTACGGAGAATTGCCGTCCGTGCAGTCGCGTCAATTCCCTTGAAACA  1500
TCATGTAATTTAATATACCCACAACAACGGAACAATATGCCATACCGAGAGTAGACGCAT  1560
GATGTGGATGATCAATTTCCACGCTCAGGAGATCTAGGTTCCCGAGCCCAGTCCTGAATC  1620
GGCCAACCCTCAAGCGGTACGTGGGCAAGGCGTGATACACGGCATGCCTTCTGATTCGTA  1680
GGCCTAGACAGCATCGGCTCCAGGTATAAGTATCTTATACATGCCCTCGTTCTAATAGTC  1740
ATCCAGCTCACCAGCATCACATTCACTCATTCAACCAACCAACTACCAAACACCCCAAAA  1800
^
AACCAAACATGCGTTTCGTTCCCGACCTTAGCTTCTCGGCTGCTGCCGTCGCCCTTCTGG  1860
         M  R  F  V  P  D  L  S  F  S  A  A  A  V  A  L  L     17
CTTCCACTGCTTCGGCCCAGAGCTGCAAGCTTCCCACCTCTTACAAGTGGACCTCCTCCG  1920
A  S  T  A  S  A  Q  S  C  K  L  P  T  S  Y  K  W  T  S  S     37
GCGCTCTTGCCCAGCCCAAGTCTGGATGGGCCAACTTGAAGGATTTCACCATCTCCAGCC  1980
G  A  L  A  Q  P  K  S  G  W  A  N  L  K  D  F  T  I  S  S     57
TTAACGGCAAGCACATTGTCTATGCTACCGACCACGACACTGGATCCAAGTACGGATCCA  2040
L  N  G  K  H  I  V  Y  A  T  D  H  D  T  G  S  K  Y  G  S     77
TGGCTTTCAGCCCCTTCGGTAGCTTCAGCGAGATGGCCTCTGCTTCCCAGACCGCCACCC  2100
M  A  F  S  P  F  G  S  F  S  E  M  A  S  A  S  Q  T  A  T     97
CCTTCACTGCTGTTGCCCCTACCCTCTTCCGTTTCGCTCCCAAGAACATTTGGGTCTTGG  2160
P  F  T  A  V  A  P  T  L  F  R  F  A  P  K  N  I  W  V  L    117
```

Sequence continued next page.

Figure 26. Nucleotide and deduced amino acid sequence of *C. carbonum ARF1* gene.

The open reading frame encodes a protein of 325 amino acids; ^ transcription start site; * stop codon; # polyadenylation site.

Sequence continued from previous page.

```
CCTACCAGTGGGGACCTACCACCTTCTCCTACAGGACCTCCAGCGACCCCACCAACCCCA  2220
A  Y  Q  W  G  P  T  T  F  S  Y  R  T  S  S  D  P  T  N  P     137
ACAGCTGGGGTGGTGTCCAGACTCTCTTCTCTGGCAAGATCTCCGGCAGCAGCACTGGTG  2280
N  S  W  G  G  V  Q  T  L  F  S  G  K  I  S  G  S  S  T  G     157
CTATTGACCAGACTGTCATTGGTGACGCCATCAACATGTACCTCTTCTTCGCCGGTGACA  2340
A  I  D  Q  T  V  I  G  D  A  I  N  M  Y  L  F  F  A  G  D     177
ACGGCAAGATCTACCGTAGCAGCATGCCCAAGGCCAACTTCCCTGGCAGCTTCGGCACTG  2400
N  G  K  I  Y  R  S  S  M  P  K  A  N  F  P  G  S  F  G  T     197
CTTCCACCGTCATCATGAGCGACAGCACCAACAACCTCTTCGAGGCTGTTCAGGTCTACA  2460
A  S  T  V  I  M  S  D  S  T  N  N  L  F  E  A  V  Q  V  Y     217
CCGTCAAGGGTGGCGGTTACCTCATGATCGTTGAGGCTGTTGGCTCTGGCGGCCGTTACT  2520
T  V  K  G  G  G  Y  L  M  I  V  E  A  V  G  S  G  G  R  Y     237
TCCGCTCTTTCACTGCCTCCAGCCTCAGCGGTAGCTGGACCCCCAACGCTGCCACCGAGA  2580
F  R  S  F  T  A  S  S  L  S  G  S  W  T  P  N  A  A  T  E     257
GCAACCCCTTCGCCGGCAAGGCCAACAGCGGTGCCACCTGGACCAACGACATCTCCCACG  2640
S  N  P  F  A  G  K  A  N  S  G  A  T  W  T  N  D  I  S  H     277
GAGATCTTGTCAAGGTCACCAACGACGAGACCATGACCGTCGACCCTTGCAACCTGCAGC  2700
G  D  L  V  K  V  T  N  D  E  T  M  T  V  D  P  C  N  L  Q     297
TGTTGTACCAGGGCCGTGCCCCCAACTCTGGCGGCGACTACGACCGTCTCCCCTACAGGC  2760
L  L  Y  Q  G  R  A  P  N  S  G  G  D  Y  D  R  L  P  Y  R     317
CCGGTCTCCTTACCCTCAAGAAGTAGAGCCTTCTTTGCTCGGCTCCTGGCGAGGTACCTT  2820
P  G  L  L  T  L  K  K  *                                       325
CATTGTAAATGAATTTTTGTGTGGGAGATTGTATATAGCTCAGTTTGTGGTGGAATTCGT  2880
ATATATATTATTTTGGGCCACTGGCTTTGGCTCTTTGGCATTGCGCGAGGCATCAATTGA  2940
TTCTTCAACCTATCTTTGAAAATCCATCTATCTTACCTTTTGAGTGACTTCTTGTGAAAC  3000
      #
ATGACTCGATGGCTTGCCTGCCTATTGCAACTCCTTGGAGAAAATCCGCCGACGCTGCTT  3060
ATGCTGGTATATCTCGCTCCTACACACTTCTAGATTCTTCCTCTCTCTAATCGCAATCTC  3120
CTGCAAGATGGCCATGCTCTTGGCAGATAACAACGGGACCGCCCTTTGCACTGGGTCCAG  3180
GCCTCGTGCAGTTCCATAAACGCCCAAGCCATACCAACAAGGGCATTCCGGTGCACTAGC  3240
CACCGCATACTCGTCGCAACCATTCATTCCGCAAAAGCAGCTGACTCGGGCTCGCCCAGC  3300
GACACTGCCTCGCGCAAATTACACGGCTGCTGGTAGCCTGCATTAAGACACAACTCTAGG  3360
CCAAGTTCCACGATGCCAACCGCCTCGCCATCATCAG                          3397
```

sequences revealed that the *ARF1* gene has no introns. The product of *ARF1* (called Arf1p) has a predicted signal peptide cleavage site between amino acids 23 and 24 (Nielson et al., 1997). Its deduced amino acid sequence has about 64 to 66% identity with *A. niger*, *Aspergillus sojae*, *S. coelicolor*, and *S. lividans* α-L-arabinofuranosidases (Figure 27).

A 264-bp *ARF2* fragment was also amplified by PCR using degenerate oligonucleotides that were synthesized based on the conserved regions of arabinofuranosidases from *Aspergillus niger* (Flipphi et al., 1993), *Emericella nidulans* (Gielkens et al., 1999), and *Trichoderma reesei* (Margolles-Clark et al., 1996). The PCR product was used as a probe to obtain the genomic copy of *ARF2*, which was sequenced on both strands (Figure 28). The *ARF2* cDNA was also amplified from a cDNA library, and the transcriptional start determined by 5'-RACE. The *ARF2* DNA sequence encodes a predicted protein (Arf2p) of 503 amino acid residues with a molecular mass of 52.2 kDa. *ARF2* gene has one 59-bp intron. Arf2p also has a predicted signal peptide of 21 amino acids residues. Arf2p has 66 to 70% amino acid identity with *A. niger*, *E. nidulans*, *T. reesei* arabinofuranosidases (Figure 29).

Hybridization of *ARF1* or *ARF2* probe to *C. carbonum* genomic DNA digested with different restriction enzymes (*Hin*dIII, *Pst*I, *Sal*I, or *Xho*I) resulted in a single band in each case (Figure 30), indicating the existence of a single copy of *C. carbonum ARF1* and *ARF2*.

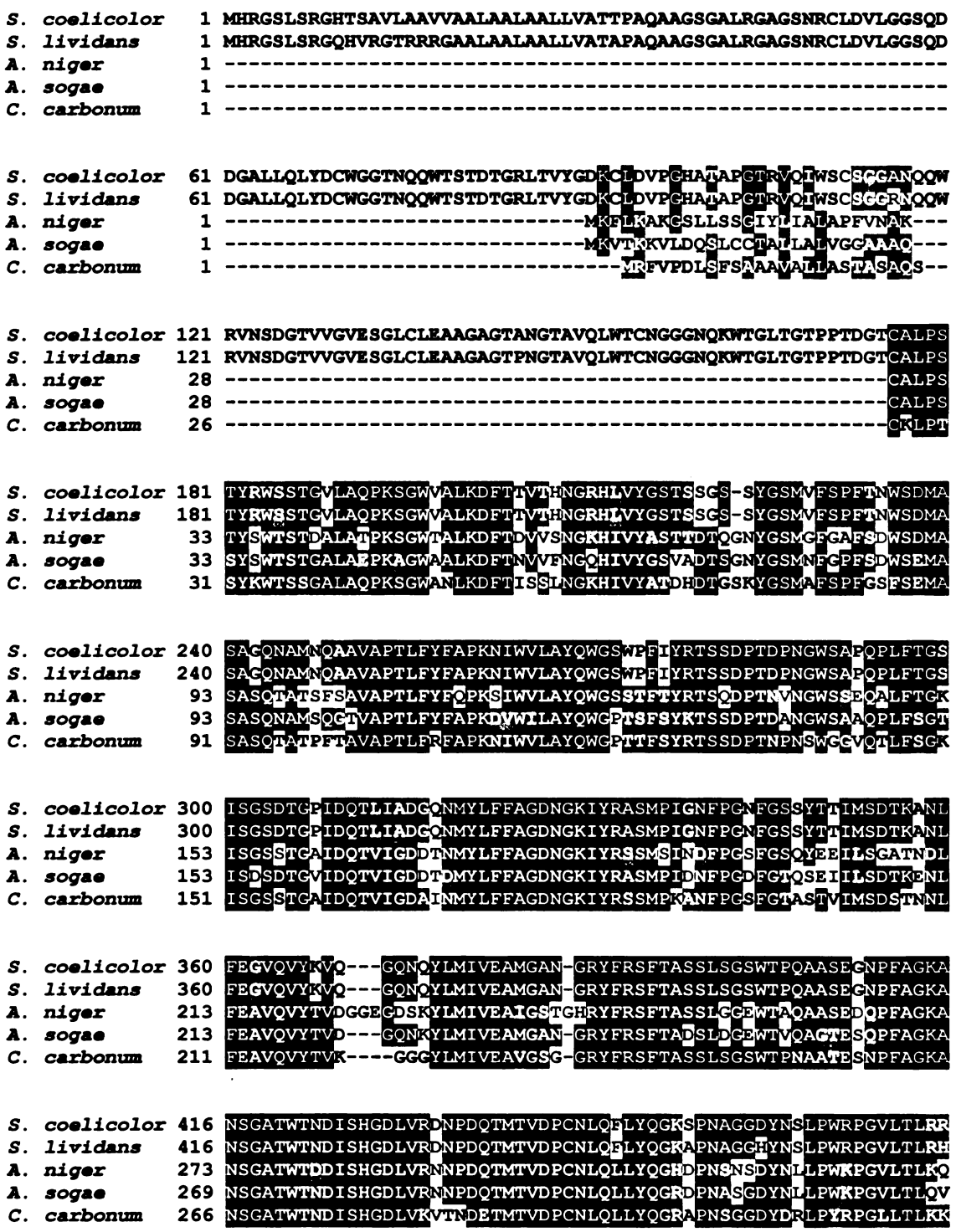

Figure 27. Comparison of Deduced Amino Acid Sequences of *C. carbonum ARF1* and α-L-arabinofuranosidases of *A. niger*, *A. sogae*, *S. coelicolor*, and *S. lividans*.

Residues that are identical in at least three sequences are indicated by black shading while conserved substitutions are in gray shading.

```
AGAGGAGCACAGAGGACTTGGGTTTGTCTTTTTTCTTCATATCCAAAAAAAACCATGTCT    60
      ^                                                  M  S     2
TCAAAATCGCTTCTCGTTGCGCTTGGGCTTGTAGCCACAGGTTCATTGGTGCATGCAGGG   120
 S  K  S  L  L  V  A  L  G  L  V  A  T  G  S  L  V  H  A  G    22
CCTTGTGACATTTACGCCAAAGGCAACACACCATGCATTGCTGCACATGCCACCACTCGT   180
 P  C  D  I  Y  A  K  G  N  T  P  C  I  A  A  H  A  T  T  R    42
GCTCTGTATAATTCATACAGTGGCCCGCTCTACCAAGTCAAGCGTGGCTCAGACGGTGCC   240
 A  L  Y  N  S  Y  S  G  P  L  Y  Q  V  K  R  G  S  D  G  A    62
ACAACTGACATTGCTCCACTGTCCGCCGGCGGTGTCGCCAATGCAGCTGCTCAAGACAAG   300
 T  T  D  I  A  P  L  S  A  G  G  V  A  N  A  A  A  Q  D  K    82
TTCTGTGCCAACACGACATGTCTCATTTCCATCATCTACGACCAATCTGGCAAAGGAAAC   360
 F  C  A  N  T  T  C  L  I  S  I  I  Y  D  Q  S  G  K  G  N   102
CACCTCACACAAGCACCACCAGGCGCTTTCAAGGGCCCAGACGTCGGCGGTTACGACAAT   420
 H  L  T  Q  A  P  P  G  A  F  K  G  P  D  V  G  G  Y  D  N   122
CTGGCAAGTGCTATCGGTGCACCTGTTTCGCTAGGCGGCAAAAAGGCATATGGCGTCTTC   480
 L  A  S  A  I  G  A  P  V  S  L  G  G  K  K  A  Y  G  V  F   142
ATCTCCCCTGGAACCGGGTACCGCAATAACAACGTAAAAGGCTCGGCCGTAAAAGACGAG   540
 I  S  P  G  T  G  Y  R  N  N  N  V  K  G  S  A  V  K  D  E   162
CCCCAAGGCATATACGCCGTCTTGGACGGTACACATTACAATGGCGGCTGCTGCTTCGAC   600
 P  Q  G  I  Y  A  V  L  D  G  T  H  Y  N  G  G  C  C  F  D   182
TATGGCAATGCTGAGACAAACAACCTTGACACTGgtaagtatatcacctcccatcaatttt  660
 Y  G  N  A  E  T  N  N  L  D  T                              193
ttgatatcagccaattgaccacccaaaaaaacagGCAACGGCCACATGGAAGCCATCTACT  720
                                   G  N  G  H  M  E  A  I  Y   202
TTGGTGACAACACCGTGTGGGGCAGCGGCGCTGGCAACGGCCCCTGGGTCATGGCTGACC  780
F  G  D  N  T  V  W  G  S  G  A  G  N  G  P  W  V  M  A  D   222
TAGAAAACGGCCTCTTCTCCGGTGCAAACCCAAAGCAGAACACTCAAAACCCATCCGTCT  840
L  E  N  G  L  F  S  G  A  N  P  K  Q  N  T  Q  N  P  S  V   242
CAAACCGCTTCCTAACGACTGTTGTCAAGGGCAAGCCTGGCGTCTGGGCCATCCGCGCCG  900
S  N  R  F  L  T  T  V  V  K  G  K  P  G  V  W  A  I  R  A   262
GAGACGCCACCACAGGCGGACTCTCAACATACTACAACGGCTCGCGCCCAAGTGTGTCGG  960
G  D  A  T  T  G  G  L  S  T  Y  Y  N  G  S  R  P  S  V  S   282
GCTACAACCCCATGAGCAAAGAAGGCGCCATTATCCTCGGTATCGGCGGCGACAACAGCA 1020
G  Y  N  P  M  S  K  E  G  A  I  I  L  G  I  G  G  D  N  S   302
ACGGCGCACAGGGAACCTTCACCGAGGGCGCAATGACGTTCGGGTATCCCAGCGACGCAA 1080
N  G  A  Q  G  T  F  T  E  G  A  M  T  F  G  Y  P  S  D  A   322
TCGAAAACGAAGTCCAGGCCAACCTTGTTGCGGCTGGATACTCCACTGGACGCGGATTGA 1140
I  E  N  E  V  Q  A  N  L  V  A  A  G  Y  S  T  G  R  G  L   342
TGACTAGCGGACCCGCATACACTGTTGGATCCAGTGTATCTCTCAGGGCCACGACATCTG 1200
M  T  S  G  P  A  Y  T  V  G  S  S  V  S  L  R  A  T  T  S   362
```

Sequence continued next page.

Figure 28. Nucleotide and deduced amino acid sequence of *C. carbonum ARF2*.

The open reading frame encodes a protein of 503 amino acids; ^ transcription start site; * stop codon; # polyadenylation site. An introns of 59 bp is typed in *italic lowercase*.

Sequence continued from previous page.

```
GCTACACGGACCGCTATCTCGCGCACTCCGGAGCGACAGTGAACACCCAAGTCGTATCGT  1260
G  Y  T  D  R  Y  L  A  H  S  G  A  T  V  N  T  Q  V  V  S     382
CGTCGAGCACTGCGCTGCTCAAGCGCCAGGCGAGCTGGATTGTCCGCGCTGGCTTCACCA  1320
S  S  S  T  A  L  L  K  R  Q  A  S  W  I  V  R  A  G  F  T     402
ACAGCGAGTGCTTTGCGTTCGAATCAAAGGATACGGCTGGAAGCTTTCTCCGCCACGCAA  1380
N  S  E  C  F  A  F  E  S  K  D  T  A  G  S  F  L  R  H  A     422
ATTTCGTGCTGCAGGTCAATGCTAATGATGGATCCAAGGGGTTCAAGGAAGATGCGACAT  1440
N  F  V  L  Q  V  N  A  N  D  G  S  K  G  F  K  E  D  A  T     442
TTTGTCCTCAGGCGGGTCTTACGGGTAAGGGTAGCTCGATTAGAACTTGGGCGTACCCGA  1500
F  C  P  Q  A  G  L  T  G  K  G  S  S  I  R  T  W  A  Y  P     462
CGAGGTGGATTCGCCATTTTAACAATGTTGGGTATATTTCGAGCAATGGTGGTGTCAAGG  1560
T  R  W  I  R  H  F  N  N  V  G  Y  I  S  S  N  G  G  V  K     482
ACTTTGATAATGTCAGCTCGTTCAACGACGATATCACGTGGCTTGTTGAGAGTGCCCTGG  1620
D  F  D  N  V  S  S  F  N  D  D  I  T  W  L  V  E  S  A  L     502
CTTGAGGGGACCGGGTAGTATGTGGTGGAATTTTATGTAAGTATGAATGCTTCCGACGTA  1680
A  *                                                           503
TATAAGTTGGTTTTGTCAGAATATGTTGATGAATGGTATATTCCTATGTATATATGGACG  1740
                                    #
GGATTGCCTCTACTATGTAAGCTGCATTTGTTAGCAAATATAAAGATACCAAAGTACTCT  1800
AGTAAACTTCTGTTAAATTTCCTCACTTACCTTGAATTTTGTAATTCATGATCGGAAGCG  1860
GGCATTGTATCATATCGCATTTGAACACGAGAAATCAACATCCAGTTCACACACACACTG  1920
CATATGAACATTGAACAAGTTTTGTTTAAGCTACTGGTACAAGTTATAGTACTGGCTATC  1980
AAGACTGCTTACAAAAAAAGACCCTGACATCGCCTAGTCTATGGCTAAATCTAGGGGATAT  2040
ACACATCAACAGGGCTGGGAATAACAGAAAGAAGAAGATCCTTTTAGTCAATAAGCAGAC  2100
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CGGCACC                                                        2167
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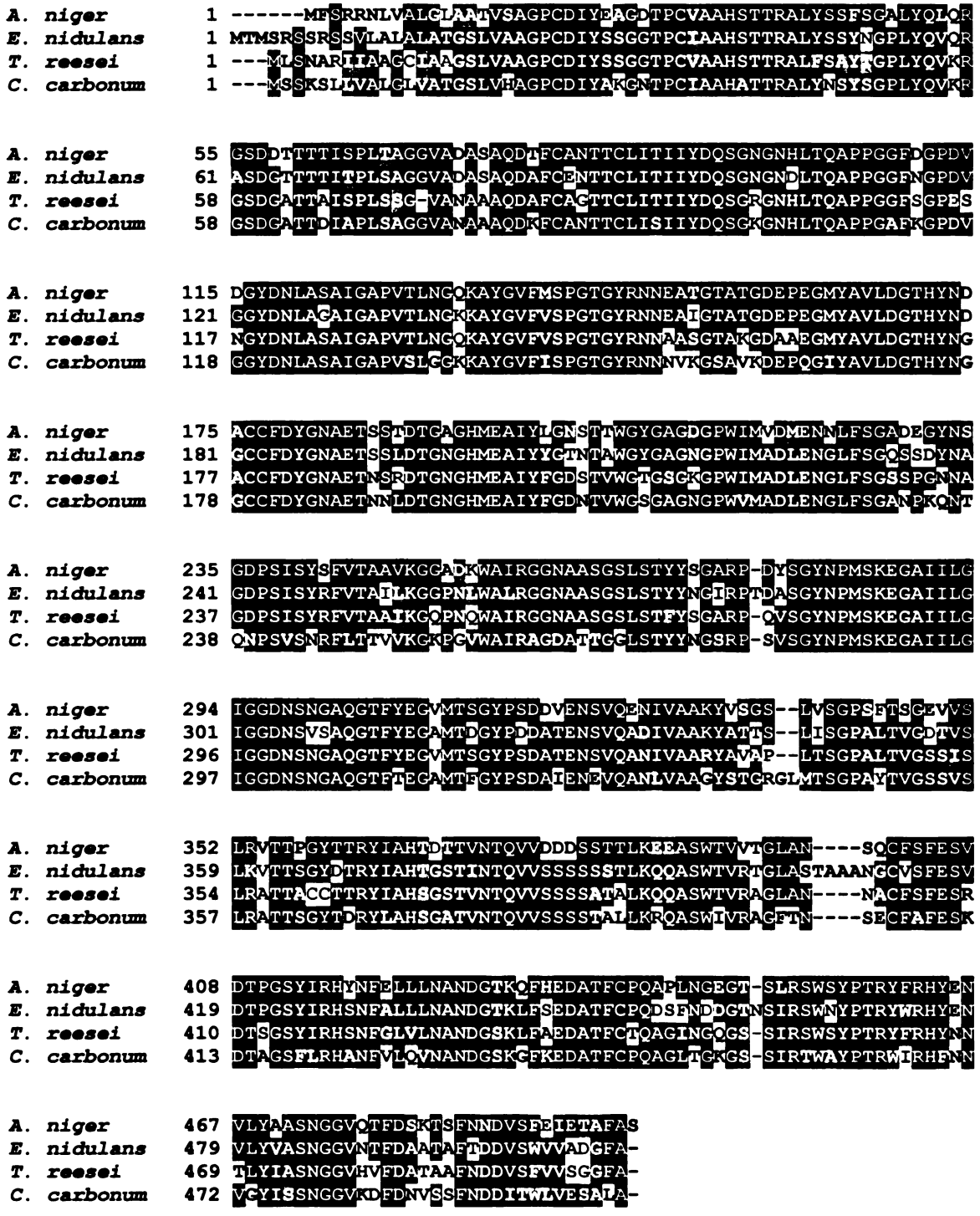

Figure 29. Comparison of Deduced Amino Acid Sequences of *C. carbonum ARF2* and α-L-arabinofuranosidases of *A. niger*, *E. nidulans*, and *T. reesei*.

Residues that are identical in at least two sequences are indicated by black shading while conserved substitutions are in gray shading.

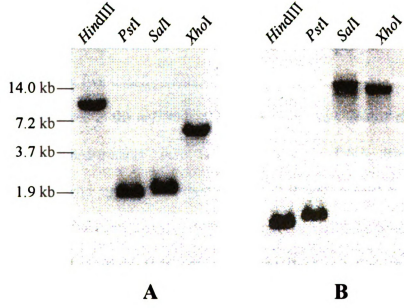

Figure 30. Copy number of *ARF1* (A) and *ARF2* (B) in *C. carbonum*

Southern blot analysis of total genomic DNA from *C. carbonum* digested with *Hin*dIII, *Pst*I, *Sal*I, or *Xho*I.

Targeted Disruption of *ARF1* and *ARF2*

Five hygromycin-resistant transformants were isolated and these were purified to nuclear homogeneity by two rounds of single-spore isolation on V8-hyg plates. All were determined by DNA gel blot analysis to have undergone gene replacement. Genomic DNA from both the mutants and the wild type were digested with *Sac*I and probed with the deleted 1.0-kb *Hin*dIII-*Eco*RI *ARF1* fragment. All five transformants showed no hybridization whereas there is a 2.5-kb band seen in the wild type lane (Figure 31A). To confirm the integration of the hygromycin resistance gene, a similar blot was probed with a fragment of *hphI*. Only the five replacement mutants exhibited hybridization. As control, a *SNF1* genomic fragment was used as probe, and hybridization was observed with the wild type and the five transformants.

ARF2 was disrupted in the *arf1* mutant in order to create a double mutant. Three phleomycin-resistant transformants were isolated. Genomic DNA from both the transformants and the wild type were digested with *Pst*I and probed with an *ARF2* fragment. All three transformants and *arf1* mutant showed a 1.4-kb hybridization, while two transformants have an additional 8.4 kb band. The third transformant also has an additional band, but of size 8.2 kb (Figure 31B) indicating that the resistance gene, *sh-ble,* integrated into the *ARF2* gene in one position for the first two transformants, and in a different position for the third transformant. To confirm the integration of the phleomycin resistance gene, a similar blot was probed with a fragment of *sh-ble*. Only the three transformants exhibited hybridization. As control, a *SNF1* genomic fragment was used as probe, and hybridization was observed with the wild type and the three transformants.

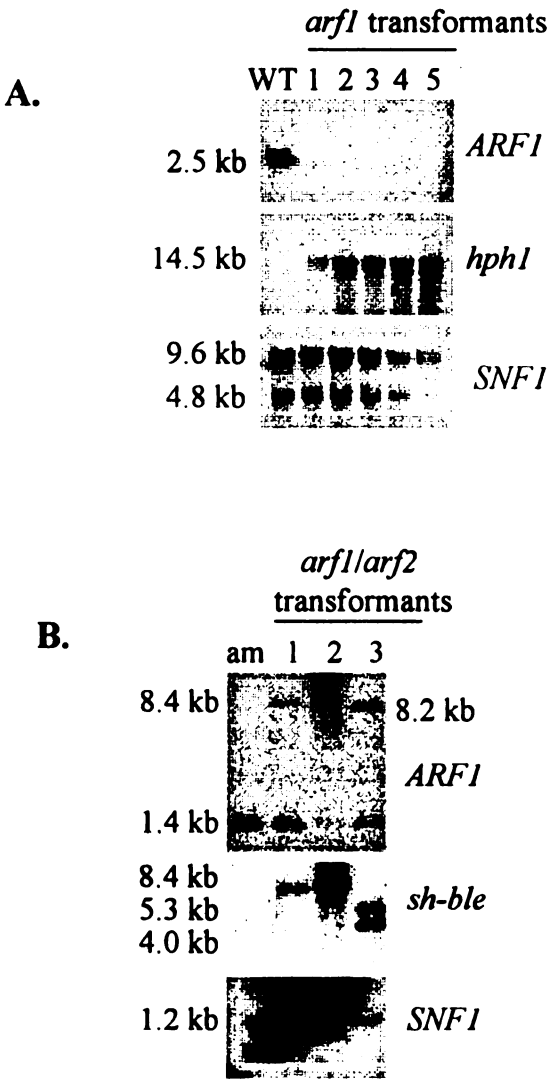

Figure 31. Screening of *arf1* and *arf2* Disruption Transformants.

(A) DNA blotting of wild type (WT) and five *arf1* disruptant transformants. DNA was digested with *Sac*I. Similar blots were probed with *ARF1*, *hphI*, or *SNF1* as a control.
(B) The *ARF2* gene was disrupted in the *arf1* mutant to obtain an *arf1/arf2* double mutant.DNA from *arf1* mutant (am) and three *arf1/arf2* disruptant transformants was digested with *Pst*I. Similar blots were probed with *ARF2*, *sh-ble*, or *SNF1* as a control.

Effect of the *ARF1* and *ARF2* mutations on enzyme activities

In preliminary experiments, all five *arf1* mutant strains displayed similar phenotypes with regard to growth and enzymatic activities (data not shown) and the *arf1* mutant T708 (arbitrarily chosen) was used for all further experiments. Strains 367-2A (wild type) and T708 (*arf1*) were grown in liquid still culture with purified maize cell walls as the sole carbon source. After seven days of growth, α-L-arabinofuranosidase activity was measured. Activity in the culture filtrate of the *arf1* mutant decreased by 3.6 ± 1.76 % (Figure 32).

The *arf1* mutant of *C. carbonum* had residual arabinofuranosidase activity indicating the presence of one or more additional α-L-arabinofuranosidases. After concentration by rotary evaporation, dialysis, and passage through an anion-exchange column to remove acidic proteins and pigments, culture filtrates of the *C. carbonum arf1* mutant were analyzed by cation-exchange HPLC. Two peaks of arabinofuranosidase activity were observed (Figure 33B). In the wild type *C. carbonum* strain, three major peaks were present (Figure 33A). From this result, it can be inferred that the *ARF1* gene encodes the third arabinofuranosidase peak eluting at 29 to 31 min (Figure 33A). Each of the peaks were further analyzed to determine whether they have other activities. Fractions 24, 27 and 30 were assayed for arabinofuranosidase, β-D-xylopyranosidase and β-D-glucopyranosidase activities. While fractions 24 and 30 exhibit predominantly arabinofuranosidase activity, fraction 27 had significant β-D-xylopyranosidase activity in addition to a lesser amount of α-L-arabinofuranosidase activity. Fraction 24 had a small

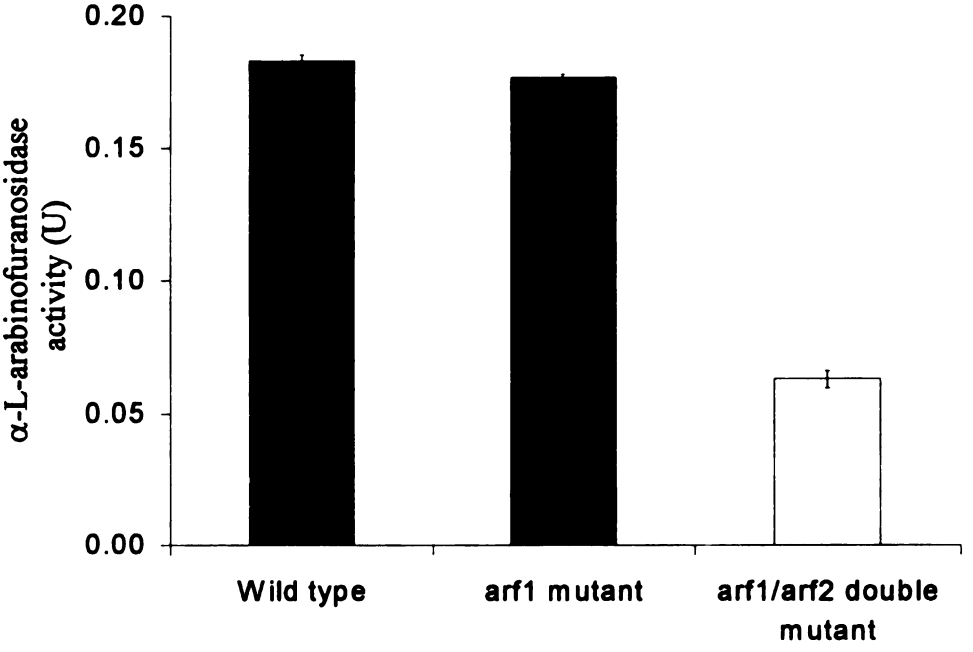

Figure 32. Effect of *ARF1* and *ARF2* mutations on α-L-arabinofuranosidase activity.

Arabinofuranosidase activity of *C. carbonum* wild type, *arf1* mutant and *arf1/arf2* double mutant after growth for 7 days in liquid cultures containing 2 % corn cell walls.

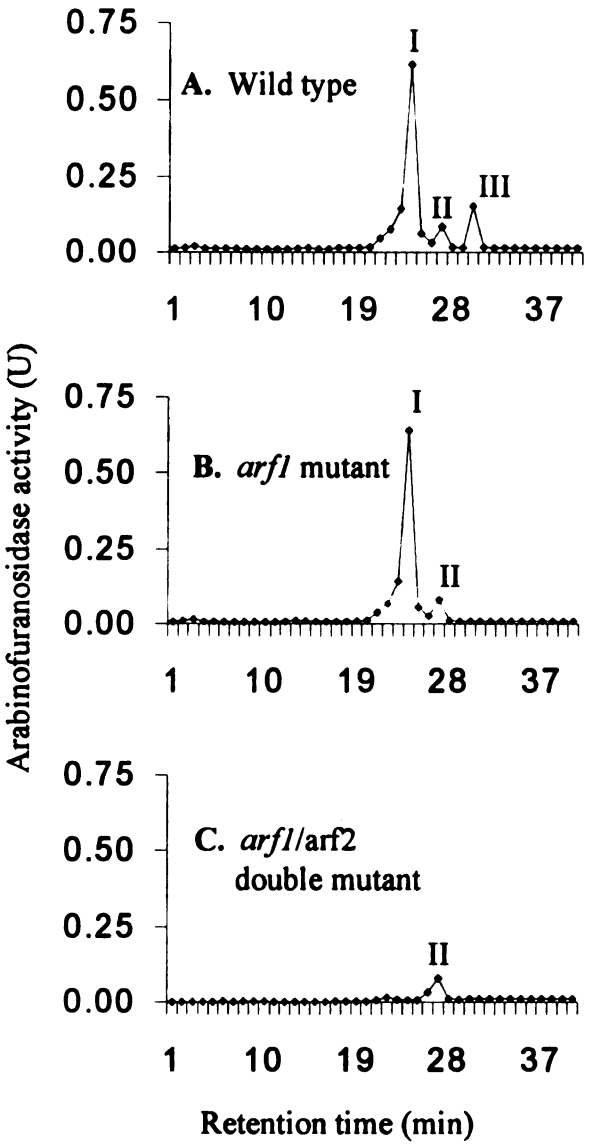

Figure 33. Cation-exchange HPLC analysis of α-L-arabinofuranosidase activity of wild-type, *arf1* mutant and *arf1/arf2* double mutant.

Culture filtrates were harvested after 7 days of growth on 0.8% corn cell walls plus 0.2% sucrose as the carbon source. Equal amounts of protein were loaded onto the HPLC column in each case.

amount of β-D-xylopyranosidase activity, and β-D-glucopyranosidase activity was barely detectable in all three fractions (Figure 34).

Culture filtrates of the three *C. carbonum arf1/arf2* transformants were also analyzed by cation-exchange HPLC. Two transformants, 1 and 2 (Figure 31B), have similar HPLC profiles as the *arf1* mutant (not shown) indicating that the *sh-ble* resistance gene integrated into the *C. carbonum* genome ectopically. In the culture filtrate of the third transformant, the first (major) arabinofuranosidase peak in addition to the third peak disappeared (Figure 33C). From this result, it can be inferred that the *ARF2* gene encodes the major arabinofuranosidase peak eluting at 20 to 25 min (Figure 33A). Activity in the culture filtrate of the *arf1/arf2* double mutant decreased by 65.7 ± 2.22 % (Figure 32).

Effect of the *ARF1* and *ARF2* mutations on growth

To assess if the reduced α-L-arabinofuranosidase activity secreted by the *arf1* mutant and *arf1/arf2* double mutant had any effect on growth on cell wall components including xylose, D-arabinose, L-arabinose, xylan, or corn cell walls, the mutants and wild type were grown on agar media supplemented with the above named compounds as the sole carbon source. On D-arabinose medium, the *arf1* mutant has about 37% decrease in growth, but the difference is smaller (0 to 13%) than on the other media (Figure 35). Growth of the *arf1/arf2* double mutant was reduced 78% in the D-arabinose medium, while reduction in the other media ranged from 39 to 56%.

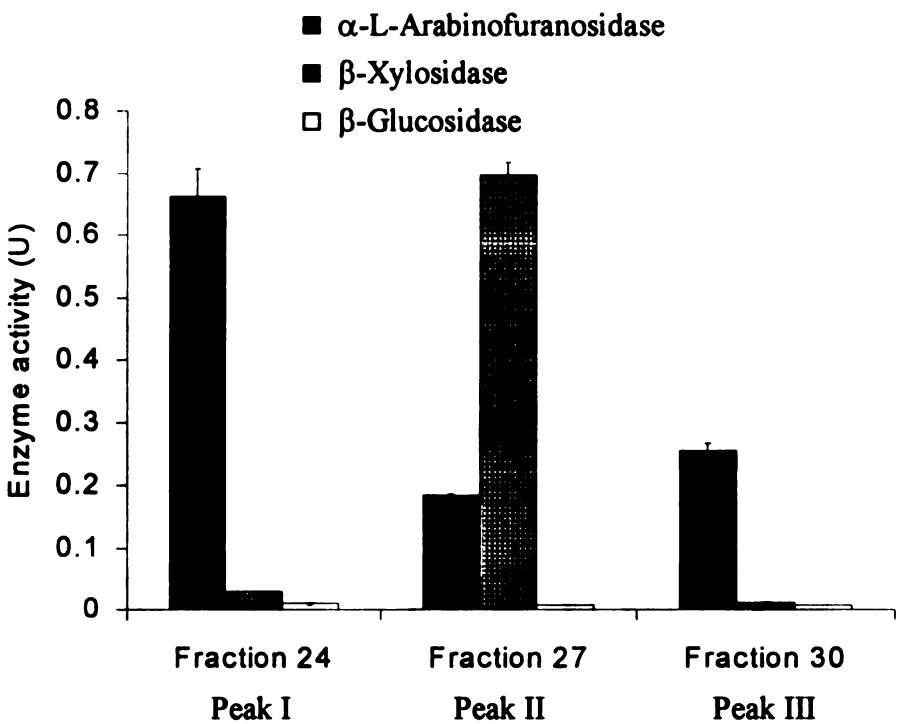

Figure 34. α-L-Arabinofuranosidase, β-xylosidase and β-glucosidase activities in the three peaks of the wild-type culture filtrate.

Fractions 24, 27 and 30 were from culture filtrates of wild type fractionated by cation-exchange HPLC.

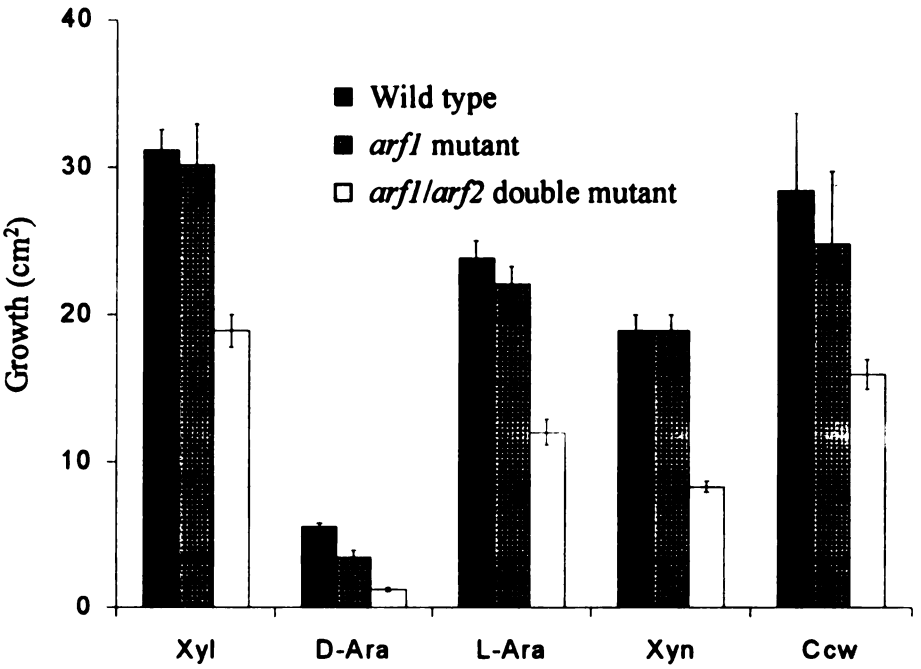

Figure 35. Growth of *C. carbonum* Wild type, *arf1* mutant, and *arf1/arf2* double mutant.

Fungi were grown on agar in 15-cm Petri plates. Basal salts medium was supplemented with 1% (w/v) xylose (Xyl), D-arabinose (D-Ara), L-arabinose (L-Ara), noxylan (Xyn), or corn cell walls (Ccw) as sole carbon source. Growth was measured after 4 days.

Effect of the *ARF1* and *ARF2* mutations on pathogenicity

Maize plants were infected with *C. carbonum* wild-type, *arf1* mutant and *arf1/arf2* double mutant. There was practically no difference between the plants inoculated with the wild type or the mutants (Figure 36). The wild type and the mutants were indistinguishable with regard to lesion size, color, and rate of lesion formation that they caused. Thus, *ARF1* and/or *ARF2* do not make a significant contribution to the virulence of *C. carbonum.*

DISCUSSION

The survival of a pathogens depends on its ability to have access to and utilize nutrients in the plant. The role played by the CWDEs is of interest because of their ability to degrade the cell wall polymers of host plants to provide materials that can be used for growth of the pathogen. The impetus behind this work was the observation that *C. carbonum* carrying a *ccsnf1* mutation has reduced virulence and does not express *ARF1*, a gene encoding arabinofuranosidase, suggesting that this enzyme may be important in virulence.

This study has shown that *C. carbonum* has three α-L-arabinofuranosidase activities and two are encoded by *ARF1* and *ARF2*. These enzymes can act together or substitute with each other in removing arabinose residues from side chains of cell wall xylans. Such redundancy in enzyme activities where two or more chromatographically

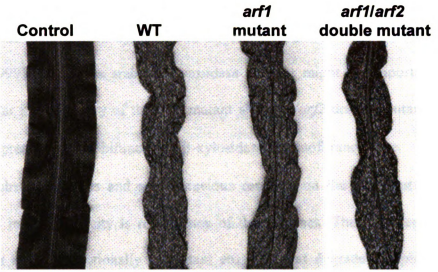

Figure 36. Pathogenicity Assay of *arf1* Mutant and *arf1*/*arf2* Double Mutant.

Plants of genotype *hm*/*hm* (inbred Pr) were inoculated with wild type, *arf1* mutant, and *arf1*/*arf2* double mutant spores.

separable proteins have similar enzymatic activities is common in *C. carbonum* and other fungi (Yao and Koller, 1995; Walton 1994). *C. carbonum* also secretes at least four endo-1,4-xylanases (Apel-Birkhold et al., 1996; Scott-Craig et al., unpublished) and three proteases (Murphy and Walton, 1996). From the position of elution compared to the major arabinofuranosidase peak, the smallest peak eluting at 26 to 28 min (Figure 29) which was also shown to have β-D-xylopyranosidase activity, corresponds to the β-xylosidase which has been previously described by Ransom and Walton (1997) and Wegener et al. (1999). Insofar as arabinofuranosidase activity might be important in virulence, the similar pathogenicity of the *arf1* mutant and *arf1/arf2* double mutant can be attributed to the presence of the bifunctional β-xylosidase/arabinofuranosidase.

The main obstacle of this and some previous research on the contribution of individual CWDEs in pathogenicity is redundancy of the enzymes. The occurrence of multiple genes that encode functionally redundant enzymes that degrade the physical barriers in the host is a common feature of fungal pathogens that infect plants. And this is probably why the single or sometimes multiple disruption mutants of *C. carbonum* can still grow and be pathogenic (Apel et al., 1993; Apel-Birkhold and Walton, 1996; Gorlach et al., 1998; Murphy and Walton, 1996; Scott-Craig et al., 1990, 1998; Wegener et al., 1999). The alternative requires the disruption of a regulatory gene that promotes the expression of several CWDE genes as demonstrated in our previous experiment in which the *ccSNF1* gene was knocked out resulting in reduced *C. carbonum* virulence on maize (Tonukari et al., 2000).

REFERENCES

Apel, P. C, Panaccione, D. G., Holden, F. R., and Walton, J. D. (1993). Cloning and targeted gene disruption of *XYL1*, a β-1,4-xylanase gene from the maize pathogen *Cochliobolus carbonum*. Mol. Plant-Microbe Interact. **6**, 467-473.

Apel-Birkhold, P. C., and Walton, J. D. (1996). Cloning, disruption, and expression of two endo-β-1,4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. Appl. Environ. Microbiol. **62**, 4129-4135.

Carpita, N.C., and Gibeaut, D.M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J. **3**, 1-30.

Celenza, J. L., and Carlson, M. (1984). Cloning and genetic mapping of *SNF1*, a gene required for expression of glucose-repressible genes in *Saccharomyces cerevisiae*. Mol. Cell Biol. **4**, 49-53.

Flipphi, M.J., van Heuvel, M., van der Veen, P., Visser, J., and de Graaff, L.H. (1993) Cloning and characterization of the *abfB* gene coding for the major alpha-L-arabinofuranosidase (ABF B) of *Aspergillus niger*. Curr. Genet. **24**, 525-532.

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

Gielkens, M., Gonzalez-Candelas, L., Sanchez-Torres, P., van de Vondervoort, P., de Graaff, L., Visser, J., and Ramon, D. (1999). The abfB gene encoding the major alpha-L-arabinofuranosidase of Aspergillus nidulans: nucleotide sequence, regulation and construction of a disrupted strain. Microbiology **145**, 735-741.

Gielkens, M.M., Visser, J. and de Graaff, L.H. (1997). Arabinoxylan degradation by fungi: characterization of the arabinoxylan-arabinofuranohydrolase encoding genes from *Aspergillus niger* and *Aspergillus tubingensis*. Curr. Genet. **31**, 22-29.

Gorlach, J.M., Van Der Knaap, E., and Walton, J.D. (1998). Cloning and targeted disruption of *MLG1*, a gene encoding two of three extracellular mixed-linked glucanases of *Cochliobolus carbonum*. Appl. Environ. Microbiol. **64**, 385-391.

Howell, H.E. (1975). Correlation of virulence with secretion in vitro of three wall-degrading enzymes in isolates of *Sclerotinia fructigena* obtained after mutagen treatment. J. Gen. Microbiol. **90**, 32-40.

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

Margolles-Clark, E., Tenkanen, M., Nakari-Setala, T., and Penttila, M. (1996). Cloning of genes encoding alpha-L-arabinofuranosidase and beta-xylosidase from *Trichoderma reesei* by expression in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **62**, 3840-3846.

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

Nielsen, H., Engelbrecht, J., Brunak, S., and G. von Heijne. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10**, 1-6.

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

Ransom, R.F., and Walton, J.D. (1997). Purification and characterization of extracellular β-xylosidase and α-arabinosidase from the plant pathogenic fungus *Cochliobolus carbonum*. Carbohydr. Res. **297**, 357-364.

Redenbach, M., Kieser, H.M., Denapaite, D., Eichner, A., Cullum, J., Kinashi, H. and Hopwood, D.A. (1996). A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. Mol. Microbiol. **2**, 77-96.

Rehnstrom, A.L., Free, S.J., and Pratt, R.G. (1994). Isolation, characterization and pathogenicity of *Sclerotinia trifoliorum* arabinofuranosidase-deficient mutants. Physiol. Mol. Plant Pathol. **44**, 199-206.

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

Scott-Craig, J.S., Cheng, Y.Q., Cervone, F., De Lorenzo, G., Pitkin, J.W., and Walton, J.D. (1998). Targeted mutants of *Cochliobolus carbonum* lacking the two major extracellular polygalacturonases. Appl. Environ. Microbiol. **64**, 1497-1503.

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

Sunna, A., and Antranikian, G. (1997). Xylanolytic enzymes from fungi and bacteria. Crit. Rev. Biotechnol. **17**, 39-67.

Sweigard, J. A., Chumley, F. G., and Valent, B. (1992). Disruption of a *Magnaporthe grisea* cutinase gene. Mol. Gen. Genet. **232,** 183-190.

Tonukari, N.J., Scott-Craig, J.S. and Walton, J.D. (2000) The *Cochliobolus carbonum SNF1* gene is required for cell wall-degrading enzyme expression and virulence on maize. Plant Cell **12**, 237-248.

Van Wert, S.L., and Yoder, O.C. (1992). Structure of the *Cochliobolus heterostrophus* glyceraldehyde-3-phosphate dehydrogenase gene. Curr. Genet. **22,** 29-35.

Vincent, P., Shareck, F., Dupont,C., Morosoli, R. and Kluepfel, D. (1997). New alpha-L-arabinofuranosidase produced by *Streptomyces lividans*: cloning and DNA sequence of the *abfB* gene and characterization of the enzyme. Biochem. J. **322**, 845-852.

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

Wegener, S., Ransom R.F., and Walton, J.D. (1999). A unique eukaryotic β-xylosidase gene from the phytopathogenic fungus *Cochliobolus carbonum*. Microbiology **145**, 1089-1095.

Chapter Five

Conclusion and future directions

CONCLUSION

Pathogenesis is a complex process and an understanding of the underlying biochemical mechanisms is dependent on the characterization of the fungal gene products that influence the progression of infection of the host. Successful penetration of living plant tissue by fungal pathogens is also preceded by an exchange of signals between both organisms and may depend on signaling pathways for fungal development and virulence (Knogge, 1998). A better understanding of pathogenicity will occur when the full cascade of signaling events between and within the fungal parasite and its host plant during penetration is uncovered. The application of molecular technologies to the study of fungal-plant interactions offers a new and more definitive approach for examining the virulence process and the role played by CWDEs in disease development. The softening of the plant cell walls for penetration by fungal hyphae, as well as provision of nutrients for growth are possible roles of CWDEs in plant disease development.

This work showed that full induction of CWDEs in *C. carbonum* requires a gene, *ccSNF1*, which is a structural and functional homolog of *S. cerevisiae SNF1*. The demonstration that the *C. carbonum ccsnf1* mutant makes less CWDEs and exhibits significantly reduced virulence strongly suggest the importance of these enzymes as virulence factors. The deficiencies in penetration of the plant surface by the *ccsnf1* mutant may have arisen because they are unable to secrete an adequate amount or the required threshold of CWDEs needed to degrade the cell walls of the maize epidermis. Nevertheless, partial induction of several of the CWDEs occurs in the *ccsnf1* mutant indicating that a *SNF1*-independent pathway also regulates induction.

Repression of xylanase genes was observed with glucose as the sole carbon source, whereas expression was seen when the culture medium contained xylan. The xylanase genes are substrate-induced, but differentially expressed. An ortholog of *CREA*, a gene encoding a carbon catabolite repressor, was cloned and consensus CreAp binding sites are present in *C. carbonum* CWDE genes.

Deletion of *ARF1* results in complete disappearance of a peak of α-L-arabinofuranosidase activity in culture filtrate fractionated by HPLC. Nevertheless, the mutant had more than 96 % of wild-type levels of total α-L-arabinofuranosidase activity and growth was normal on xylan but somewhat reduced on xylose, arabinose or corn cell walls. The disruption of a second arabinofuranosidase gene, *ARF2*, making an *arf1/arf2* double mutant, leads to the disappearance of the two major arabinofuranosidase activity peaks in culture filtrate fractionated by HPLC. Growth of the *arf1/arf2* double mutant was reduced in the above sugars, but more strongly on arabinose. Both the *arf1* mutant and *arf1/arf2* double mutant have similar virulence as the wild type *C. carbonum* but growth is reduced. The remaining activities was due to a bifunctional β-xylosidase/α-L-arabinofuranosidase (Ransom and Walton, 1997; Wegener et al., 1999) which seems adequate for virulence.

A major decrease in virulence may require disruption of genes that encode more than one class of the functionally redundant barrier-degrading enzymes. Therefore, emphasis of future research will be to consider all the important genes encoding the major enzymatic activity including xylanase, pectinase, β-xylosidase, α-L-arabinofuranosidase, and glucanase. The alternative requires the disruption of a regulatory gene that promotes the expression of several CWDE genes as demonstrated in

our previous experiment in which the *ccSNF1* gene was knocked out resulting in reduced *C. carbonum* virulence on maize.

SUGGESTIONS FOR FUTURE STUDIES

Effects of *SNF1* mutation

The phenomenon of glucose repression is concerned with the repression of a large number of genes when glucose is an abundant carbon source. *S. cerevisiae* can use several different sugars for growth, but it selectively ferments glucose when less desirable carbon sources are also available (Ronne, 1995). This is achieved by glucose down-regulation of the transcription of genes involved utilization of these alternate carbon sources. In fungi, the extracellular CWDEs are prominent among the glucose-repressed genes (Hensel and Holden, 1996).

It is possible that ccSnf1p regulates other genes, in addition to CWDEs, that are glucose repressed. Only a handful (fewer than 25) of *C. carbonum* genes have been isolated, and it is therefore not possible at present to know all the genes controlled by ccSnf1p. Nevertheless, such studies can be conducted in *S. cerevisiae* because sequencing of all its 16 chromosomes has been completed and nearly all the genes identified (http://genome-www.stanford.edu/Saccharomyces). Analysis of the global expression of all genes in *snf1* mutant (compared to wild type) in the presence and absence of glucose using the microarray technology (Lee and Lee, 2000; Schena et al., 1998; Shalon et al.,

1996) would provide valuable insights into the different genes regulated by Snf1p and their possible function in fungal activities.

Regulation of Snf1p

Regulation of gene expression is an important mechanism for adaptation to the nutritional environment and there is a complex signaling network that interconnects transduction pathways from sugars and nutrient signals (Sheen et al., 1999). Because glucose down-regulates fungal cell wall degrading enzymes, it may be that the fungi use glucose not only as nutrient but also as signal molecule. The mRNA of the enzyme that hydrolyzes sucrose into glucose and fructose, beta-fructosidase (invertase), accumulates in plants after pathogen attack (Sturm and Chrispeels, 1990). Sucrose is the main sugar that is transported in plants, and its hydrolysis during infection by the induced beta-fructosidase may have the effect of releasing large amounts of glucose, which, when absorbed by the fungus, would lead to repression of CWDEs. This may be a manner by which plants resist fungal pathogens. Snf1p, which is required for CWDE expression (Tonukari et al., 2000) is not active in the presence of glucose (Hardie, 1999).

Therefore, it is necessary that the specific factors that control Snf1p in filamentous fungi be identified and characterized, because any factor that inhibits Snf1p or its positive effector(s) may also inhibit the expression of CWDEs. Isolation of such factors as well as other components of the *C. carbonum* Snf1-CreA pathway (Figure 21) could be achieved by using the yeast two-hybrid screen with either Snf1p or CreAp as bait. A possible candidate is Snf4p which acts as a positive effector of the kinase activity

of Snf1. The yeast *snf4* mutant does not express invertase and cannot grow on glucose (Celenza and Carlson, 1989). Random mutagenesis using, for example, the restriction enzyme-mediated integration (REMI) (Bolker et al., 1995; Maier and Schafer, 1999; Riggle and Kumamoto, 1998), followed by screening transformants on replica plates of glucose and corn cell walls media, could also be used to find genes in the glucose repression pathway. Transformants that grew well on glucose and poorly on corn cell walls would be candidates for further characterization of the mutated gene.

Identification of Fungal Pathogenicity Genes

Targeted gene disruptions and deletions have allowed the generation of specific mutants defective in various properties that have been implicated in pathogenesis. Some of these factors, such as ccSnf1p (Tonukari et al., 2000), branched-chain-amino-acid transaminase (Cheng et al., 1999), alanine racemase (Cheng and Walton, 2000), fatty acid synthase (Ahn and Walton, 1997), and ToxEp (Ahn and Walton, 1998) have been demonstrated to be required for pathogenicity in *C. carbonum*. But others, like the individual CWDE genes, are not essential for *C. carbonum* virulence (Apel et al., 1993; Apel-Birkhold and Walton, 1996; Gorlach et al., 1998; Murphy and Walton, 1996; Scott-Craig et al., 1990, 1998; Wegener et al., 1999). Further studies in cloning and deletion of other genes predicted to be necessary for virulence in *C. carbonum* would further clarify our understanding of the nature of fungal pathogenicity. Complementary approaches for identification of pathogenicity genes involve the generation of random mutants with subsequent characterization of the induced mutations. These approaches do not require *a*

priori knowledge of gene function, and are likely to be of great value in the molecular dissection of phytopathogenicity. Useful mutants will include those that exhibit non- or reduced virulence, and in which the deleted gene encodes proteins or catalyzes the production of chemical substances that are secreted by the fungi and may have targets in the plant.

Proteins that inactivate CWDEs

Plants defend themselves against pathogens by other mechanisms, which can be constitutive or induced (Hunt et al., 1996). Chitinases and beta-1,3-glucanases, which are induced in response to infection, have received considerable attention due to their probable antimicrobial activity through hydrolysis of the fungal cell wall components, chitin and beta-1,3-glucans (Beffa and Meins, 1996; Collinge et al., 1993; Powell et al., 2000). Enzyme inhibitors such as protease inhibitors (Joshi et al., 1998) and specific polygalacturonase inhibitors (Mahalingam et al., 1999; Stotz et al., 1993; Yao et al., 1999) that target CWDEs may be useful antifungal agents. This interplay of invading CWDEs and cellular inhibitors may play a vital role in the spread of infection and host range.

Constitutive overexpression of a protein involved in plant defense is one strategy for increasing plant tolerance to fungal pathogens. Making the plant produce more of its natural protectants is one of the reigning paradigms in crop improvement (Grison et al., 1996; Shelton et al., 2000). Identification and overexpression of the CWDE inhibitor proteins in plants could provide a means for the development of plant varieties with

increased resistance to certain fungal pathogens. A useful approach that could be applied for the identification of CWDE-inhibitors would be screening for susceptible plants in a population of *A. thaliana* random mutants (Parinov and Sundaresan, 2000) using non-HC-toxin-producing strains of *C. carbonum*. The use of *A. thaliana* is suggested here because it is naturally resistant to *C. carbonum*, is a widely studied model plant, and a majority of its genes have been identified. This will reveal a variety of genes that make most plants naturally resistant to plant pathogens.

PERSPECTIVES

Although the goal of this study has been to understand the molecular mechanisms of plant fungal pathogenesis, the potential application of such knowledge to design plants that are resistant to fungi is of great importance. Moreover, as the use of chemical control of pathogenic fungi and other microorganisms become increasingly restricted, there is an urgent need to develop crop varieties with resistance to these pathogens. Achieving this goal will require identification and characterization of the genes involved in pathogenicity and host specificity so that such factors could be targeted for the development of efficient and durable disease control. To obtain a clearer picture of fungal pathogenesis, the mechanisms of susceptibility and resistance ought to be aggressively analyzed and well understood. Resistance to CWDEs may be part of the constitutive and/or acquired resistance that plants possesses against various pathogens. A broad characterization of the host defense apparatus will reveal the combination of factors that

give a pathogen the capacity to ignore or bypass host defenses. Hence it should be of biological interest to further explore why most plants are immune to a pathogen that can devastate other plants. From a biotechnology perspective, further research into the expression of more CWDE-inhibitors will be important in the development of better agronomic crops with increased resistance to fungal pathogens.

REFERENCES

Ahn, J.H., and Walton, J.D. (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**, 207-214.

Ahn, J.H., and Walton, J.D. (1998). Regulation of cyclic peptide biosynthesis and pathogenicity in *Cochliobolus carbonum* by TOXEp, a novel protein with a bZIP basic DNA-binding motif and four ankyrin repeats. Mol. Gen. Genet. **260**, 462-469.

Apel, P.C., Panaccione, D.G., Holden, F.R., and Walton, J.D. (1993). Cloning and targeted gene disruption of *XYL1*, a β-1,4-xylanase gene from the maize pathogen *Cochliobolus carbonum*. Mol. Plant-Microbe Interact. **6**, 467-473.

Apel-Birkhold, P.C., and Walton, J.D. (1996). Cloning, disruption, and expression of two endo-β-1, 4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. Appl. Environ. Microbiol. **62**, 4129-4135.

Beffa, R., and Meins, F. Jr. (1996). Pathogenesis-related functions of plant beta-1,3-glucanases investigated by antisense transformation--a review. Gene **179**, 97-103.

Bolker, M., Bohnert, H.U., Braun, K.H., Gorl, J., and Kahmann, R. (1995). Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). Mol. Gen. Genet. **248**, 547-552.

Celenza, J.L., and Carlson, M. (1989). Mutational analysis of the Saccharomyces cerevisiae SNF1 protein kinase and evidence for functional interaction with the SNF4 protein. Mol. Cell Biol. **9**, 5034-5044.

Cheng, Y.Q., Ahn, J.H., and Walton, J.D. (1999). A putative branched-chain-amino-acid transaminase gene required for HC-toxin biosynthesis and pathogenicity in *Cochliobolus carbonum*. Microbiology **145**, 3539-3546.

Cheng, Y.Q., and Walton, J.D. (2000). A eukaryotic alanine racemase gene involved in cyclic peptide biosynthesis. J. Biol. Chem. **275**, 4906-4911.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., and Vad, K. (1993). Plant chitinases. Plant J. **3**, 31-40.

Gorlach, J.M., Van Der Knaap, E., and Walton, J.D. (1998). Cloning and targeted disruption of *MLG1*, a gene encoding two of three extracellular mixed-linked glucanases of *Cochliobolus carbonum*. Appl. Environ. Microbiol. **64**, 385-391.

Grison, R,, Grezes-Besset, B., Schneider, M., Lucante, N., Olsen, L., Leguay, J.J., and Toppan, A. (1996). Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. Nat. Biotechnol. **14**, 643-646.

Hardie, D.G. (1999). Roles of the AMP-activated/SNF1 protein kinase family in the response to cellular stress. Biochem. Soc. Symp. **64**,13-27.

Hensel, M., and Holden, D. W. (1996). Molecular genetic approaches for the study of virulence in both pathogenic bacteria and fungi. Microbiology **142**, 1049-1058.

Hunt, M.D., Neuenschwander, U.H., Delaney, T.P., Weymann, K.B., Friedrich, L.B., Lawton, K.A., Steiner, H.Y., and Ryals, J.A. (1996). Recent advances in systemic acquired resistance research. Gene **179**, 89-95.

Joshi, B.N., Sainani, M.N., Bastawade, K.B., Gupta, V.S., and Ranjekar, P.K. (1998). Cysteine protease inhibitor from pearl millet: a new class of antifungal protein. Biochem. Biophys. Res. Commun. **246**, 382-387.

Knogge, W. (1998). Fungal pathogenicity. Curr. Opin. Plant Biol. **1**, 324-328.

Lee, P.S., and Lee, K.H. (2000). Genomic analysis. Curr. Opin. Biotechnol. **11**, 171-175.

Mahalingam, R., Wang, G., Knap, and H.T. (1999). Polygalacturonase and polygalacturonase inhibitor protein: gene isolation and transcription in *Glycine max-Heterodera glycines* interactions. Mol. Plant Microbe Interact. **12**, 490-498.

Maier, F.J., and Schafer, W. (1999). Mutagenesis via insertional- or restriction enzyme-mediated-integration (REMI) as a tool to tag pathogenicity related genes in plant pathogenic fungi. Biol. Chem. **380**, 855-864.

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

Parinov, S., and Sundaresan, V. (2000). Functional genomics in Arabidopsis: large-scale insertional mutagenesis complements the genome sequencing project. Curr. Opin. Biotechnol. **11**, 157-161.

Powell, A.L., van Kan, J., ten Have, A., Visser, J., Greve, L.C., Bennett, A.B., and Labavitch, J.M. (2000). Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant Microbe Interact. **13**, 942-950.

Ransom, R.F., and Walton, J.D. (1997). Purification and characterization of extracellular β-xylosidase and α-arabinosidase from the plant pathogenic fungus *Cochliobolus carbonum*. Carbohydr. Res. **297**, 357-364.

Riggle, P.J., and Kumamoto, C.A. (1998). Genetic analysis in fungi using restriction-enzyme-mediated integration. Curr. Opin. Microbiol. **1**, 395-399.

Ronne, H. (1995). Glucose repression in fungi. Trends genet. **11**, 12-17.

Schena, M., Heller, R.A., Theriault, T.P., Konrad, K., Lachenmeier, E., and Davis, R.W. (1998). Microarrays: biotechnology's discovery platform for functional genomics. Trends Biotechnol. **16**, 301-306.

Scott-Craig, J.S., Cheng, Y.Q., Cervone, F., De Lorenzo, G., Pitkin, J.W., and Walton, J.D. (1998). Targeted mutants of *Cochliobolus carbonum* lacking the two major extracellular polygalacturonases. Appl. Environ. Microbiol. **64**, 1497-1503.

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

Shalon, D., Smith, S.J., and Brown, P.O. (1996). A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Res. **6**, 639-645.

Sheen, J., Zhou, L.,and Jang, J.C. (1999). Sugars as signaling molecules. Curr. Opin. Plant Biol. **2**, 410-418.

Shelton, A.M., Tang, J.D., Roush, R.T., Metz, T.D., and Earle, E.D. (2000). Field tests on managing resistance to Bt-engineered plants. Nat. Biotechnol. **18**, 339-342.

Stotz, H.U., Powell, A.L., Damon, S.E., Greve, L.C., Bennett, A.B., and Labavitch, J.M. (1993). Molecular characterization of a polygalacturonase inhibitor from *Pyrus communis* L. cv Bartlett. Plant Physiol. **102**, 133-138.

Sturm, A., and Chrispeels, M.J. (1990). cDNA cloning of carrot extracellular beta-fructosidase and its expression in response to wounding and bacterial infection. Plant Cell **2**, 1107-1119.

Tonukari, N.J., Scott-Craig, J.S. and Walton, J.D. (2000). The *Cochliobolus carbonum SNF1* gene is required for cell wall-degrading enzyme expression and virulence on maize. Plant Cell **12**, 237-248.

Wegener, S., Ransom R.F., and Walton, J.D. (1999). A unique eukaryotic β-xylosidase gene from the phytopathogenic fungus *Cochliobolus carbonum*. Microbiology **145**, 1089-1095.

Yao, C., Conway, W.S., Ren, R., Smith, D., Ross, G.S., and Sams, C.E. (1999). Gene encoding polygalacturonase inhibitor in apple fruit is developmentally regulated and activated by wounding and fungal infection. Plant Mol. Biol. **39**, 1231-1241.