EXPLORING THE ROLE OF AUTOPHAGY AND CELL WALL DEGRADING ENZYMES IN THE LIFE CYCLE AND PATHOGENIC DEVELOPMENT OF THE BASIDIOMYCETE FUNGAL PLANT PATHOGEN *USTILAGO MAYDIS*

by

MARINA NADAL

(Under the Direction of Scott Gold)

ABSTRACT

The plant pathogen *Ustilago maydis,* of the Basidiomycota is responsible for corn smut disease and an important model organism for this fungal phylum. One of the main aims in our laboratory is to investigate the genetic determinants involved in the processes associated with *U. maydis* morphogenesis and pathogenic development. In the work reported here, I was particularly interested in addressing the roles of autophagy and the regulation of cell wall degrading enzymes (CWDE) as key controlling factors of *U. maydis* development and virulence. Using a reverse genetic approach and a combination of other techniques, we firstly showed here that the *U. maydis* autophagy related genes *atg1* and *atg8,* are associated with autophagy dependent processes. Deletion of *atg8* resulted in a lower survival capacity during carbon starvation conditions, abnormal morphogenesis, and most importantly severe reduction of virulence. Interestingly, Δ*atg1* strains yielded phenotypes similar to the Δ*atg8* strains but of lower magnitude. Surprisingly, *atg1* deletion in the Δ*atg8* background generated an additive phenotype.

We then focused on the study of *U. maydis* CWDE genes as potential virulence factors regulated by carbon source availability. Little is known about the mode that *U. maydis* employs

to penetrate and spread in the corn plant and CWDE involvement in these processes has been hypothesized but never thoroughly tested. CWDE genes in fungi are often subject to transcriptional glucose repression. In yeast, one of the main players controlling this process is SNF1, which encodes a protein kinase. *SNF1* homologue disruption in the phytopathogenic fungi *Fusarium oxysporum* and *Cochliobolus carbonum* led to a reduction in the expression of several CWDE genes accompanied by a decrease in virulence. In this work we showed that in *U. maydis* Snf1 acts as either a negative or positive regulator of particular CWDE genes and is not required for metabolism of alternative carbon sources. Unlike in Ascomycete plant pathogens, deletion of *snf1* did not profoundly affect virulence in *U. maydis.*

INDEX WORDS: autophagy, pathogenicity, budding, autophagosomes, galls, teliospores, nutrient stress, CWDE, glucose repression, pathogenicity, carbon utilization, morphogensis

EXPLORING THE ROLE OF AUTOPHAGY AND CELL WALL DEGRADING ENZYMES IN THE LIFE CYCLE AND PATHOGENIC DEVELOPMENT OF THE BASIDIOMYCETE FUNGAL PLANT PATHOGEN *USTILAGO MAYDIS*

by

MARINA NADAL

B.S., Universidad Nacional de La Plata, Argentina, 2001

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2009

EXPLORING THE ROLE OF AUTOPHAGY AND CELL WALL DEGRADING ENZYMES IN THE LIFE CYCLE AND PATHOGENIC DEVELOPMENT OF THE BASIDIOMYCETE FUNGAL PLANT PATHOGEN *USTILAGO MAYDIS*

by

MARINA NADAL

Major Professor: Scott Gold

Committee: Sarah Covert
Anthony Glenn
Michael Scanlon

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2009

DEDICATION

This is for my mother, Beatriz Siemieniuk, and my husband William Bunney who have sacrificed so much for me and continue to do so even now, and to whom this achievement might signify even more than it does to me.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Professor Scott Gold, for his guidance and assistance during the many years of my research. I would also like to thank the members of my committee for the time and effort they invested in me as a developing scientist. The University of Georgia and particularly the Department of Plant Pathology have my gratitude for the support they afforded me as I worked toward becoming the scientist I wish to be.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

The fungus *Ustilago maydis* is a member of the class Ustilaginomycetes within the phylum Basidiomycota (Hibbett, *et al.* 2007) and the causal agent of corn smut disease of maize. *U. maydis* is well established as a model organism for the study of pathogenicity, mating and the important phenomenon of fungal dimorphism. Among the assets that have made *U. maydis* such an excellent system are: the extensive repertoire of genetic tools, an easily cultivable haploid phase and importantly, its taxonomical proximity to other smut fungi, e.g. *Tilletia*, *Urocystis* as well as the rusts.

U. maydis is one of a small number of fungi that during their lifecycles experience a dimorphic switch that is tightly linked to the sexual development and pathogenic behavior (Banuett 1991, Bolker 2001, Nadal, *et al.* 2008). As a saprobe, *U. maydis* exists as a population of single haploid cells called "sporidia", which reproduce asexually by budding. As a pathogen, its thallus adopts the organization of a dikaryotic mycelium, dwelling within the tissues of maize, its exclusive host.

The first step toward *U. maydis* pathogenic development is the mating of two compatible haploid cells in the presence of the host to establish an infectious dikaryon that penetrates the plant. As *U. maydis* grows and colonized the plant tissues, it induces hypertrophy and hyperplasia of the maize cells (Bolker 2001). This re-programming of the plant cell cycle leads to the development of tumor-like structures, termed galls, the most remarkable symptom of corn smut disease. Although all above ground organs of maize are theoretically susceptible to gall

formation, in the field the galls develop most frequently and dramatically on the ears and generate the economical impact of the disease. My research has focused on two aspects of *U. maydis* biology as key factors influencing the development and pathogenic capacity of this fungus. One of my objectives has been to explore the process of autophagy in *U. maydis* as a potential crucial pathway acting to ensure survival during nutritional stress conditions as well as promoting those programmed developmental changes required to complete its life cycle. My other objective was the study of the *U. maydis snf1* gene's potential role as a key factor controlling glucose repression and cell wall degrading enzyme (CWDE) expression.

Autophagy is a central cellular degradative pathway that serves the eukaryotic cell to recycle cellular materials (Reggiori and Klionsky 2002). The pathway has been extensively studied and is best understood in the yeast *S. cerevisiae,* where the genetic elements have been identified and characterized (Kawamata, *et al.* 2008, Suzuki and Ohsumi 2007). In this yeast, autophagy primarily enables the yeast cells to endure periods of resource scarcity (Takeshige, *et al.* 1992). In addition to its role guaranteeing cellular adaptability to nutrient stress, autophagy is also an integral part of the developmental program in many species, facilitating the morphological changes that shape their bodies and allow completion of their life cycles. In filamentous *Aspergillus oryzae*, loss of autophagy leads to defects in conidiation and conidial germination (Kikuma, *et al.* 2006). Among the pathogenic fungi, appressorium development of the rice blast fungus *Maganporthe grisea* is tightly linked to autophagy (Veneault-Fourrey, *et al.* 2006). Degradation of three of the four nuclei within *M. grisea* germinating conidia is a prerequisite for full appressorium development and autophagy acts to ensure that nuclear collapse occurs. Autophagy deficient mutants of *M. grisea* strains are unable to complete appressoria development and to penetrate the plant surface, and therefore completely non pathogenic.

Most plant pathogens spend a great deal of their life dwelling and reproducing within the internal tissues of their host. The plant cell wall imposes a protective barrier to the external environment that pathogens must overcome if they are to succeed in colonizing their hosts. Many pathogenic fungi rely on the secretion of extracellular CWDE that can degrade cell wall elements and thus help in the penetration as well as the colonization of the host tissues (Walton 1994). Due to gene and enzymatic activity redundancy, the analysis of CWDE single gene deletion mutants is generally not an effective approach for addressing the importance of these enzymes as virulence factors. An interesting aspect of CWDE in fungi is that they are usually under transcriptional glucose repression. In *S. cerevisiae*, the *SNF1* gene is a key element controlling glucose repression, acting to release gene expression when glucose is depleted (Celenza and Carlson 1984). The *SNF1* gene encodes the catalytic α-subunit of a serine-threonine protein kinase that is activated when a drop in glucose level occurs. One of the principal targets of activated Snf1p is the DNA binding transcription repressor Mig1, which binds the promoter of several glucose repressed genes. When phosphorylated by Snf1p, Mig1p loses the ability to bind to the promoters of glucose repressed genes and is exported to the cytoplasm (Ahuatzi, *et al.* 2007, De Vit, *et al.* 1997). The disruption of the *SNF1* homologue in the phytopathogenic fungi *Fusarium oxysporum* and *Cochiobolus carbonum* leads to a reduction in the expression of several CWDE genes and the resulting strains exhibited a severe reduction in their virulence (Ospina-Giraldo, *et al.* 2003, Tonukari, *et al.* 2000).

The host penetration method of *U. maydis* is not quite clear; nor is it fully understood how the fungus proliferates inside the host tissue. Independent reports show that *U. maydis* might employ both mechanical and enzymatic approaches to penetrate and proliferate within its host

(Doehlemann, *et al.* 2008, Snetselaar and Mims 1994). However, no conclusive answers on the relevance of CWDE as a virulence factor have yet been obtained.

The long term goal of my research has been to improve our understanding of *U. maydis'* pathogenic nature and thus contribute to the development of strategies aimed at mitigating fungal diseases of important crops. To achieve this goal, my specific research objectives were:

1. To generate *U. maydis* autophagy deficient mutant strains and determine the role of this pathway in morphogenesis and pathogenic development.
2. To address the relevance of *U. maydis SNF1* homologue, *snf1*, as a regulator of CWDE expression.

References

Ahuatzi D., Riera A., Pelaez R., Herrero P. & Moreno F. (2007) Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. *J Biol Chem* **282**: 4485-4493.

Banuett F. (1991) Identification of genes governing filamentous growth and tumor induction by the plant pathogen Ustilago maydis. *Proc Natl Acad Sci USA* **88**: 3922-3926.

Bolker M. (2001) *Ustilago maydis*--a valuable model system for the study of fungal dimorphism and virulence. *Microbiology* **147**: 1395-1401.

Celenza J.L. & 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.

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

Doehlemann G., Wahl R., Vranes M., de Vries R.P., Kämper J. & Kahmann R. (2008) Establishment of compatibility in the Ustilago maydis/maize pathosystem. *Journal of Plant Physiology* **165**: 29-40.

Hibbett D.S., Binder M., Bischoff J.F., Blackwell M., Cannon P.F., Eriksson O.E., Huhndorf S., James T., Kirk P.M., Lucking R., Thorsten Lumbsch H., Lutzoni F., Matheny P.B., McLaughlin D.J., Powell M.J., Redhead S., Schoch C.L., Spatafora J.W., Stalpers J.A., Vilgalys R., Aime M.C., Aptroot A., Bauer R., Begerow D., Benny G.L., Castlebury L.A., Crous P.W., Dai Y.C., Gams W., Geiser D.M., Griffith G.W., Gueidan C., Hawksworth D.L., Hestmark G., Hosaka K., Humber R.A., Hyde K.D., Ironside J.E., Koljalg U., Kurtzman C.P., Larsson K.H., Lichtwardt R., Longcore J., Miadlikowska J., Miller A., Moncalvo J.M., Mozley-Standridge S., Oberwinkler F., Parmasto E., Reeb V., Rogers J.D., Roux C., Ryvarden L., Sampaio J.P., Schussler A., Sugiyama J., Thorn R.G., Tibell L., Untereiner W.A., Walker C., Wang Z., Weir A., Weiss M., White M.M., Winka K., Yao Y.J. & Zhang N. (2007) A higher-level phylogenetic classification of the Fungi. *Mycol Res* **111**: 509-547.

Kawamata T., Kamada Y., Kabeya Y., Sekito T. & Ohsumi Y. (2008) Organization of the pre-autophagosomal structure responsible for autophagosome formation. *Mol Biol Cell* **19**: 2039-2050.

Kikuma T., Ohneda M., Arioka M. & Kitamoto K. (2006) Functional analysis of the ATG8 homologue Aoatg8 and role of autophagy in differentiation and germination in Aspergillus oryzae. *Eukaryot Cell* **5**: 1328-1336.

Nadal M., Garcia-Pedrajas M.D. & Gold S.E. (2008) Dimorphism in fungal plant pathogens. *FEMS Microbiol Lett* **284**: 127-134.

Ospina-Giraldo M.D., Mullins E. & Kang S. (2003) Loss of function of the Fusarium oxysporum SNF1 gene reduces virulence on cabbage and Arabidopsis. *Curr Genet* **44**: 49-57.

Reggiori F. & Klionsky D.J. (2002) Autophagy in the eukaryotic cell. *Eukaryot Cell* **1**: 11-21.

Snetselaar K.M. & Mims C.W. (1994) Light and electron microscopy of Ustilago maydis hyphae in maize. *Mycological Research* **98**: 347-355

Suzuki K. & Ohsumi Y. (2007) Molecular machinery of autophagosome formation in yeast, Saccharomyces cerevisiae. *FEBS Lett* **581**: 2156-2161.

Takeshige K., Baba M., Tsuboi S., Noda T. & Ohsumi Y. (1992) Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J Cell Biol* **119**: 301-311.

Tonukari N.J., Scott-Craig J.S. & 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.

Veneault-Fourrey C., Barooah M., Egan M., Wakley G. & Talbot N.J. (2006) Autophagic fungal cell death is necessary for infection by the rice blast fungus. *Science* **312**: 580-583.

Walton JD (1994) Deconstructing the Cell Wall. *Plant Physiology* **104**: 1113-1118.

CHAPTER 2

LITERATURE REVIEW

The *U. maydis* life cycle is characterized by a dimorphic switch that the fungus experiences as it abandons its harmless saprobic lifestyle to adopt the more devastating parasitic one. Dimorphism is a widespread phenomenon among fungal pathogens, and it extends beyond the field of plant pathology (Nadal, *et al.* 2008). Like *U. maydis*, several human pathogenic fungi such as *Candida albicans* and *Blastomyces dermatitidis* also shift their morphologies when living as pathogens within their hosts.

During the saprobic phase of its life cycle, *U. maydis* exists as a population of haploid yeast cells, called "sporidia", that perpetuate themselves asexually by budding. Mating is the first step toward the pathogenic life style and *U. maydis* has a tetrapolar mating system controlled by the ***a*** and ***b*** loci. The ***a*** locus consists of two tightly linked genes: *mfa* and *pra* encoding a lipopeptide pheromone and a seven-transmembrane domain receptor, respectively (Spellig, *et al.* 1994). This system is involved in cell recognition and fusion and compatible sporidia must carry different alleles of both genes in order to mate (Banuett 1995, Bolker, *et al.* 1992, Gold, *et al.* 1994). The ***b*** locus also contains two tightly linked genes encoding two homeodomain proteins, bE and bW, which heterodimerize to form a transcription factor necessary to maintain a stable dikaryon within the plant (Brachmann, *et al.* 2001, Gillissen, *et al.* 1992). A functional bE/bW transcription factor is only established when bE and bW are derived from different *b* alleles (Kamper, *et al.* 1995). Therefore, successful mating and establishment of the stable infectious dikaryon requires compatible sporidia to differ at both mating type loci. During mating, plasmogamy takes place but karyogamy is delayed to a later developmental stage. Mitotic

division in the initial dikaryotic cell is suspended with the nuclei arrested at the G2 state while this cell elongates and eventually forms a poorly differentiated appressorium from which it penetrates the plant. Only inside the host does mitosis resume and the dikaryotic mycelium proliferates (Banuett and Herskowitz 1989) by the coordinated division of both independently inherited nuclei.

The colonization of the maize tissue by the dikaryon is characterized by early intracellular and later intercellular *in planta* growth. A remarkable characteristic of *U. maydis* intracellular growth is that as fungal hyphae push across the cells, they only penetrate the walls to invaginate the plasma membranes without piercing it. As a result, host cytoplasm is never in direct contact with the fungal hyphae and the plant cells are not killed immediately (Snetselaar and Mims 1994). In this aspect, *U. maydis* resembles other biotrophic fungi such as the economically important and less tractable rust species. Rust fungi inside their host produce a short yet highly specialized "feeding" organ, the haustorium (Mendgen and Hahn 2002, Mims, *et al.* 2002). Like the *U. maydis* intracellular hyphae, rust haustoria penetrate the host cell walls while leaving the plasma membrane intact to ensure the long life of the plant cell and the abundance of nutrients (Hahn and Mendgen 2001).

Within the galls, the dikaryotic hyphae proliferate, branch out and eventually experience a series of dramatic developmental changes that culminate with the differentiation of teliospores (Banuett and Herskowitz 1996). Kariogamy appears to occur during this stage of teliospore formation and the mature diploid teliospore is thought to be the environmentally resistant stage of the fungus, overwintering in the soil and plant debris. In spring the teliospore germinates, producing a promycelium into which the nucleus moves and undergoes meiosis, typically generating four haploid basidiospores, thus reinitiating the cycle.

Throughout its lifecycle, *U. maydis* is continuously challenged by a changing environment. Being able to perceive those changes and modify its cellular metabolism accordingly is critical for its success as a species.

A main strategy exploited by eukaryotic cells to cope with nutrient stress conditions is the recycling of their own cellular material through autophagy. During autophagy, double membrane vesicles called autophagosomes begin to form *de novo* in the cytoplasm, randomly engulfing nearby cytoplasmatic material (Cebollero and Reggiori 2009). Mature autophagosomes are then docked to the lytic compartment, vacuoles in fungi and plants or lysosomes in animals. Within these lytic compartments, the autophagosomes and their contents are recycled by resident hydrolases.

The pathway was initially described in *S. cerevisiae* as a cellular response to nutrient stress conditions and the study of autophagy has historically been limited to the morphological aspects. In recent years, many of the autophagy genes (*ATG* genes) have been identified in *S. cerevisiae* and since then, orthologs of the *ATG* genes have been identified and characterized in other organisms. Autophagy is now known to participate in a great number of developmental processes in organisms ranging from plants to humans. Within the Fungal Kingdom, autophagy was shown to be critical for *Aspergillus oryzae* conidiation and conidial germination (Kikuma, *et al.* 2006). Among the pathogenic fungi, appressorium development of the rice blast fungus *Maganporthe grisea* is tightly linked to autophagy (Veneault-Fourrey, *et al.* 2006). Degradation of three of the four nuclei of *M. grisea* germinating conidia is a prerequisite for full appressorium development and autophagy ensures that nuclear collapse occurs. Autophagy deficient mutants of *M. grisea* are unable to form haustoria capable of plant surface penetration and are therefore non-pathogenic.

Another important pathway determining cellular behavior based on environmental fluctuations is glucose repression. This mechanism is extensively employed by diverse organisms to ensure that no energy is wasted on the transcription of genes involved in the utilization of alternative carbohydrates when the preferred carbon source, glucose, is readily available in the environment (Carlson 1999, Gancedo 1998, Ronne 1995). A main element controlling glucose repression in *S. cerevisiae* and humans is the highly conserved serine/threonine protein kinase encoded by the *SNF1* and AMPK genes, respectively (Hedbacker and Carlson 2008). In *S. cerevisiae*, Snf1p is the catalytic α-subunit of the Snf1 heterotrimeric complex that also includes the activating γ-subunit Snf4p; and one of the three β-scaffolding subunits Sip1p, Sip2p or Gal83p (Vincent and Carlson 1999). One of the principal targets of the Snf1 complex is the DNA binding transcription repressor Mig1, which sits on the promoter of many genes preventing their transcription while glucose is present in the media. A drop in glucose level leads to Snf1p activation and Snf1 complex assembly. The phosphorylation of Mig1p by activated Snf1p prevents the repressor from binding to the promoters and promotes its export to the cytoplasm (Ahuatzi, *et al.* 2007, De Vit, *et al.* 1997, Smith, *et al.* 1999).

The mutation of *SNF1* homologues in the phytopathogenic ascomycete fungi, *Fusarium oxysporum* and *Cochiobolus carbonun*, resulted in a substantial reduction of the transcription of several CWDE genes (Ospina-Giraldo, *et al.* 2003, Tonukari, *et al.* 2000). These observations revealed the existence of glucose repression of CWDE genes in these fungi, controlled by the corresponding *SNF1* homologue. An important characteristic of the *SNF1* mutants is that they had reduced virulence, suggesting the role of CWDE as constituting an important virulence factor for these species in particular and for plant pathogenic fungi in general.

Within *S. cerevisiae*, an important connection has been established between the aforementioned pathways controlling nutritional regulation. When *SNF1* is deleted in this species, cells from the mutant strain are unable to trigger autophagy when faced with a stressful low nutrient environment. Research results indicate that *SNF1* control of autophagy is most likely achieved by positively regulating Atg1p or Atg13 or both, which are themselves involved in the early events of this pathway through the recruitment of other Atg proteins required for autophagosome formation (Wang, *et al.* 2001).

The present work aimed to characterize these two nutrient regulation mechanisms in the basidiomycetaous *U. maydis* in an effort to improve our understanding of its biology as a plant pathogen and potentially help to the development of the strategies for fungal disease control.

References

Ahuatzi D., Riera A., Pelaez R., Herrero P. & Moreno F. (2007) Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. *J Biol Chem* **282**: 4485-4493.

Banuett F. (1995) Genetics of Ustilago maydis, a fungal pathogen that induces tumors in maize. *Annu Rev Genet* **29**: 179-208.

Banuett F. & Herskowitz I. (1989) Different a alleles of Ustilago maydis are necessary for maintenance of filamentous growth but not for meiosis. *Proc Natl Acad Sci U S A* **86**: 5878-5882.

Banuett F. & Herskowitz I. (1996) Discrete developmental stages during teliospore formation in the corn smut fungus, Ustilago maydis. *Development* **122**: 2965-2976.

Bolker M., Urban M. & Kahmann R. (1992) The a mating type locus of U. maydis specifies cell signaling components. *Cell* **68**: 441-450.

Brachmann A., Weinzierl G., Kamper J. & Kahmann R. (2001) Identification of genes in the bW/bE regulatory cascade in Ustilago maydis. *Mol Microbiol* **42**: 1047-1063.

Carlson M. (1999) Glucose repression in yeast. *Curr Opin Microbiol* **2**: 202-207.

Cebollero E. & Reggiori F. (2009) Regulation of autophagy in yeast Saccharomyces cerevisiae. *Biochim Biophys Acta*.

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

Gancedo J.M. (1998) Yeast carbon catabolite repression. *Microbiol Mol Biol Rev* **62**: 334-361.

Gillissen B., Bergemann J., Sandmann C., Schroeer B., Bolker M. & Kahmann R. (1992) A two-component regulatory system for self/non-self recognition in Ustilago maydis. *Cell* **68**: 647-657.

Hedbacker K. & Carlson M. (2008) SNF1/AMPK pathways in yeast. *Front Biosci* **13**: 2408-2420.

Mims C.W., Rodriguez-Lother C. & Richardson E.A. (2002) Ultrastructure of the host-pathogen interface in daylily leaves infected by the rust fungus Puccinia hemerocallidis. *Protoplasma* **219**: 221-226.

Nadal M., Garcia-Pedrajas M.D. & Gold S.E. (2008) Dimorphism in fungal plant pathogens. *FEMS Microbiol Lett* **284**: 127-134.

Ospina-Giraldo M.D., Mullins E. & Kang S. (2003) Loss of function of the Fusarium oxysporum SNF1 gene reduces virulence on cabbage and Arabidopsis. *Curr Genet* **44**: 49-57.

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

Smith F.C, Davies S.P., Wilson W.A., Carling D. &Hardie D.G. (1999) The SNF1 kinase complex from Saccharomyces cerevisiae phosphorylates the transcriptional repressor protein Mig1p in vitro at four sites within or near regulatory domain 1. *FEBS Lett* **453**: 219-223.

Snetselaar K.M. & Mims C. (1994) Light and electron microscopy of Ustilago maydis. Infection hyphae and developing teliospores.

Mycological Research **98**.

Spellig T., Bolker M., Lottspeich F., Frank R.W. & Kahmann R. (1994) Pheromones trigger filamentous growth in Ustilago maydis. *Embo J* **13**: 1620-1627.

Tonukari N.J., Scott-Craig J.S. & 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.

Veneault-Fourrey C., Barooah M., Egan M., Wakley G. & Talbot N.J. (2006) Autophagic fungal cell death is necessary for infection by the rice blast fungus. *Science* **312**: 580-583.

Vincent O. & Carlson M. (1999) Gal83 mediates the interaction of the Snf1 kinase complex with the transcription activator Sip4. *EMBO J* **18**: 6672-6681.

Wang Z., Wilson W.A., Fujino M.A. & Roach P.J. (2001) Antagonistic controls of autophagy and glycogen accumulation by Snf1p, the yeast homolog of AMP-activated protein kinase, and the cyclin-dependent kinase Pho85p. *Mol Cell Biol* **21**: 5742-5752.

CHAPTER 3

THE AUTOPHAGY GENES *ATG8* AND *ATG1* AFFECT MORPHOGENESIS AND PATHOGENICITY IN *USTILAGO MAYDIS*

Nadal M. and Gold S. Submitted to *Molecular Plant Pathology*, 08/16/09.

Abstract

Autophagy is a complex degradative process in which cytosolic material including organelles are randomly sequestered within double membranous vesicles termed autophagosomes. In *Saccharomyces cerevisiae*, the autophagy genes *ATG1* and *ATG8* are crucial for autophagy induction and autophagosome assembly, respectively and their deletion impacts the autophagic potential of the corresponding mutant strains. The *ATG8* ortholog of *Aspergillus oryzae* is required for conidiation and conidial germination. In the rice blast fungus *Magnaporthe grisea,* deletion of *ATG1* or *ATG8* orthologs resulted in the loss of autophagy induction along with proper appresorium development, and consequent virulence loss The plant pathogen *Ustilago maydis,* of the Basidiomycota is responsible for corn smut disease and an important model organism for this fungal phylum.In the related human pathogenic fungus *Cryptococcus neoformans,* RNAi suppression of the Atg8 ortholog expression resulted in the lack of autophagy activity and loss of virulence. We were interested in the role of autophagy in development and virulence of *U. maydis*. Using a reverse genetic approach, we show that the *U. maydis ATG8* ortholog*, atg8,* is associated with autophagy dependent processes. Deletion of *atg8* drastically reduced the survival of *U. maydis* mutant strains during carbon starvation conditions, impacted morphogenesis, and greatly reduced virulence. To corroborate the role of autophagy in *U. maydis* development, we also deleted the *ATG1* ortholog, *atg1*.

Deletion of *atg1* yielded phenotypes similar to the Δ*atg8* strains but of lower magnitude. Notably,, *atg1* deletion in the Δ*atg8* background generated an additive phenotype.

Introduction

Autophagy is a well conserved cellular degradative pathway that allows eukaryotic cells to recycle cytoplasmic components as needed and eliminate obsolete proteins and organelles (Reggiori and Klionsky 2002). During autophagy, cytoplasmic material is randomly sequestered into double-membrane vesicles called autophagosomes, which are targeted to the lytic compartment, vacuole (fungi and plants) or lysosome (animals). After degradation by resident hydrolases, the contents are recycled.

Initially described as a cellular response to nutrient stress conditions, the study of autophagy has historically been limited to the descriptive analysis of its morphological aspects. However, in recent years many of the autophagy genes have been identified, primarily through extensive genetic screening of *S. cerevisiae* autophagy deficient mutants. Since then, orthologs of the autophagy *ATG* genes have been identified and characterized in other organisms. Autophagy has now been linked to a great variety of developmental processes, including: seed germination, leaf senescence, fungal conidiation, spore germination and appresorium development, among others (Bassham 2007, Iwamoto, *et al.* 2008, Kikuma, *et al.* 2007, Kikuma, *et al.* 2006, Liu and Lin 2008). In humans, the lack of proper autophagic activity is associated with several health disorders, ranging from tumor development and cellular aging to attenuated defense against pathogen invasion (Huang and Klionsky 2007, Sachdeva and Thompson 2008, Vellai 2009).

The induction of autophagy results in the *de novo* formation of double-membrane vesicles called autophagosomes. The first distinguishable step towards autophagosome formation is the

appearance of cup-shaped double-membrane structures referred to as isolating membranes, pre-autophagosomes or phagofores in the cytoplasm. These structures expand, randomly engulfing cytoplasmatic material, ultimately maturing into double-membrane vesicles. Autophagosomes are then docked to the lytic compartment and there their outer membranes fuse with the membrane of this organelle, releasing the inner, single-membrane vesicles, now termed autophagic bodies, into the lumen. Autophagic bodies are degraded by hydrolases present in the lumen of the lytic compartments and the building blocks of the molecules are then recycled (Suzuki and Ohsumi 2007).

The process of autophagosome formation is complex and requires the orchestrated actions of a subset of autophagic proteins. To date, 31 autophagy related genes (*ATG* genes) have been discovered and described in *S. cerevisiae* and 18 of them are specifically involved in autophagosome formation (Kawamata, *et al.* 2008, Klionsky, *et al.* 2003, Suzuki, *et al.* 2007). *ATG1* encodes a Ser/Thr protein kinase that functions early during autophagy induction through its association with the Atg13 and Atg17 proteins (Kabeya, *et al.* 2005, Kamada, *et al.* 2000). It is thought that the Atg1 complex is involved in the recruitment of other Atg proteins required for autophagosome assembly to a specific perivacuolar location known as the phagophore assembly site (PAS). Additionally, Atg1 kinase activity might be required for disassembly of the PAS once autophagosomes have formed (Cebollero and Reggiori 2009, Cheong, *et al.* 2008). Another key player is *ATG8*, encoding a ubiquitin-like protein (Ichimura, *et al.* 2000) required for autophagosome formation (Mizushima, *et al.* 1998). Atg8p is processed post-transcriptionally by the cystein protease encoded by *ATG4* (Kirisako, *et al.* 2000) and subsequently covalently linked to the phagophore phospholipid, phosphatidylethanolamine (PE) in a ubiquitin-like reaction catalyzed by the E1-like activating enzyme Atg7 and the E2-like conjugating enzyme Atg3

(Geng and Klionsky 2008, Ichimura, *et al.* 2000). It is thought that Atg8 bound to PE facilitates membrane hemifusion leading to autophagosome maturation (Nakatogawa, *et al.* 2007).

Within the Kingdom Fungi, several reports have addressed the role of autophagy in species other than *S. cerevisiae*. In *Aspergillus oryzae*, deletion of the *ATG8* ortholog leads to defects in conidiation and conidial germination (Kikuma, *et al.* 2006). In the rice blast fungi *Magnaporthe grisea,* deletion of the *ATG1* or *ATG8* orthologs resulted in the loss of autophagy induction, proper appresorium development and pathogenicity (Liu, *et al.* 2007, Veneault-Fourrey, *et al.* 2006). In the basidiomycetous human pathogen *C. neoformans*, RNAi silencing of the *ATG8* ortholog affects the ability of the fungus to trigger autophagy and resulted in the loss of virulence (Hu, *et al.* 2008).

Ustilago maydis is a plant pathogenic fungus that belongs to the phylum Basidiomicota and is responsible for corn smut disease. A central feature of *U. maydis* biology is its dimorphic switch, from a saprobic haploid yeast phase to a parasitic filamentous dikaryon (Banuett 1991, Bolker 2001, Nadal, *et al.* 2008). The first step toward pathogenic development is the mating of two compatible haploid cells, termed sporidia, on the plant surface to form a dikaryotic cell. Mitotic division in the initial dikaryotic cell is arrested while its tip elongates and eventually forms a poorly differentiated appressorium from which it penetrates the plant. Once inside the plant, mitosis resumes and the dikaryotic filament proliferates and branches within the host tissues (Banuett and Herskowitz 1989). Later, the dikaryotic filaments experience a series of dramatic developmental changes that culminate in the differentiation of teliospores (Banuett and Herskowitz 1996). We hypothesized that in *U. maydis* autophagy is critical for undergoing the developmental programs required to complete its life cycle. Based on previous reports regarding the critical role of *ATG8* in other pathogenic fungi, to test this hypothesis we initially deleted

atg8, the *ATG8* homolog in *U. maydis* and found that this gene is required for triggering autophagy during nutritional stress. Consistent with this result, the Δ*atg8* strains*'* ability to survive during carbon starvation conditions was drastically reduced. We also showed that *atg8* is required for the wild type budding pattern of haploid sporidia and its deletion resulted in an abnormal lateral budding phenotype. Most importantly, deletion of *atg8* resulted in a substantial decrease in *U. maydis* virulence accompanied by a reduction in teliospore production. In an effort to better characterize the process of autophagy in *U. maydis*, we subsequently deleted the *ATG1* ortholog, *atg1*. The phenotypes of Δ*atg1* mutants resembled those observed in Δ*atg8* strains but were less severe. However, double mutant Δ*atg1*Δ*atg8* strains exhibited a more pronounced attenuation of disease symptoms than did either single mutant strains, suggesting additive action.

Materials and Methods

Strains, media and growth conditions: *U. maydis* strains utilized in this study are listed in Table 3.1. Fungal cultures were grown on potato dextrose agar (PDA) or in potato dextrose broth (PDB) (Difco, Franklin Lakes, NJ). Nitrate minimal medium (Holliday 1974) without glucose (MM-C) was employed for gene expression experiments, survival assays and autophagy induction. MM-C employed for vacuolar evaluation of autophagosome accumulation was amended with 1mM PMSF (Sigma, St. Louis, MO) to inhibit autophagomose degradation by hydrolyses. *U. maydis* cultures for protoplast production were grown in yeast extract peptone sucrose medium (YEPS) (1% yeast extract, 2% bacto-peptone, 2% sucrose). *U. maydis* transformants were selected on YEPS medium amended with 1M sorbitol (YEPS-S) and either 3 μg/ml of carboxin (Gustafson, Mc Kinney, TX) or 150 μg/ml of hygromycin B (Calbiochem, San

Diego, CA). Fungal cultures were grown at 30°C, and for liquid cultures, agitation was 250 rpm. *Escherichia coli* DH5α cells were used for transformation during deletion construct assembly. Luria Bertani (LB)-medium containing 50 μg/μl kanamycin A (Research Products International Corp., Chicago, IL) was employed for selecting *E. coli* transformants. Mating assay were performed on complete medium containing 1% charcoal (Sigma, St. Louis, MO) (Holliday 1965).

Gene deletion: All *U. maydis* deletion mutants were generated using DelsGate methodology (Garcia-Pedrajas, *et al.* 2008). The *atg8* and *atg1* ORFs were completely replaced with DelsGate deletion constructs carrying the carboxin (*cbx*) or hygromycin (*hyg*) resistance selectable markers, respectively. Gene replacement was assessed by PCR followed by Southern blot hybridization. For each Southern blot hybridization, 5 μg of genomic DNA from wild type and either Δ*atg8* or Δ*atg1* strains was digested with *Eco*RI or *Eco*47III (New England Biolabs, Ipswich, MA), respectively and resolved on a 0.7% agarose gel. DNA was transferred overnight to Hybond XL (Amersham Pharmacia Biotech, Piscataway, NJ) nylon membrane in 0.4M NaOH and UV light cross-linked. For the *atg8* and *atg1* probes, 1kb of the corresponding gene 3' flank was amplified and DIG labeled using a Roche DIG-High Prime Labeling and Detection kit (Roche, Indianapolis, IN). Probe labeling and hybridization procedures were performed according to the manufacturer's instructions. Double Δ*atg1*Δ*atg8* mutants were generated by deleting *atg1* in a Δ*atg8* background and confirmed as above.

qRT-PCR quantification: Total RNA was extracted using a Spectrum Plant Total RNA kit (Sigma, St. Louis, MO). cDNA was synthesized using the SuperScript III First Strand Synthesis

System for RT-PCR (Invitrogen, Carlsbad, CA) using oligo-dT as primer and according to the manufacturer's recommendations. Transcript abundance was quantified by qRT-PCR using SYBR-GREEN methodology (BioRad, Hercules, CA) with gene specific primers designed through the Integrated DNA technologies (IDT) website (http://www.idtdna.com/Home/Home.aspx). Reactions were performed on a Cepheid SmartCycler I (Cepheid, Sunnyvale, CA). *atg1* and *atg8* transcript relative expressions levels were calculated according ΔCT calculations ($2^{-\Delta\Delta CT}$ method) (Pfaffl 2001) with primer pairs: atg1f: TCAACACTCTCGCAGAGACCCTTT and atg1r: TTCCCACCGTCATCTCAAAGAGCA, and atg8f: TCGGATCTCACTGTGGGCCAAT TT and atg8r: AACCATCCTCGTCCTTGTGCTCTT, respectively, and normalized to a reference cyclophilin gene, *cpr1* (um03726) using primers: ppif: ACGCCGATTCACTTCGTC and ppir: AACGACGATCCCTCGTAACCGAAA. Means of gene expression fold-increase and their corresponding standard error were calculated based on three biological replicates.

Electron microscopy: Cells were initially fixed in 2.5% glutaraldehyde in buffer (50 mM phosphate buffer, pH 7.0) at 4°C overnight, then fixed in 1% OsO_4 in buffer for 2 h at 4°C and left overnight en-bloc staining with 0.5% aqueous uranyl acetate. After fixation, cells were dehydrated in a graded ethanol series (25 to 100%) and incubated in 100% acetone for 10 minutes, twice. Cells were included in Spurr's resin (Electron Microscopy Sciences, Philadelphia) by serial gradient replacements (33%, 66% Spurr's in acetone, and 100% Spurr's) and incubated for 48 h at 60°C for polymerization. Thin sections were cut using an ultramicrotome (Reichert-Jung, Wien, Austria) equipped with a Diatome histoknife (Diatome AG, Biel, Switzerland), picked up on slot grids and allowed to dry. Samples were post-stained

with uranyl acetate and lead citrate, and viewed and photographed using an EM 902A transmission electron microscope (Zeiss, Oberkochen, Germany). For each strain, more than 100 cells were viewed. On average, 30 cells per strains were in the proper orientation for vacuole evaluation for the presence of autophagic bodies.

Starvation survival assay: Cultures were grown on PDB to stationary phase, harvested by centrifugation, washed with H_2O and resuspended in carbon starvation medium (MM-C). Samples were taken at the indicated times, diluted and plated on PDA. Colonies were counted after 3 days at 30ºC. Percentage of survival was estimated based on number of colonies formed at time 0. Mean and standard error of the percentage of surviving cells for each strain was calculated based on the two biological replicates.

Mating and pathogenicity analysis: Mating plate assays were used to determine mutant mating abilities. Indicated strains were grown over night in PDB and equal volumes of each of the mating strains were co-spotted on 1% charcoal-containing complete medium plates that were sealed with parafilm and incubated at room temperature in the dark for 24 h. White dikaryotic filaments indicated a successful mating reaction.

For pathogenicity tests, 7 day old Golden Bantam maize seedlings were co-inoculated with strain mixtures of 10^6 cells/ml. Plants were kept in a growth chamber with 16 h day at 28°C/8 h night at 20°C cycles. Symptom development was scored 7, 10 and 14 days after inoculation and each plant individually was assigned a disease rating based on the following disease scale: 0 no symptoms; 1: anthocyanin production and/or chlorosis; 2: small leaf galls; 3: small stem galls; 4: large stem galls; and 5: plant death (Gold and Kronstad, 1994). Disease index calculated as the

average disease rating. For each mutant strain considered, three independent biological replicates of pathogenicity tests were conducted. A non parametric statistical test was performed to simultaneously evaluate the differences among treatments (Shah and Madden 2004).

For teliospore isolation, the ears of Tom Thumb maize plants were inoculated with the same wild type and Δ*atg8* strain combinations used in the pathogenicity tests and at the same cell concentration. Nine plants per treatment were used and at least two ears per plant were inoculated. The experiment was repeated twice. Teliospore production was assessed after complete gall development (between 3 and 5 weeks, depending on particular treatment).

Results

Identification and deletion of *U. maydis ATG8* and *ATG1* orthologs.

In order to investigate potential roles of autophagy in *U. maydis* development, we sought to create mutant strains in which the pathway was no longer functional. Considering that *ATG8* and *ATG1* orthologs are essential for correct autophagic activity in organisms as distantly related as plants and fungi, we decided to delete the *U. maydis atg8* and *atg1* genes to generate the hypothesized autophagy deficient strains. The *U. maydis atg8* and *atg1* genes were identified using the *S. cerevisiae* Atg8 and Atg1 protein sequences, respectively to search the *U. maydis* genome using the BLAST-homology search algorithm (http://mips.gsf.de/genre/proj/ustilago).

A single highly related ($3e^{-54}$) *U. maydis* gene (um05567), named here *atg8*, encoding a 118 amino acid protein was identified. The *U. maydis* and *S. cerevesiae* Atg8 protein sequences shared 76% identity and 91% similarity. Alignment of the *U. maydis* Atg8 protein with other fungal, plant and human (LC3) Atg8 proteins showed a high degree of amino acid conservation, suggesting that um05567 indeed encodes the *U. maydis* Atg8 homolog (Fig.3.1). Residue G116

of *S. cerevisiae* Atg8 is of vital importance for protein function (Kirisako, *et al.* 2000) Examination of *U. maydis* the Atg8 sequence indicates that residue G116 and the sequence context in which it is located have been conserved (Fig. 3.1, asterisk).

The *U. maydis* gene identified as most closely related to *S. cerevisiea ATG1* was um06363 ($2.9e^{-83}$) (Fig. 3.2). The um06363 gene, named here *atg1*, encodes a predicted 990 amino acid serine threonine protein kinase (http://smart.embl-heidelberg.de/).

The Δ*atg8* and Δ*atg1* deletion strains were generated using the DelsGate methodology (Garcia-Pedrajas, *et al.* 2008) by complete replacement of the corresponding gene ORF with the plasmid sequences containing the carboxin (*cbx*) or hygromycin (*hyg*) resistant selectable marker, respectively. Several potential Δ*atg8* and Δ*atg1* mutants were identified by PCR screening of transformants. For each mating type background, we confirmed the deletion of *atg8* and *atg1* in at least two independent mutant strains by Southern blot (Fig. 3.3). Employing the same approach, double mutant Δ*atg1*Δ*atg8* strains were generated by deleting *atg1* in a Δ*atg8* background.

atg8 and _atg1_ are required for wild-type budding of haploid sporidia.

U. maydis wild-type sporidia divide by budding, with buds emerging at or near the tips of cigar-shaped cells. During the late exponential growth phase of liquid grown wild type cells apical, budding predominates (Fig. 3.4A). By the time wild-type cells reach the stationary phase, they have ceased to divide and virtually no emerging buds are observed (Fig. 3.4B and C). Like in the wild type, the Δ*atg8*, Δ*atg1* and Δ*atg1*Δ*atg8* strains also displayed a high degree of budding during exponential growth. However, in addition to buds emerging apically, cells from Δ*atg8*, Δ*atg1* and Δ*atg1*Δ*atg8* strains displayed frequent lateral budding (Fig. 3.4D, E and F).

During the exponential growth phase, the average percentage of Δ*atg8*, Δ*atg1* and Δ*atg1*Δ*atg8* cells with lateral buds were respectively 6%, 7% and 12%, more than fifteen-fold higher than the 0.4% of wild-type. As cultures reached stationary phase, the percentages of Δ*atg8*, Δ*atg1* and Δ*atg1*Δ*atg8* cells bearing lateral buds increased substantially to 25%, 10% and 29%, respectively, with Δ*atg8* and Δ*atg1*Δ*atg8* cells exhibiting more frequent lateral budding than did Δ*atg1* strains (Fig. 3.4B). Moreover, in some of the cells more than one lateral bud was present or the cell bore multiple apical buds. Additionally at that stage, approximately 1% and 6.5% of the Δ*atg8* and Δ*atg1* cells bore an apical bud, respectively. Similar results were obtained when several independent deletion mutant strains were observed. These results indicate that deletion of either *atg8* or *atg1* seriously affects the budding process of haploid *U. maydis.* Not only is the bud site selection altered, but there is a clear separation defect judging from the percentage of mutant cells still with attached buds at stationary phase, a stage at which complete mother-daughter cell detachment was observed in wild type.

atg8 and _atg1_ transcripts accumulated during carbon stress conditions.

In many organisms such as *S. cerevisiae* and *Arabidopsis thaliana,* the exposure of cells to nutrient stress conditions (low nitrogen or carbon) brings about a rapid accumulation of several *ATG* gene transcripts (Kirisako, *et al.* 1999, Rose, *et al.* 2006). To investigate if *U. maydis* behaves in a similar manner, we examined the levels of *atg8* and *atg1* transcripts by qRT-PCR as cells were starved for carbon. Wild-type cells grown in PDB to the exponential phase (O.D. ~ 0.4, approximately 10^7 cells/ml) were transferred to minimal medium lacking any carbon source (MM-C) and incubated for 8 h. cDNA was synthesized from RNA samples collected immediately before the shift to MM-C and then 30 min, 1 h, 2 h, 4 h and 8 h after transfer.

Transcript abundance was estimated relative to levels of transcripts right before cells were transferred to MM-C by qRT-PCR. As expected, an increased accumulation of *atg8* transcript was observed in wild-type *U. maydis* cells upon transfer to MM-C. After 4 and 8 h of carbon starvation, *atg8* transcript levels increased more than 13 and 25 times the initial value, respectively. A similar pattern of transcript accumulation but of reduced amplitude was observed for *atg1* (Fig. 3.5). These results indicated that as in other systems, nutrient stress conditions are sufficient to induce the transcript accumulation of *U. maydis* autophagy genes *atg8* and *atg1*.

atg8 deletion prevents vacuolar accumulation of autophagosomes in _U. maydis_.

When cells undergo autophagy, autophagic bodies tend to accumulate within the vacuole. Monitoring the accumulation of autophagic bodies is thus a reliable and standard method used to evaluate the autophagic activity within a cell (Baba, *et al.* 1994, Galluzzi, *et al.* 2009). In order to determine if *atg8* is important for autophagy in *U. maydis*, we evaluated the ability of the wild-type and Δ*atg8* strains to accumulate autophagic bodies in the vacuoles when cells were exposed to carbon stress conditions. Wild-type and Δ*atg8* cells were grown in PDB until they reached the exponential growth phase (O.D. ~ 0.4, approximately 10^7 cells/ml) and then transferred to minimal medium lacking a carbon source (MM-C) in the presence of the proteinase inhibitor PMSF (1mM). After 5 h of incubation in the MM-C, cells were collected by centrifugation and prepared for transmission electron microscopy. Transmission electron micrographs showed that wild-type cells had accumulated numerous autophagic bodies within their vacuoles (Fig. 3.6). On the contrary, no autophagic bodies were observed in the vacuoles of Δ*atg8* cells. These results indicate that carbon stress conditions are sufficient to trigger autophagy in *U. maydis* and that the *atg8* gene is necessary for proper autophagosome accumulation under these conditions.

atg8 and _atg1_ deletions affect _U. maydis_ survival during carbon starvation.

To determine if deletion of *atg1* and *atg8* affected the ability of *U. maydis* to survive under carbon starvation conditions, we analyzed the capacity of the wild-type, Δ*atg8*, Δ*atg*1and Δ*atg1*Δ*atg8* strains to survive in MM-C for a period of 5 days. The three mutant strains showed a severe loss of viability under carbon starvation when compared to wild-type cells (Fig. 3.7). After 3 days of incubation in MM-C on average only 8.5%, 16.5% and 7.5% of *atg8*, Δ*atg1* and Δ*atg1*Δ*atg8* cells survived, respectively, while more than 50% of the wild-type cells remained alive (Fig. 3.7). Moreover, by day 4 only 3% of cells from Δ*atg1* and none from Δ*atg8* and Δ*atg1*Δ*atg8* strains had survived but approximately 36% of wild-type cells remained viable (Fig. 3.7). Similar results were obtained when several independent deletion mutant strains were observed. These results clearly indicate that the autophagy genes *atg8* and *atg1* are required for survival of *U. maydis* during long-term exposure to conditions of carbon stress.

Mating is unaffected by _atg8_ and _atg1_ deletion.

In *U. maydis*, the mating of compatible haploid sporidia is a prerequisite for dikaryon establishment and pathogenic development. When compatible sporidia are co-spotted on charcoal plates, an initial dikaryotic cell is established that elongates at its tip. This reaction results in a "fuzzy" white colony that is easily distinguished from those in which mating did not occur. Mating reactions of compatible Δ*atg1*Δ*atg8* strains on charcoal mating plates were indistinguishable from that of compatible wild-type strains (Fig. S3.1). These results indicate that Δ*atg1*Δ*atg8* strains are competent to mate with comparable efficiency to wild-type strains. Likewise, mating of single Δ*atg8* or Δ*atg1* mutants was unaffected (data not shown).

***atg8* and *atg1* are required for complete symptom development during pathogenic growth.**

In order to test the pathogenic capacity of the Δ*atg8* strains, maize seedlings were co-inoculated with pair-wise combinations of compatible wild-type and Δ*atg8* strains and disease progression was monitored. Two sets of independent compatible mutants were tested for their virulence, and for each pair three biological replicates of the pathogenicity test were conducted. Results for 10 days after inoculation (dai) employing Δ*atg8* mutant strains MN8.1 and MN8.11 are summarized in Table 3.2**.** Similar results were obtained when independent compatible Δ*atg8* strains MN8.4 and MN8.16 were employed (data not shown). Results 14 and 21 dai reflected the same trend among the treatments (data not shown). Dikaryons formed between combinations of Δ*atg8* strains always resulted in less severe disease symptoms, reflected in the lower disease index, than did inoculations with other strain combinations. Moreover, by the end of the experiment (21 dai) plants inoculated with compatible Δ*atg8* strains had developed considerably fewer galls than those inoculated with any of the other treatments (Fig. 3.8). These results clearly indicate that the *atg8* gene of *U. maydis* is required for full symptom development during infection of maize seedlings.

To evaluate the importance of *atg1* gene during *U. maydis* pathogenic development, we conducted pathogenicity tests with the Δ*atg1* strains, MN12.1 and MN29.4 in the same manner as for the Δ*atg8* strains. The pathogenicity test results showed that the Δ*atg1* mutant strains are slightly less virulent than wild type with no substantial reduction in the number of galled plants observed (Table 3.3). However, when plants were co-inoculated with a mixture of compatible Δ*atg1*Δ*atg8* double mutant strains, MN81.5 and MN81.0, the disease symptoms were drastically reduced, to an even greater extent than infection with compatible Δ*atg8* strains. Remarkably,

Δ*atg1*Δ*atg8* dikayons completely failed to induce galls (Table 3.4 and Fig. 3.8). Similar results were obtained when independent compatible Δ*atg1*Δ*atg8* strains MN81.8 and MN81.10 were employed (data not shown).

Deletion of *atg8* affects gall formation and teliospore production in ears of mature maize.

We inoculated the ears of maturing maize plants with the same strain combinations utilized in the pathogenicity test to better evaluate gall development and teliospore production. We used 9 plants per treatment and at least 2 ears per plant were inoculated. Three weeks after inoculation, numerous galls had formed on the ears of plants co-inoculated with wild-type strains or with combinations of compatible wild-type and Δ*atg8* strains. Of the 9 plants inoculated with compatible Δ*atg8* strains, one possessed a single ear in which a few very immature galls were present while the remaining 8 plants were gall free. The galls in this single ear lacked the characteristic dark coloration indicative of massive teliospore production and very few black teliospores were present (Fig. 3.9, arrow). Plants co-inoculated with any other strain combination bore numerous ears in which abundant teliospores developed (Fig. 3.9). This experiment was repeated twice with similar results.

In order to investigate if the few teliospores produced in the plant co-inoculated with Δ*atg8* strains were capable of germination and meiosis, we observed the segregation of the mating type locus of the progeny. Analysis of 20 teliospore haploid progeny isolated from the plants co-inoculated with these strains included descendents with the parental mating types, *a1b1* and *a2b2* (4 and 3, respectively) as well as the recombinants *a1b2* and *a2b1* (5 and 8, respectively). The frequency of recombinants was similar to the parents *a1b1* and *a2b2* and indicated that meiosis

did take place. These results indicate that teliospore formation in Δ*atg8* strains is severely compromised but that those that do form are functional.

Discussion

Historically recognized as a cellular nutrient stress adaptation mechanism, autophagy has more recently been associated with developmental events in a great variety of species (Levine and Klionsky 2004). When challenged with a low-nutrient environment, eukaryotic cells undergo adaptations to survive while stressful conditions prevail. In many species, this is attained by the recycling of cytosolic components through the autophagic pathway. This "self-eating" process helps the cell to survive while external resources remain unavailable. However, prolongation of the nutrient stress conditions will inevitably result in cell death. In recent years, a rich body of compelling evidence identified autophagy as an integral part of the cellular machinery that facilitates the programmed developmental changes that occur during organ or tissue remodeling in many organisms (Mizushima 2007). In this work, we have explored the process of autophagy in the plant pathogenic fungus *U. maydis.* Using a reverse genetic approach, we were able to show that a tight connection exists between autophagy and several aspects of *U. maydis* biology.

We identified *U. maydis* autophagy genes, *atg8* and *atg1*, as unique homologs of the well characterized *S. cerevisiae ATG8* and *ATG1* genes, respectively, based on sequence similarity. In *S. cerevisiae,* Atg8 is posttranslationally modified at its C-terminus by cystein protease Atg4 to generate $Atg8^{G116}$. The resulting $Atg8^{G116}$ is then covalently bound to the lipid PE in an ubiquitin-like reaction catalyzed by Atg7 and Atg3 (Ichimura, *et al.* 2000, Kirisako, *et al.* 2000). Examination of the protein sequence indicates that residue G116 and the sequence context in

which it is located has been conserved in *U. maydis* Atg8, reinforcing the notion that *atg8* is indeed, the functional ortholog of *ATG8.*

When exploring the *U. maydis* genome in search of the *ATG1* homolog, several genes were identified that encode proteins related to *ATG1*. Based on sequence similarity to *S. cerevisiae ATG1*, we established that *U. maydis atg1* was gene um06363. Supporting the hypothesis of um06363 being a unique *atg1* ortholog is the fact that the second most similar protein was the well characterized PKA catalytic subunit, Adr1 encoded by um04456 ($1.4e^{-28}$). In addition to um06363 and um04456, other *U. maydis* genes encoding proteins related to Ser/Thr kinase showed some degree of similarity to *S. cerevisiae ATG1*. However when the BLAST search was performed using the *S. cerevisiea* Atg1 protein sequence from which the kinase domain was removed, only *U. maydis* gene um06363 was identified as the homolog. Therefore, these observations strongly suggest that *atg1* is the *U. maydis* true ortholog of the *S. cerevisiae ATG1* gene.

Because *atg8* and *atg1* are required for normal autophagic activity in many other systems, it is very likely that the phenotypes observed in Δ*atg8* and Δ*atg1* as well as Δ*atg1*Δ*atg8* cells result from failure to properly trigger this pathway during budding and pathogenic growth. The observations made with the transmission electron microscope clearly indicate that *atg8* is required for autophagosome formation and its deletion seriously impaired autophagic activity when cells were challenged with a low nutrient environment.

While living as a saprobe, *U. maydis* exists as haploid yeast termed sporidia, which divide by apical budding. During exponential growth of *U. maydis* in culture, sporidia actively divide with new buds typically emerging at or near the tip of the cell. Very occasionally, wild type cells bud laterally from the middle of the mother cell rather than at the tip. At the stationary phase, wild-

type cells cease to divide and complete detachment of the daughter from mother cells occurs. Thus in wild-type cultures, an exponential growth phase of actively budding cells precedes a stationary phase in which cells no longer divide and no buds are observed.

The deletion of autophagy genes, *atg8* or *atg1,* alters the budding process in *U. maydis.* In the early exponential phase when resources are not limiting, Δ*atg8* and Δ*atg1* cells actively divide by budding at their tips but additionally, frequently bud laterally. During the exponential growth phase, Δ*atg8,* Δ*atg1* and Δ*atg1*Δ*atg8* showed at least fifteen-fold higher frequency of lateral buds than did wild type. As cultures reached stationary phase, the percentages of the mutant cells bearing lateral buds increased substantially. Additionally at that stage and opposed to wild type, some of the Δ*atg8* and Δ*atg1* cells still bore an apical bud as well. Although the Δ*atg1* mutant strains displayed this abnormal budding phenotype, at stationary phase the frequency of Δ*atg1* cells with lateral buds was lower than that observed in the Δ*atg8* or Δ*atg1*Δ*atg8* cells. The fact that at stationary phase many of the Δ*atg8,* Δ*atg1* and Δ*atg1*Δ*atg8* cells still carried buds indicated a detachment defect. Thus, the increased number of lateral buds in the mutant cells appears to be the result of a combination of two defects. The *atg8* and *atg1* deletions did not only alter the site of bud emergence but also negatively affected the process of mother-daughter cell separation.

In *U. maydis,* when haploid sporidia are faced with carbon depletion the induction of autophagy is evident by the accumulation of autophagic bodies within the cell vacuoles and the induction of at least two autophagy related genes, *atg1* and *atg8*. The absence of autophagic bodies from vacuoles of carbon starved Δ*atg8* mutant cells suggested that autophagy activity was lost. Moreover, this lack of normal autophagic activity is consistent with the reduced survival capacity of both Δ*atg8* and Δ*atg1* mutants under conditions of nutrient stress.

When living parasitically, *U. maydis* grows as a branching filamentous dikaryon within the maize plant. Only within the host does sexual reproduction occur and therefore, pathogenic development is inexorably linked to its life cycle. Because nutrient availability is generally limited in the host plant, the ability of the fungus to trigger autophagy might be crucial for its overall pathogenic development. From the time when two haploid sporidia mate on the plant surface until a competent infection filament reaches the interior of the maize tissue, cells are likely to experience extreme nutrient deprivation, and must rely on their stored energy supplies and the recycling of their macromolecules for securing the components needed to continue cellular activities. Because the plant tissue itself might represent a relatively nutrient-poor environment, even in later stages of infection, *U. maydis* might continue to depend on the ability to mobilize and recycle its own cellular material for completing its life cycle. Therefore, an important point when considering *U. maydis* pathogenic development is that the ability to trigger autophagy might be crucial during the early as well as later stages of plant infection when nutrient availability is most likely limiting.

As expected, we observed a reduction in virulence in Δ*atg8,* Δ*atg1* and Δ*atg1*Δ*atg8* strains compared to wild type infections. Interestingly, the reduced virulence phenotypes displayed by each of these mutants reflected the same trend observed with regard to altered bud site selection and cell separation in budding pattern. Both, virulence reduction and altered budding pattern were greater in Δ*atg8* than in Δ*atg1* strains. But when *atg1* was deleted in Δ*atg8* strains, the resulting Δ*atg1*Δ*atg8* double mutant virulence was even lower than Δ*atg8* single mutants to the point that gall formation was completely suppressed.

A particularly important aspect of *U. maydis* pathogenic development in light of our observations is that teliospore production involves drastic remodeling of the fungal hyphae.

Firstly, intracellular hyphae ramify profusely within the tumor cells and then begin to fragment while karyogamy takes place within individual cells. Later in development, hyphal fragments round-up and a sculpted, darkly pigmented cell wall is deposited around cytoplasmic regions containing single diploid nuclei. Our results suggest that the reduction and loss of gall formation, respectively, during infection of the Δ*atg8* and Δ*atg1*Δ*atg8* strains are due to a lack of autophagic activity during tumor development. Furthermore, the relatively low number of teliospores produced in the few Δ*atg8* galled ears indicates a significant role for autophagy in the sporulation process.

Our interpretation is that the *atg8* and *atg1* genes are necessary for the full integrity of the autophagy pathway during budding of haploid wild type sporidia and dikaryotic pathogenic development of *U. maydis.* The *atg8* gene plays a crucial role by ensuring full autophagic activity. On the other hand, the *atg1* gene appears to play a less significant role, likely because its deletion does not dramatically impair the pathway. The role of *atg1* becomes more apparent in Δ*atg8* strains, suggesting an additive effect. The simultaneous deletion of *atg8* and *atg1* likely affected two, not fully co-dependent stages of the pathway and thus resulted in the additive phenotype observed in the double Δ*atg1*Δ*atg8* mutant. Alternatively, Atg8 or Atg1 or both could have additional roles in independent pathways other than autophagy, controlling budding and pathogenic development in *U. maydis.*

It has been suggested that many remodeling steps that occur during development are triggered by nutrient starvation and that autophagy facilitates nutrient recycling during these events (Mizushima 2007). When mutations that impair autophagy are introduced in diploid *S. cerevisiae* strains, nutrient stress induced sporulation is blocked (Tsukada and Ohsumi 1993). In the social amoeba species *Dictyostelium discoideum,* the same kind of mutations lead to a

differentiation lacking fruiting bodies, a process regularly induced by starvation, high temperatures or overcrowding (Otto, *et al.* 2003). In the filamentous ascomycetous fungus, *A. oryzae*, deletion of the Aoatg8 gene results in several developmental defects including a loss of conidiation and conidial germination (Kikuma, *et al.* 2007). As in these organisms, the sporulation phenotypes of Δ*atg8* and Δ*atg1*Δ*atg8* strains could be explained by autophagy playing a critical role in nutrient mobilization during teliospore development in *U. maydis*.

In many organisms including insects and humans, autophagy is directly involved in the programmed cell death that takes place during developmental changes which are not necessarily triggered by starvation (Baehrecke 2003, Baehrecke 2002). In *Drosophila melanogaster*, the steroid hormone 20-hydroxyecdysone triggers synchronous autophagic cell death of the larval salivary gland cells during organ development (Baehrecke 2003). Therefore, a role of autophagic programmed cell death during teliospore formation cannot be ruled out. Further experiments need to be conducted to determine precisely what role autophagy plays during gall and teliospore development in *U. maydis*.

An important aspect of autophagy in the context of this work is its connection to glycogen accumulation (Wang, *et al.* 2001). Glycogen is a widespread energy storage molecule in fungi and animals and autophagy deficient mutants of *S. cerevisiae* experience a reduction in its accumulation. In *Ustilago nuda*, a species closely related to *U. maydis*, glycogen along with lipids constitutes the main energy reserve of teliospores (Van Laere and Fransen 1989). In *M. grisea*, conidial glycogen storage is mobilized during germination to the developing appressorium. This seems to contribute to the production of the high glycerol concentration associated with the turgor pressure needed during this organ's full development (Thines, *et al.*

2000). Therefore, the reduced virulence of *U. maydis* mutant strains could potentially result from a defect in glycogen metabolism associated with a lack of autophagic activity.

In this work we present substantial evidence to support the hypothesis that *U. maydis* autophagy genes *atg8* and *atg1* are important for several aspects of *U. maydis* biology, including proper budding of haploid sporida, nutrient starvation survival and pathogenic development. Most importantly, *atg8* and *atg1* are required for *U. maydis* to exist as an effective pathogen in the relatively low nutrient environment of the plant host.

References

Baba, M., Takeshige, K., Baba, N. and Ohsumi, Y. (1994) Ultrastructural analysis of the autophagic process in yeast: detection of autophagosomes and their characterization. *J Cell Biol*, **124**, 903-13

Baehrecke, E. H. (2003) Autophagic programmed cell death in Drosophila. *Cell Death Differ*, **10**, 940-5

Baehrecke, E. H. (2002) How death shapes life during development. *Nat Rev Mol Cell Biol*, **3**, 779-87

Banuett, F. (1991) Identification of genes governing filamentous growth and tumor induction by the plant pathogen Ustilago maydis. *Proc Natl Acad Sci U S A*, **88**, 3922-6

Banuett, F. and Herskowitz, I. (1989) Different a alleles of Ustilago maydis are necessary for maintenance of filamentous growth but not for meiosis. *Proc Natl Acad Sci U S A*, **86**, 5878-5882

Banuett, F. and Herskowitz, I. (1996) Discrete developmental stages during teliospore formation in the corn smut fungus, Ustilago maydis. *Development*, **122**, 2965-76

Bassham, D. C. (2007) Plant autophagy--more than a starvation response. *Curr Opin Plant Biol*, **10**, 587-93

Bolker, M. (2001) Ustilago maydis--a valuable model system for the study of fungal dimorphism and virulence. *Microbiology*, **147**, 1395-401

Cebollero, E. and Reggiori, F. (2009) Regulation of autophagy in yeast Saccharomyces cerevisiae. *Biochim Biophys Acta*,

Cheong, H., Nair, U., Geng, J. and Klionsky, D. J. (2008) The Atg1 kinase complex is involved in the regulation of protein recruitment to initiate sequestering vesicle formation for nonspecific autophagy in Saccharomyces cerevisiae. *Mol Biol Cell*, **19**, 668-81

Doehlemann, G., van der Linde, K., Assmann, D., Schwammbach, D., Hof, A., Mohanty, A., Jackson, D. and Kahmann, R. (2009) Pep1, a secreted effector protein of Ustilago maydis, is required for successful invasion of plant cells. *PLoS Pathog*, **5**, e1000290

Galluzzi, L., Aaronson, S. A., Abrams, J., Alnemri, E. S., Andrews, D. W., Baehrecke, E. H., Bazan, N. G., Blagosklonny, M. V., Blomgren, K., Borner, C., Bredesen, D. E., Brenner, C., Castedo, M., Cidlowski, J. A., Ciechanover, A., Cohen, G. M., De Laurenzi, V., De Maria, R., Deshmukh, M., Dynlacht, B. D., El-Deiry, W. S., Flavell, R. A., Fulda, S., Garrido, C., Golstein, P., Gougeon, M. L., Green, D. R., Gronemeyer, H., Hajnoczky, G., Hardwick, J. M., Hengartner, M. O., Ichijo, H., Jaattela, M., Kepp, O., Kimchi, A., Klionsky, D. J., Knight, R. A., Kornbluth, S., Kumar, S., Levine, B., Lipton, S. A., Lugli, E., Madeo, F., Malorni, W., Marine, J. C., Martin, S. J., Medema, J. P., Mehlen, P., Melino, G., Moll, U. M., Morselli, E., Nagata, S., Nicholson, D. W., Nicotera, P., Nunez, G., Oren, M., Penninger, J., Pervaiz, S., Peter, M. E., Piacentini, M., Prehn, J. H., Puthalakath, H., Rabinovich,

G. A., Rizzuto, R., Rodrigues, C. M., Rubinsztein, D. C., Rudel, T., Scorrano, L., Simon, H. U., Steller, H., Tschopp, J., Tsujimoto, Y., Vandenabeele, P., Vitale, I., Vousden, K. H., Youle, R. J., Yuan, J., Zhivotovsky, B. and Kroemer, G. (2009) Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. *Cell Death Differ*,

Garcia-Pedrajas, M. D., Nadal, M., Kapa, L. B., Perlin, M. H., Andrews, D. L. and Gold, S. E. (2008) DelsGate, a robust and rapid gene deletion construction method. *Fungal Genet Biol*, **45**, 379-88

Geng, J. and Klionsky, D. J. (2008) The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. *EMBO Rep*, **9**, 859-64

Holliday, R. (1965) Induced Mitotic Crossing-over in Relation to Genetic Replication in Synchronously Dividing Cells of Ustilago Maydis. *Genet Res*, **10**, 104-20

Holliday, R. (1974) *Ustilago maydis*. *In Handbook of Genetics (ed. R. C. King)*, 575–95

Hu, G., Hacham, M., Waterman, S. R., Panepinto, J., Shin, S., Liu, X., Gibbons, J., Valyi-Nagy, T., Obara, K., Jaffe, H. A., Ohsumi, Y. and Williamson, P. R. (2008) PI3K signaling of autophagy is required for starvation tolerance and virulenceof Cryptococcus neoformans. *J Clin Invest*, **118**, 1186-97

Huang, J. and Klionsky, D. J. (2007) Autophagy and human disease. *Cell Cycle*, **6**, 1837-49

Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda, T. and Ohsumi, Y. (2000) A ubiquitin-like system mediates protein lipidation. *Nature*, **408**, 488-92

Iwamoto, J., Takeda, T., Matsumoto, H., Sato, Y. and J, K. Y. (2008) Beneficial effects of combined administration of alendronate and alfacalcidol on cancellous bone mass of the tibia in orchidectomized rats: a bone histomorphometry study. *J Nutr Sci Vitaminol (Tokyo)*, **54**, 11-7

Kabeya, Y., Kamada, Y., Baba, M., Takikawa, H., Sasaki, M. and Ohsumi, Y. (2005) Atg17 functions in cooperation with Atg1 and Atg13 in yeast autophagy. *Mol Biol Cell*, **16**, 2544-53

Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M. and Ohsumi, Y. (2000) Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol*, **150**, 1507-13

Kawamata, T., Kamada, Y., Kabeya, Y., Sekito, T. and Ohsumi, Y. (2008) Organization of the pre-autophagosomal structure responsible for autophagosome formation. *Mol Biol Cell*, **19**, 2039-50

Kikuma, T., Arioka, M. and Kitamoto, K. (2007) Autophagy during conidiation and conidial germination in filamentous fungi. *Autophagy*, **3**, 128-9

Kikuma, T., Ohneda, M., Arioka, M. and Kitamoto, K. (2006) Functional analysis of the ATG8 homologue Aoatg8 and role of autophagy in differentiation and germination in Aspergillus oryzae. *Eukaryot Cell*, **5**, 1328-36

Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T. and Ohsumi, Y. (1999) Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J Cell Biol*, **147**, 435-46

Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T. and Ohsumi, Y. (2000) The reversible modification regulates

the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J Cell Biol*, **151**, 263-76

Klionsky, D. J., Cregg, J. M., Dunn, W. A., Jr., Emr, S. D., Sakai, Y., Sandoval, I. V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M. and Ohsumi, Y. (2003) A unified nomenclature for yeast autophagy-related genes. *Dev Cell*, **5**, 539-45

Levine, B. and Klionsky, D. J. (2004) Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*, **6**, 463-77

Liu, X. H. and Lin, F. C. (2008) Investigation of the biological roles of autophagy in appressorium morphogenesis in Magnaporthe oryzae. *J Zhejiang Univ Sci B*, **9**, 793-6

Liu, X. H., Lu, J. P., Zhang, L., Dong, B., Min, H. and Lin, F. C. (2007) Involvement of a Magnaporthe grisea serine/threonine kinase gene, MgATG1, in appressorium turgor and pathogenesis. *Eukaryot Cell*, **6**, 997-1005

Mizushima, N. (2007) Autophagy: process and function. *Genes Dev*, **21**, 2861-73

Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klionsky, D. J., Ohsumi, M. and Ohsumi, Y. (1998) A protein conjugation system essential for autophagy. *Nature*, **395**, 395-8

Nadal, M., Garcia-Pedrajas, M. D. and Gold, S. E. (2008) Dimorphism in fungal plant pathogens. *FEMS Microbiol Lett*, **284**, 127-34

Nakatogawa, H., Ichimura, Y. and Ohsumi, Y. (2007) Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell*, **130**, 165-78

Otto, G. P., Wu, M. Y., Kazgan, N., Anderson, O. R. and Kessin, R. H. (2003) Macroautophagy is required for multicellular development of the social amoeba Dictyostelium discoideum. *J Biol Chem*, **278**, 17636-45

Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*, **29**, e45

Reggiori, F. and Klionsky, D. J. (2002) Autophagy in the eukaryotic cell. *Eukaryot Cell*, **1**, 11-21

Rose, T. L., Bonneau, L., Der, C., Marty-Mazars, D. and Marty, F. (2006) Starvation-induced expression of autophagy-related genes in Arabidopsis. *Biol Cell*, **98**, 53-67

Sachdeva, U. M. and Thompson, C. B. (2008) Diurnal rhythms of autophagy: implications for cell biology and human disease. *Autophagy*, **4**, 581-9

Shah, D. A. and Madden, L. V. (2004) Nonparametric analysis of ordinal data in designed factorial experiments. *Phytopathology*, **94**, 33-43

Suzuki, K., Kubota, Y., Sekito, T. and Ohsumi, Y. (2007) Hierarchy of Atg proteins in pre-autophagosomal structure organization. *Genes Cells*, **12**, 209-18

Suzuki, K. and Ohsumi, Y. (2007) Molecular machinery of autophagosome formation in yeast, Saccharomyces cerevisiae. *FEBS Lett*, **581**, 2156-61

Thines, E., Weber, R. W. and Talbot, N. J. (2000) MAP kinase and protein kinase A-dependent mobilization of triacylglycerol and glycogen during appressorium turgor generation by Magnaporthe grisea. *Plant Cell*, **12**, 1703-18

Tsukada, M. and Ohsumi, Y. (1993) Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. *FEBS Lett*, **333**, 169-74

Van Laere, A. and Fransen, M. (1989) Metabolism of germinating teliospores of *Ustilago nuda Archives of microbiology*, **153**, 33-37

Vellai, T. (2009) Autophagy genes and ageing. *Cell Death Differ*, **16**, 94-102

Veneault-Fourrey, C., Barooah, M., Egan, M., Wakley, G. and Talbot, N. J. (2006) Autophagic fungal cell death is necessary for infection by the rice blast fungus. *Science*, **312**, 580-3

Wang, Z., Wilson, W. A., Fujino, M. A. and Roach, P. J. (2001) Antagonistic controls of autophagy and glycogen accumulation by Snf1p, the yeast homolog of AMP-activated protein kinase, and the cyclin-dependent kinase Pho85p. *Mol Cell Biol*, **21**, 5742-52

Tables

Table 3.1. *Ustilago maydis* strains used in this study

Strain	Relevant Genotype	Source
1/2	*a1b1* (also known as strain 521)	Gold *et al.*, 1997
2/9	*a2b2* (BX7A22, near isogenic to 1/2)	Gold *et al.*, 1997
MN8.1	*a1b1* Δ*atg8::cbx*	this study
MN8.4	*a1b1* Δ*atg8::cbx*	this study
MN8.11	*a2b2* Δ*atg8::cbx*	this study
MN8.16	*a2b2* Δ*atg8::cbx*	this study
MN12.4	*a1b1* Δ*atg1::hyg*	this study
MN29.4	*a2b2* Δ*atg1::hyg*	this study
MN81.5	*a1b1* Δ*atg1::hyg* Δ*atg8::cbx*	this study
MN81.8	*a1b1* Δ*atg1::hyg* Δ*atg8::cbx*	this study
MN81.0	*a2b2* Δ*atg1::hyg* Δ*atg8::cbx*	this study
MN81.10	*a2b2* Δ*atg1::hyg* Δ*atg8::cbx*	this study

Table 3.2. Δ*atg8* pathogenicity 10 dai

Treatment*	Dikaryon	Replicates	Plants per rep.	Disease index[§]	t-grouping[ψ]
1	+/+	3	20	2.8 ± 0.41	a
2	+/-	3	20	2.7 ± 0.45	a
3	-/+	3	20	2.8 ± 0.17	a
4	-/-	3	20	1.28 ± 0.12	b

***** Treatment: paired strains are as follows (see Table 3.1 for strain genotypes) 1 = (1/2 x 2/9); 2 = (1/2 x MN8.11); 3 = (MN8.1 x 2/9); 4 = (MN8.1 x MN8.11). Inoculation was of 10^6 cells/ml for all strains.

[§]Mean ± standard error were calculated for each treatment based on independent biological replicates

ψ Statistical analysis was performed using a non parametric test of ordinal data in designed factorial experiments (Shah and Madden 2004)

Table 3.3. Δ*atg1* pathogenicity 10 dai

Treatment*	Dikaryon	Replicates	Plants per rep.	Disease index[§]	t-grouping[ψ]
1	+/+	3	20	2.56 ± 0.23	a
2	+/-	3	20	2.55 ± 0.15	a
3	-/+	3	20	2.25 ± 0.10	ab
4	-/-	3	20	1.9 ± 0.20	b

***** Treatment: paired strains are as follows (see Table 3.1 for strain's genotypes) 1 = (1/2 x 2/9); 2 = (1/2 x MN29.4); 3 = (MN12.4 x 2/9); 4 = (MN12.4 x MN29.4). Inoculation was of 10^6 cells/ml for all strains

[§]Mean ± standard error were calculated for each treatment based on independent biological replicates

ψ Statistical analysis was performed using a non parametric test of ordinal data in designed factorial experiments (Shah and Madden 2004)

Table 3.4. Δ*atg1*Δ*atg8* pathogenicity 10 dai

Treatment*	Dikaryon	Replicates	Plants per rep.	Disease index[§]	t-grouping[ψ]
1	+/+	3	20	3.05± 0.03	a
2	+/-	3	20	2.6 ± 0.07	a
3	-/+	3	20	2.86 ± 0.19	a
4	-/-	3	20	0.95 ± 0.03	b

***** Treatment: paired strains are as follows (see Table 3.1 for strain's genotypes) 1 = (1/2 x 2/9); 2 = (1/2 x MN81.0); 3 = (MN81.5 x 2/9); 4 = (MN81.5 x MN81.0). Inoculation was of 10^6 cells/ml for all strains.

[§]Mean ± standard error were calculated for each treatment based on independent biological replicates

ψ Statistical analysis was performed using a non parametric test of ordinal data in designed factorial experiments (Shah and Madden 2004)

Figures

Figure 3.1. *U. maydis* Atg8 share a high degree of homology with other fungal homologs. Atg8 orthologs are shown in the following order from top to bottom: *U. maydis, C. neoformans, S. cerevisiae*, *Podospora anserina*, *H. sapiens* and *A. thaliana*. Asterisk indicates site of proteolysis of S. cerevisiae Atg8 by cystein protease Atg4.

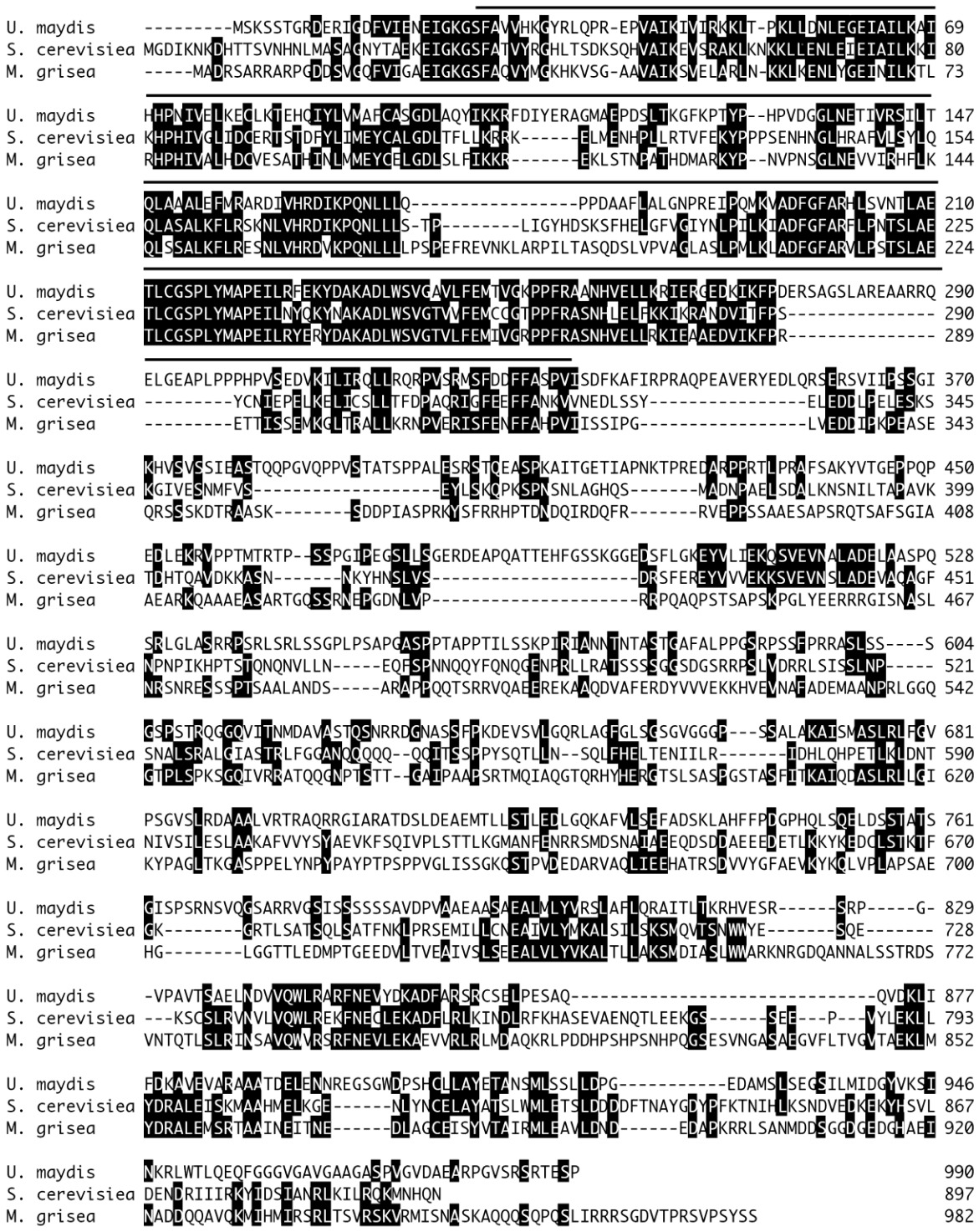

Figure 3.2. *U. maydis* Atg1 share a high degree of sequence similarity with other Atg1 fungal orthologs. Alignment of Atg1 orthologs from *U. maydis*, *S. cerevisiae* and *M. grisea*. Black bar above sequence indicates S/T kinase domain

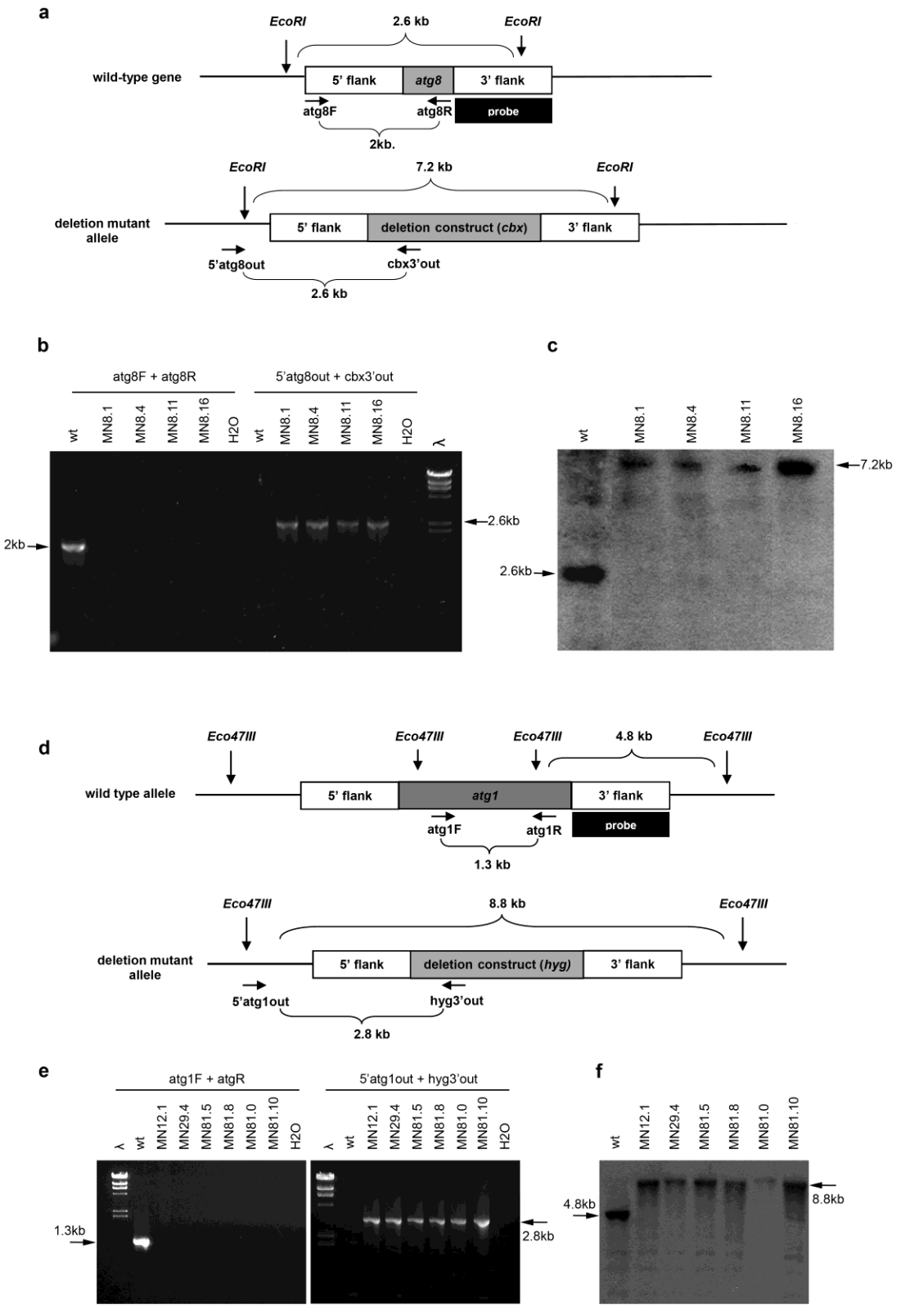
a
EcoRI
2.6 kb
EcoRI
wild-type gene
5' flank
atg8
3' flank
atg8F
atg8R
probe
2kb.
EcoRI
7.2 kb
EcoRI
deletion mutant allele
5' flank
deletion construct (cbx)
3' flank
5'atg8out
cbx3'out
2.6 kb
b
atg8F + atg8R
5'atg8out + cbx3'out
wt
MN8.1
MN8.4
MN8.11
MN8.16
H2O
wt
MN8.1
MN8.4
MN8.11
MN8.16
H2O
λ
2kb
2.6kb
c
wt
MN8.1
MN8.4
MN8.11
MN8.16
7.2kb
2.6kb
d
Eco47III
Eco47III
Eco47III
4.8 kb
Eco47III
wild type allele
5' flank
atg1
3' flank
atg1F
atg1R
probe
1.3 kb
Eco47III
8.8 kb
Eco47III
deletion mutant allele
5' flank
deletion construct (hyg)
3' flank
5'atg1out
hyg3'out
2.8 kb
e
atg1F + atgR
5'atg1out + hyg3'out
λ
wt
MN12.1
MN29.4
MN81.5
MN81.8
MN81.0
MN81.10
H2O
λ
wt
MN12.1
MN29.4
MN81.5
MN81.8
MN81.0
MN81.10
H2O
1.3kb
2.8kb
f
wt
MN12.1
MN29.4
MN81.5
MN81.8
MN81.0
MN81.10
4.8kb
8.8kb

Figure 3.3. Deletion of *atg8* and *atg1* in *Ustilago maydis*. Schematic representation of wild type and mutant Δ*atg8* (A) and Δ*atg1* (D) alleles generated by DelsGate gene replacement. The position of PCR primers used to screen transformants is indicated (arrows). Corresponding Southern probe and position of the recognition sites of restriction enzymes used to digest fungal genomic DNA are indicated. The lengths of predicted Southern blot hybridizing bands are indicated. Transformants from both mating types, a1b1 and a2b2, were initially screened for *atg8* (B) and *atg1* (E) gene replacement by PCR with the indicated primer sets. The length of PCR products is indicated on the sides of images (arrows). Gene deletion was confirmed by Southern blot hybridization. Genomic DNA from wild type and either *atg8* (C) or *atg1* (F) transformants were digested with *Eco*RI or *Eco*47III, respectively. The predicted size of hybridizing bands is indicated in kb. Strains are indicated above.

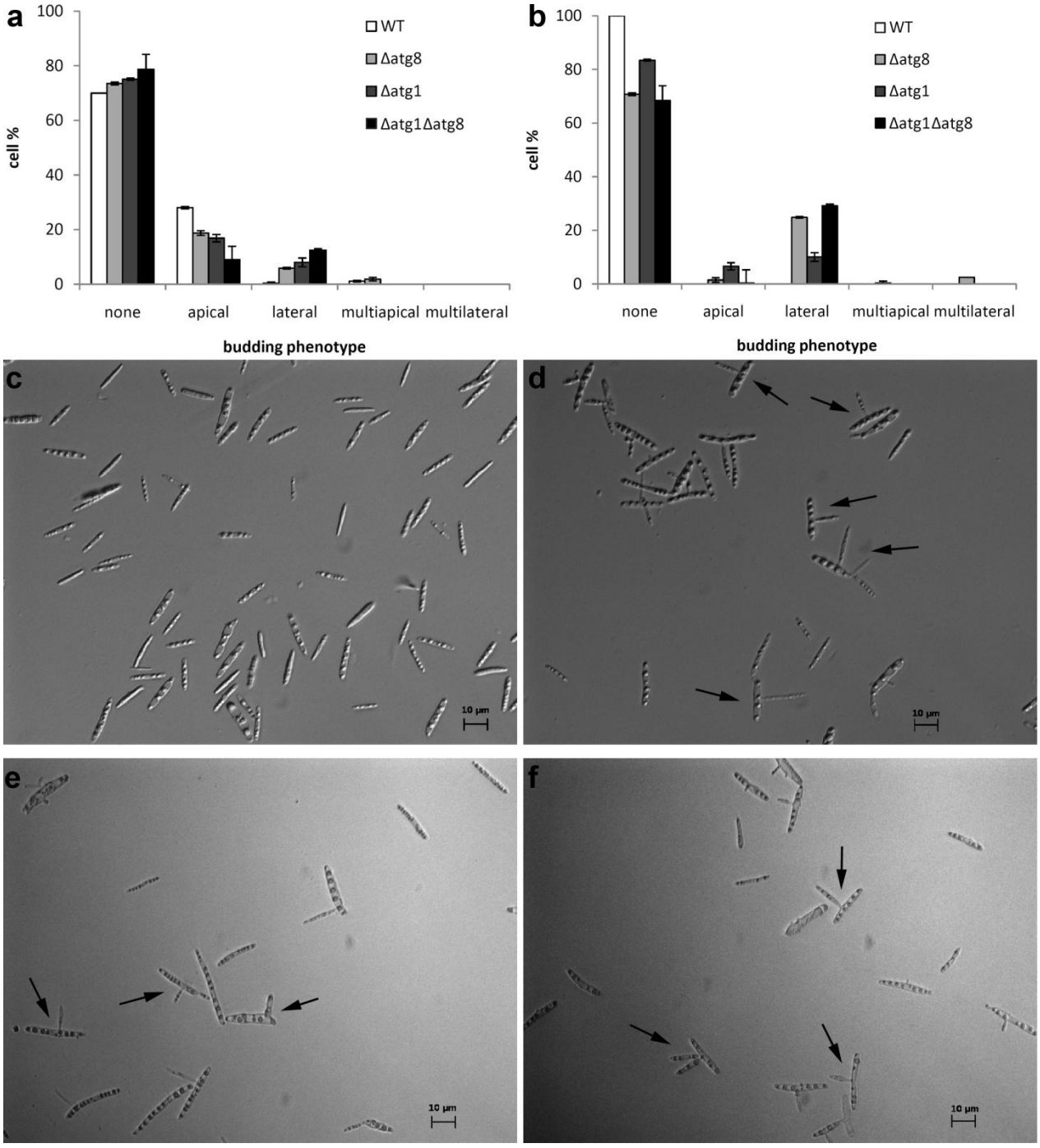

Figure 3.4. Budding patterns of wild type, Δ*atg8*, Δ*atg1* and Δ*atg1*Δ*atg8 U. maydis* haploid sporidia. Particular budding morphology of wild-type and Δ*atg8* strains was scored at the exponential growth (A) and stationary phase (B). For each strain, more than 150 cells were scored for bud location and number. Mean and standard error were calculated based on the two

biological replicates. DIC images of stationary phase cultures of wild-type (C), Δ*atg8* (D), Δ*atg1* (E) and Δ*atg1*Δ*atg8* (F). Examples of lateral buds are indicated by arrows.

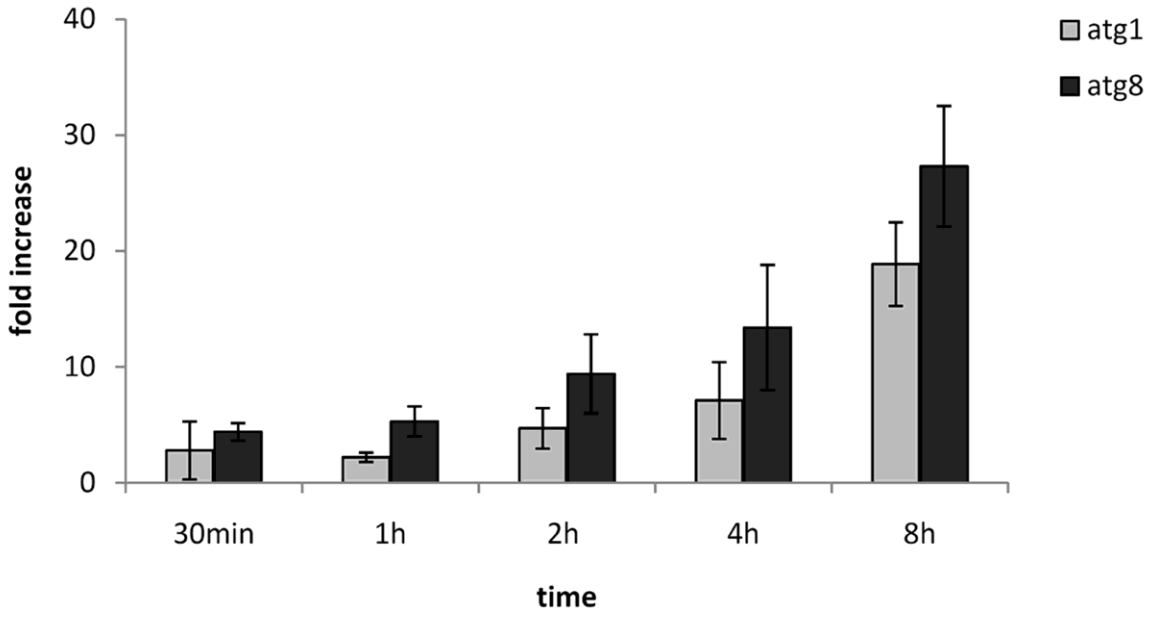

Figure 3.5. Relative expression of *U. maydis atg8* and *atg1* genes during carbon starvation. Relative levels of *atg8* and *atg1* were calculated by qRT-PCR methodology. Wild-type RNA samples were collected at the time points indicated in the graphs. Expression levels were normalized to control gene *cpr1* (Doehlemann, et al. 2009). Indicated values correspond to means of three biological replicates; bars represent standard error of biological variation.

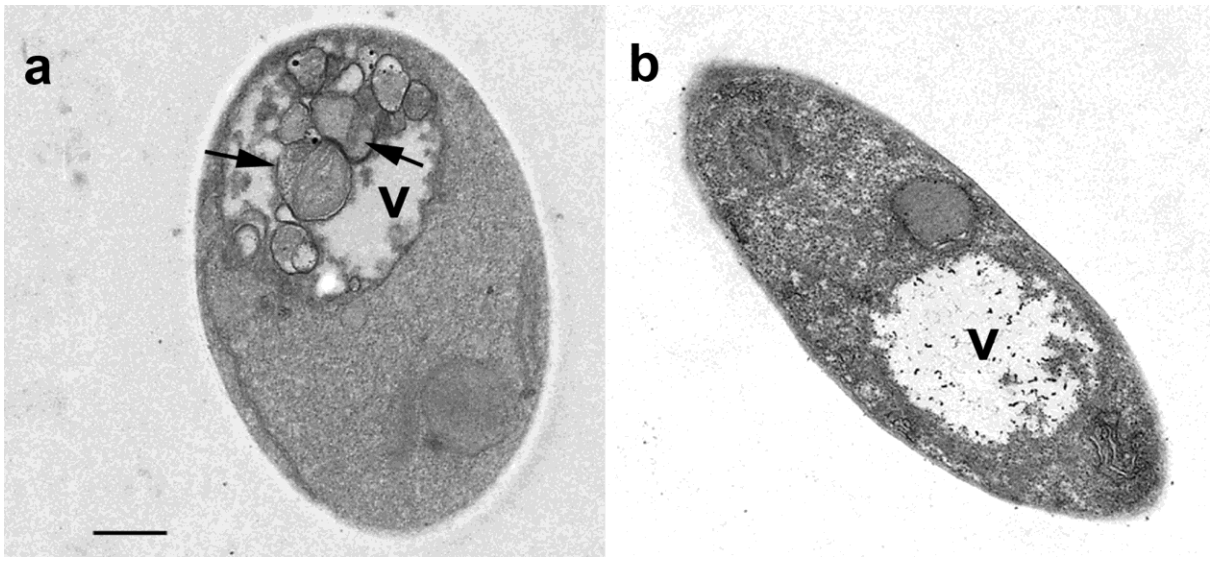

Figure 3.6. Starvation induced autophagosome accumulation in wild type and Δ*atg8* *U. maydis* cells. Wild-type and Δ*atg8* were incubated for 5 h in medium lacking a carbon source (MM-C). TEM images show the accumulation of autophagic bodies (arrows) within the vacuole (v) of wild-type cells (A). Note the absence of these structures from vacuoles of Δ*atg8* cells (B). Scale bars, 1µm.

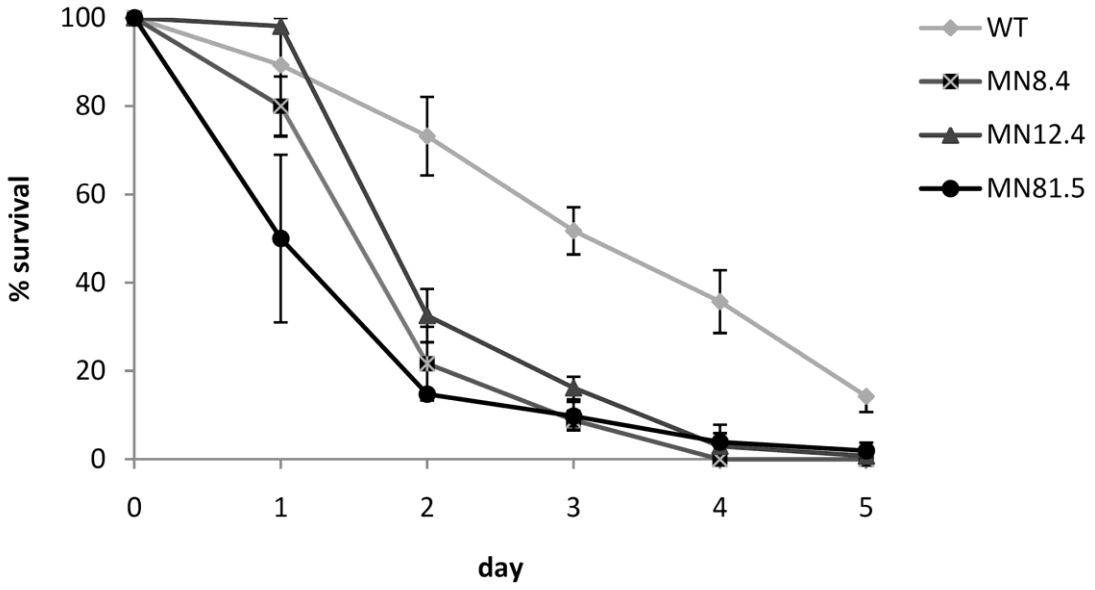

Figure 3.7. Survival of wild type, Δ*atg8*, Δ*atg1* and Δ*atg1*Δ*atg8 U. maydis* cells during carbon starvation. Wild-type, MN8.1 (Δ*atg8*), MN12.4 (Δ*atg1*) and MN81.5 (Δ*atg1*Δ*atg8*) cells were grown in liquid medium lacking a carbon source (MM-C). Cell aliquots were diluted and plated on PDA at the indicated times. Colonies were counted after 2-3 days and the percentage of survival calculated based on initial colony number (day 0) for each strain.

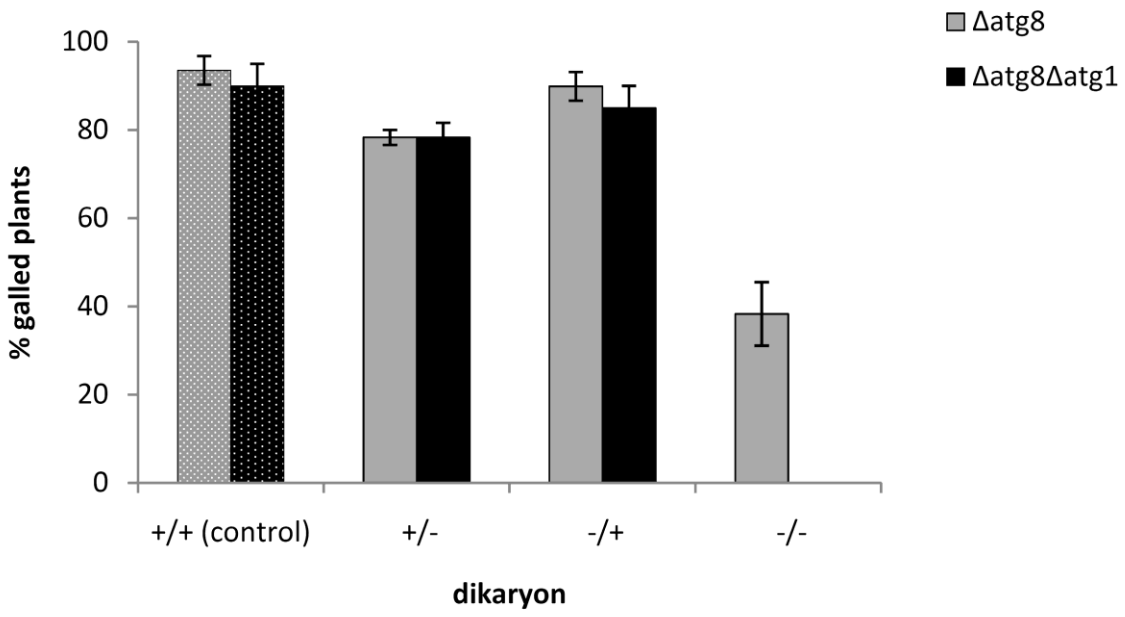

Figure 3.8. Gall production in wild type, Δ*atg8* and Δ*atg1*Δ*atg8 U. maydis* strains during maize seedling infection. Dikaryon genotype as follows: +/+ = (1/2 x 2/9); +/- = (1/2 x either MN8.1 or MN81.5); -/+ = (either MN8.11 or MN81.0 x 2/9); -/- = (MN8.1 x MN8.11 or MN8.5 x MN8.0). Average percentage of plants that developed galls 21 days after inoculation was calculated based on three independent biological replicates; bars represent standard error of biological variation

Figure 3.9. Comparison between wild type and Δ*atg8* gall development in maize ears.

Maize ears were inoculated with 10^6 cells per ml. Paired strains are as follows (see Table 3.1 for strain genotypes). 1 = (1/2 x 2/9); 2 = (1/2 x MN8.11); 3 = (MN8.1 x 2/9); 4 = (MN8.1 x MN8.11). Nine plants were used for each treatment. Note the reduced number of galls and teliospores in ears co-inoculated with compatible Δ*atg8* strains (arrow points at teliospores in galled ear. Experiment was repeated twice with similar results.

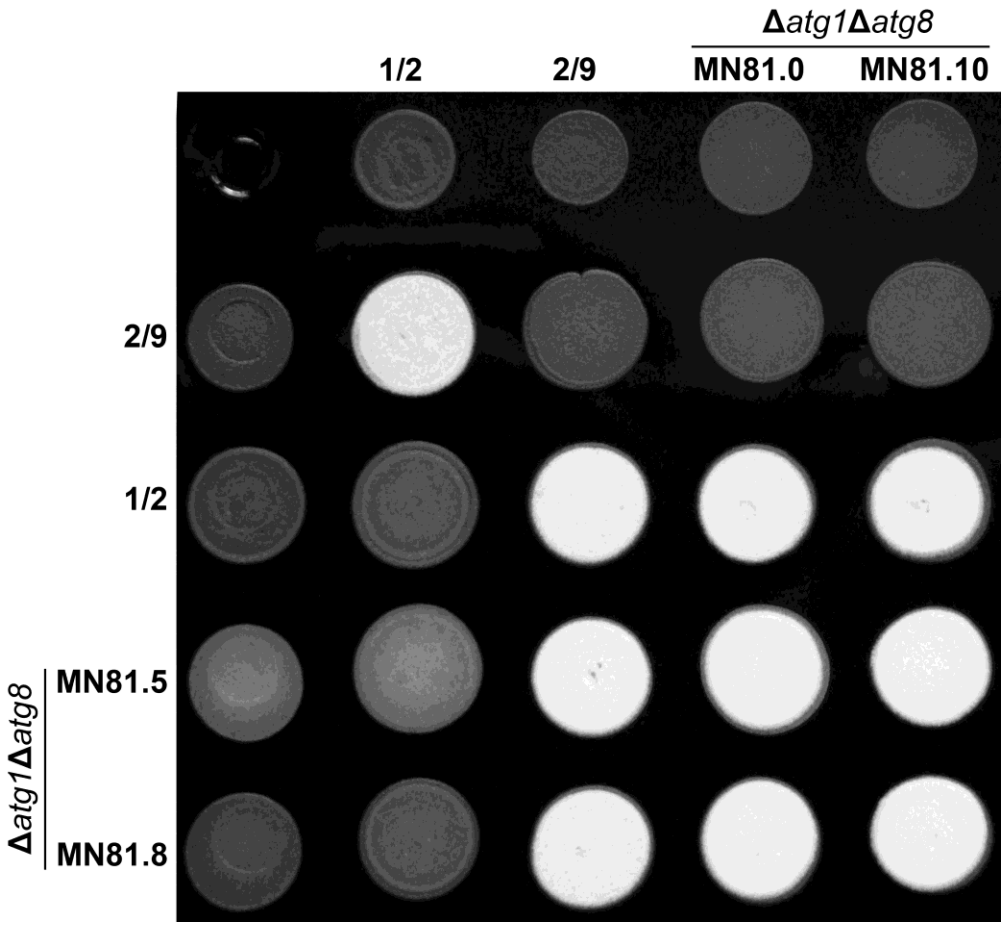

Figure Supplementary 3.1. Mating reaction comparison of wild type and Δ*atg1*Δ*atg8*. Five µl of overnight cell cultures of indicated column and row strains (indicated by the number above and to left) were spotted on charcoal-containing medium and dried in a transfer hood. Mating reactions were incubated at room temperature and photographed 24 h post inoculation. Production of white fuzzy colonies is indicative of successful mating reactions.

CHAPTER 4

THE *USTILAGO MAYDIS SNF1* GENE ACTS AS A DUAL REGULATOR OF CELL WALL DEGRADING ENZYME GENES

Nadal M. and Gold S. To be submitted to *Phytopathology*.

Abstract

Many fungal plant pathogens are known to produce extracellular enzymes that degrade cell wall elements required for host penetration and infection. Due to gene redundancy, single gene deletions generally do not address the importance of these enzymes in pathogenicity. Cell wall degrading enzymes (CWDE) in fungi are often subject to carbon catabolite repression at the transcriptional level such that when glucose is available, CWDE genes, along with many other genes, are repressed. In yeast one of the main players controlling this process is *SNF1*, which encodes a protein kinase. The Snf1/AMPK (mammals) family is critical in plants, fungi and animals to adapt to environment stress. In yeast, *SNF1* plays a key role in regulating metabolic activities in response to salt stress, heat shock, glucose depletion and starvation for other nutrients. *SNF1* homolog disruption in the phytopathogenic fungi *Fusarium oxysporum* and *Cochliobolus carbonum* led to a reduction in the expression of several CWDE genes accompanied by a decrease in virulence.

We show in this work that in *U. maydis* Snf1 acts as either a negative or positive regulator of particular CWDE genes and is not required for metabolism of alternative carbon sources. Unlike in Ascomycete plant pathogens, deletion of *snf1* did not profoundly affect virulence in *U. maydis.*

Introduction

Many plant pathogens actively force their entrance into their host employing mechanical or enzymatic methods, or a combination of both. Many pathogenic fungi produce a specialized organ termed appressorium able to generate enough turgor pressure to pierce the plant surface (Deising, *et al.* 2000, Emmett and Parbery 1975). The melanized appresorium of the rice blast fungus, *Magnaporthe grisea* can reach turgor pressures of 5.8MPa (De Jong, *et al.* 1997). In addition to producing an appressorium, the corn leaf blight fungus *C. carbonum* secretes a cocktail of cell wall degrading enzymes (CWDE) that depolymerizes the different constituents of the the plant cell wall, allowing the fungus to penetrate and spread within the host (Walton *et al.*, 1994). The importance of CWDE as virulence factors in fungi has only recently begun to be understood. Several CWDE, including pectinases and endoxylanases act not only to aid pathogens during colonization of their hosts, but they may function as elicitors to trigger plant defense mechanisms (Belien, *et al.* 2006, Juge 2006). Due to gene and enzymatic activity redundancy, reverse genetic approaches relying on single or even multiple gene deletions have not effectively addressed the importance of these enzymes in pathogenicity (Garcia-Maceira, *et al.* 2000, Scott-Craig, *et al.* 1990, Wu, *et al.* 1997).

In the basidiomycete *Ustilago maydis*, causal agent of corn smut, when two compatible haploid sporidia mate on the plant surface, the resulting dikaryotic infective hypha produces a poorly differentiated appresorium at the site of entry (Snetselaar and Mims 1993). Within the host, the fungus spreads initially intracellularly by penetrating the host cell wall and invaginating the plasma membrane; as a result the pathogen and host cytoplasms are never in direct contact (Snetselaar and Mims 1994). The occurrence of deformed rupture sites between plant cells suggests that some mechanical mechanism might be involved in the fungal ramification inside

the plant (Snetselaar and Mims 1994). However, because there is no well differentiated melanized appressorium, enzymatic digestion of plant cell wall components as a requisite for penetration are implicated.

Using confocal microscopy, Doehlemann *et al* (2008) were able to demonstrate, that in *U. maydis* later stages of gall formation, the fungal hyphae form aggregates that fill enlarged apoplastic cavities that result from degradation of the middle lamella between tumor cells (Doehlemann, *et al.* 2008). The authors also analyzed the pattern of expression of CWDE gene induction during pathogenic development to conclude that degradation of hemicellulose and cellulose may be critical for tissue colonization in all stages of disease development; but pectin degradation might only be required for cavity development. Although this information points toward a pathogenic *U. maydis* employing an enzymatic approach, reverse genetics has not been successful in addressing the question, primarily due to gene redundancy; but also because cell wall degradation might require coordinated activities of several CWDE classes rather than single enzyme types.

An interesting aspect of CWDE function in fungi is that they are usually under transcriptional glucose repression (Ruijter and Visser 1997). Glucose repression is a widespread mechanism exploited by organisms for which glucose is the preferred carbon source, such as that exhibited by the baking yeast *Saccharomyces cerevisiae* (Gancedo 1998). In *S. cerevisiae*, the *SNF1* gene is required to release glucose transcriptional repression when this sugar is no longer available as a carbon source (Celenza and Carlson 1984, Hedbacker and Carlson 2008). *SNF1* encodes the catalytic α-subunit of a serine-threonine protein kinase that forms a heterotrimeric complex with the activating γ-subunit Snf4p and one of the three β-scaffolding subunits Sip1p, Sip2p or Gal83p (Vincent and Carlson 1999). One of the principal targets of Snf1p is the DNA

binding transcriptional repressor Mig1, which binds the promoter of many genes preventing their transcription while glucose is present in the media. A drop in glucose level leads to Snf1p activation, which in turn phosphorylates Mig1p promoting its export to the cytoplasm (Ahuatzi, *et al.* 2007, De Vit, *et al.* 1997, Smith, *et al.* 1999).

The disruption of the *SNF1* homologue in the phytopathogenic ascomycete fungi, *F. oxysporum* and *C. carbonun* led to a reduction in the expression of several CWDE genes, accompanied by a decrease in virulence (Ospina-Giraldo, *et al.* 2003, Tonukari, *et al.* 2000). In this work, we explore the *SNF1* ortholog in the basidiomycete fungus *U. maydis* in connection to its role in CWDE expression and virulence. We present data indicating that in *U. maydis*, Snf1 is not necessary for releasing glucose repression of some CWDE genes or to metabolize alternative carbon sources. Most importantly, deletion of *snf1* did not profoundly affect virulence in *U. maydis.*

Materials and Methods

Strains, media and growth conditions: Fungal cultures were grown on potato dextrose agar (PDA) or in potato dextrose broth (PDB) (Difco, Franklin Lakes, NJ). Nitrate minimal medium (Holliday 1974) amended with 1% (w/v) of either oat spelt xylan, citrus pectin, sucrose, glucose or arabinose (Sigma, St. Louis, MO) was employed for growth assays and gene expression experiments. *U. maydis* cultures for protoplast production were grown in YEPS (1% yeast extract, 2% bacto-peptone, 2% sucrose). *U. maydis* transformants were selected on YEPS medium amended with 1M sorbitol (YEPS-S) and 3 μg/ml of carboxin (Gustafson, Texas). Cultures were grown at 30°C, and for liquid cultures agitation was kept at 250 rpm. *E. coli* DH5α cells were used for transformation during deletion construct assembly. Luria Bertani (LB)-

medium containing 50 μg/ml ampicillin was employed for selecting *E. coli* transformants. Mating assay were performed on complete medium containing 1% charcoal (Sigma) (Holliday 1965).

Nucleic acids procedures: The Δ*snf1* mutant strains were generated by completely replacing the *snf1* ORF with the carboxin (*cbx*) resistant gene cassette. One kb of the 5' flank region of *snf1* was amplified from genomic DNA with primers: MN1:CTCACAAGCAAAGCAG

CGT and MN2: CAGCGAACGGCGTTCTTCAATCCGTG, cloned into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) and then excised with *Eco*RI and introduced into the *Eco*RI site in pCBX5 to generate pCBX55. The orientation of the 5' flank was determinted by digesting the resulting plasmid with *Sac*I. 1 kb of the 3' flank was amplified with primers MN3: GGGCCCGAGCACCATTGAGCGTGAATGG and MN4: GGGCCCATGTTGGGCTGCTGCGAATGCG, TOPO-cloned and then excised with *Apa*I. The resulting fragment was introduced into the *Apa*I site of pCBX55. Clones carrying the appropriately orientated 3' flank were identified by *Hind*III digestion. The resulting plasmid was named pCBX553. Correct assembly of the deletion construct was further confirmed by sequencing. A linear DNA fragment containing the *cbx* flanked by 1 kb of the 5' and 3' *snf1* ORF flanks was obtained by digesting pCBX553 with *Sac*I and *KpnI*. This fragment was gel purified and used to transform wild-type *U. maydis* 1/2 (*a1b1*) and 2/9 (*a2b2*) strain protoplasts as previously described (Tsukuda, *et al.* 1988). Transformants were initially tested by PCR with primers SNF1Fw: CTCGAATTGGTCGGTGAT and SNF1Rv: TACATCTTGCAGCAGACA for the absence of *snf1* ORF, and with primers cbx3out: CATTTCTCGTGATTGTCC and snf13out: TTGCCAAACGTAGCGTTG for homologous integration of the *cbx* resistance

cassette within the *snf1* locus. Further confirmation was provided by Southern blot hybridization. Ten μg of genomic DNA from each strain was digested with *Bgl*II and resolved on a 0.7% agarose gel stained with ethidium bromide. DNA was transferred overnight by capillarity to Hybond XL (Amersham Pharmacia Biotech, Piscataway, NJ) nylon membrane in 10X SSC and cross-linked by UV. For the probe, 1kb of the *snf1* 3' flank was amplified and DIG labeled using Roche DIG-high Prime kit and Roche Wash and Block Buffer Set was used for hybridization steps (Roche, Indianapolis, Indiana). Probe labeling and hybridization procedures were performed according to the manufacturer's instructions. X-ray film (Kodak, Rochester, NY) was developed after 10 minutes of exposure.

For northern blot hybridizations total RNA was extracted using Trizol LS reagent (Invitrogen), according to the manufacturer's instructions. Fifteen μg of RNA from each sample was fractionated on a 1.2% formaldehyde agarose gel and after 4 hours of electrophoresis at 45 Volts, stained with ethidium bromide. For estimating transcripts lengths, the 0.5-10 kb RNA ladder (Invitrogen) was included. RNA was transferred to Hybond XL (Amersham Pharmacia Biotech, Piscataway, NJ) nylon membrane in 10X SSC overnight, and then the membrane was baked for 2 hours at 80°C to cross-link the RNA. For each probe, approximately 500 bp of the corresponding CWDE gene ORF was amplified and TOPO-cloned (Invitrogen). Probes were generated by PCR amplifying the corresponding segment and products were labeled with 50 μCi (5 μl of 3000 Ci/mmol, aqueous solution) of [α^{32}P]dCTP using the Rediprime II DNA Labeling System (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Sephadex G50 (Sigma) was used for purifying labeled probes. Prehybridization and hybridization were performed at 68°C in buffer containing 5 mM EDTA, 0.25 mM Na2HPO4, 0.17% H3PO4, 1% casein hydrolysate and 7% SDS (Church and Gilbert, 1984), for 4 h and overnight, respectively.

The membrane was washed in: 2X SSC, 0.05% SDS at room temperature and then at 50°C in 0.1XSSC, 0.1% SDS. Each wash was for 40 minutes and washing solutions were changed twice. The membrane was exposed to a phosphor screen (Kodak) for 24-48 hours and scanned using a Storm 860 and analyzed with Image Quant® Version 5.0 (Molecular® Dynamics).

qRT-PCR quantification of CWDE gene expression: Total RNA for qRT-PCR quantification of relative transcript accumulation was extracted using a Spectrum Plant Total RNA kit (Sigma). cDNA was synthesized using the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) using oligo-dT as primer and according to the manufacturer's recommendations. Transcript abundance was quantified by qRT-PCR using SYBR-GREEN methodology (BioRad, Hercules, CA) with gene specific primers designed through the Integrated DNA technologies (IDT) website (http://www.idtdna.com/Home/Home.aspx). Reactions were performed on a Cepheid SmartCycler I (Cepheid, Sunnyvale, CA). CWDE gene transcript relative expressions levels were calculated according ΔCt calculations ($2^{-\Delta\Delta CT}$ method) (Pfaffl 2001). Primer sequences are listed in Table 4.1. Relative gene expression was estimated by normalizing target transcript levels to the cyclophilin gene *cpr1* (um03726). Means of gene expression fold-increase and their corresponding standard error were calculated based on three biological replicates.

Functional complementation of yeast Δ*SNF1:* Yeast reference strain W303 (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11 15*) and derived Δ*SNF1* MCY4908 (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11 15*Δ*SNF1*) strain were maintained in YPD (1% yeast extract; 2% bacto-peptone; 2% glucose). The *U. maydis snf1* ORF was amplified from genomic DNA and

directionally cloned into the *URA3* containing plasmid pWS28 carrying the constitutively expressed *PGX* promoter, at the *Eco*RI and *Not*I sites to generate plasmid pWS28UM*snf1*. Yeast strain MCY4908 was transformed with either empty vector pWS28 or pWS28UM*snf1* according to standard wild type yeast transformation protocol. Transformants were first selected on synthetic dextrose minus uracil (SD-URA) medium, and then on YPS (1% yeast extract; 2% bacto-peptone; 2% sucrose) amended with 1µg/ml antimycin (Sigma) to block respiration.

Mating and pathogenicity analysis: Mating plate assays were used to assess mutant mating abilities. Indicated strains were grown overnight in PDB and equal volumes of each of the mating strains were co-spotted on 1% charcoal-containing complete medium plates that were sealed with parafilm and incubated at room temperature in the dark for 24 h. White dikaryotic filaments indicated a successful mating reaction.

For pathogenicity tests, 7 day old Golden Bantam maize seedlings were co-inoculated with strain mixtures of 10^6 cells/ml. Plants were kept in a growth chamber with 16 h day at 28°C/8 h night at 20°C cycles. Symptom development was scored 7, 10 and 14 days after inoculation and each plant individually assigned a disease rating based on the following disease scale (Gold and Kronstad, 1994) 0: no symptoms; 1: anthocyanin production and/or chlorosis; 2: small leaf galls; 3: small stem galls; 4: large stem galls; and 5: plant death. Disease index is calculated as the average disease rating. For each mutant strain considered, three independent biological replicates of pathogenicity tests were conducted. For each particular pathogenicity test a non-parametric statistical test was performed to evaluate the differences among treatments (Shah and Madden 2004).

Results

Identification and deletion of the *U. maydis SNF1* ortholog.

In order to investigate the potential role of the *U. maydis SNF1* homolog as a key regulator of glucose repression as well as CWDE expression in this fungus, we created Δ*snf1* mutant strains by completely removing the gene ORF. The *U. maydis SNF1* homolog, *snf1* (um11293), was identified based on *S. cerevisiae* Snf1 protein sequence similarity by searching the genome (http://mips.gsf.de/genre/proj/ustilago) using the protein BLAST-homology search algorithm. The identified homolog, *snf1* ($e = 1.8e^{-140}$) encodes an 841 amino acid peptide with a predicted molecular mass of ~ 92 kDa. NCBI alignment software indicates that the predicted protein encoded by um11293 shares 58% and 51% identity with Snf1p homologs from *Cryptococcus neoformans* and *S. cerevisiae*, respectively. The serine/threonine (Ser/Thr) kinase catalytic domain extends from residue 52 to 303 and exhibits a high similarity to the Snf1 Ser/Thr catalytic domain in other fungi (Fig. 4.1). A well conserved residue among Snf1p-related proteins is threonine 210 in the activation loop of the catalytic domain. Phosphorylation at this threonine by upstream kinases is indispensable for activation of the catalytic kinase domain (Mccartney and Schmidt 2001). In *U. maydis* Snf1 this threonine (Thr 207) and the sequence context in which it is located have been conserved.

Δ*snf1* mutant strains were created by completely removing the gene ORF and replacing it with the carboxin resistance gene (*cbx*). Gene replacement by homologous integration at the *snf1* locus was initially tested in transformants by PCR and further confirmed by Southern blot (Fig. 4.2). No obvious morphological phenotype differences were observed under the light microscope between liquid grown Δ*snf1* and wild-type strains.

***U. maydis snf1* is sufficient to complement a *S. cerevisiae ΔSNF1* phenotype.**

In *U. maydis* the *snf1* gene and the protein it encodes have not previously been characterized. As a way to initially describe the *U. maydis snf1* gene function, we tested if this gene is capable of rescuing the phenotype of a *S. cerevisiae ΔSNF1* mutant strain. In *S. cerevisiae*, Snf1 kinase is essential for releasing the transcriptional repression imposed on the *SUC2* gene by glucose when this sugar is depleted from the medium. *SUC2* encodes a secreted invertase responsible for hydrolyzing sucrose into glucose and fructose allowing the cells to ferment these sugars (Celenza and Carlson 1984). Consequently, deletion of *SNF1* abolishes the ability of the yeast cells to grow by fermentation on medium containing sucrose as the sole carbon source. Because the *U. maydis snf1* gene contains no introns, the complete ORF was cloned into yeast expression vector pWS28 to generate pUmsnf1. As a positive control, the *S. cerevisiae SNF1* ORF was cloned into pWS28 and the resulting plasmid named pScSNF1. Yeast transformants carrying these plasmids were initially selected on SD-ura medium (synthetic dextrose medium without uracil) for the presence of plasmid (see materials and methods) and then transferred to medium containing sucrose as the carbon source (YPS) to test their ability to grow on sucrose (Fig. 4.3). YPS plates were amended with antimycin A (1µg/ml) to block respiration and any possible growth not due to fermentation. Only those strains carrying either pUmsnf1 or pScSNF1 grew on the YPS medium, confirming that *U. maydis snf1* can functionally complement the *S. cerevisiae SNF1* mutation.

CWDE expression

Deletions of *snf1* orthologs in other plant pathogenic fungi was shown to drastically reduce or completely abolish the expression of several CWDE genes. We wanted to explore the possibility that the *U. maydis snf1* has a similar transcriptional regulatory role and thus made a first attempt to score expression of several CWDE genes encoding xylanase (um06350 and um03411); glucanases (um04368, um00235) pectin methyltransferase (um03516), α-L-arabinofuranosidase (um04309) and polygalacturonase (um02510) in media containing either glucose or an alternative carbon source was assessed by northern blots. Δ*snf1* strain 18/9 and wild-type strain 1/2 we grown overnight in PDB, washed twice with sterile water and then plated on minimal medium (MM) amended with one of the following carbon sources: glucose, xylan or pectin. After 2 days of incubation at 28° C, fungal cells were collected and total RNA extracted. Of the six tested genes, we were only able to detect transcripts corresponding to the xylanase gene um03411. For this gene, no transcript accumulation was detected when wild type or Δ*snf1* strains were grown on glucose. However, after cells were shifted to MM-xylan, transcript was detected at comparable levels in both wild type 1/2 and Δ*snf1* 18/9 strains (Fig. 4.4). Therefore, Northern blot results indicates that the expression of um03411 encoding a potential xylanase is subject to glucose repression but, contrary to the initial hypothesis, *snf1* appears to be dispensable for releasing the transcriptional repression of this gene imposed by glucose.

Because some of the CWDE might be expressed at levels difficult to detect by northen blot, we sought to quantify the relative expression of the 6 potential *U. maydis* CWDE genes in wild type and strain 18/9 using the more sensitive method of qRT-PCR (Table 4.1). For each strain, 100ml PDB cultures were grown overnight to O.D. ≈ 0.5 and then cells were shifted to minimal medium containing 1% of either glucose or alternative carbon source (xylan or pectin). After 24

hours, RNA was extracted from the cells and cDNA was synthesized. For each gene, we compared the expression on glucose (hypothetically repressive) to the expression in the alternative carbon source (hypothetically non-repressive). The qRT-PCR results suggest that expression of xylanases um03411 and um06350, and pectinases um02510 and um02523 are strongly repressed by glucose, as their transcript accumulations are dramatically higher in the non-repressive conditions in both strains than in glucose (Fig.4.5). However, the relative expression of polygalacturonase gene um02510 and endoglucanase um02523 in MM-pectin was lower in the mutant 18/9 than in the wild type strain 1/2, indicating that *snf1* is required for full induction of these genes. On the other hand, in opposition to our prediction, the induction of xylanase genes um03411 and um06350 was 2.5 and 1.7 times higher in the mutant 18/9 than in the wild type 1/2 strain, indicating that the absence of *snf1* may marginally increase transcript accumulation of these two genes.

Carbon source utilization

In *S. cerevisiae, SNF1* is indispensable for the growth on alternative carbon sources (other than glucose) and Δ*SNF1* mutants are unable to utilize sucrose or other non-fermentable sugars. Similarly, the *SNF1* homolog of *C. carbonum* and *F. oxysporum* are required for proper absorption/utilization of simple and complex carbon sources other than glucose (Ospina-Giraldo, *et al.* 2003, Tonukari, *et al.* 2000). Based on this knowledge, we decided to test the growth efficiency of our Δ*snf1* strains on different carbon sources and observe if the same held true for *U. maydis.* Two independent Δ*snf1* mutants: 18/9 and 18/11, the wild-type, 1/2, and one ectopic integrant strain, Ec7, were grown overnight in PDB and a serial dilution of each culture was spotted onto solid minimal media (MM) amended with either glucose, sucrose, galactose, xylan,

pectin or alternatively onto PDA. For each strain, its ability to utilize a particular carbon source was assessed by comparing its growth on that sugar with glucose (Fig. 4.6). According to our results, the Δ*snf1* mutation does not seem to impair *U. maydis'* ability to grow on the alternative carbon sources tested. On any of the carbon sources, the Δ*snf1* strains 18/9 and 18/11 can sustain growth similar to wild-type or ectopic strains. Therefore, contrary to the case of *S. cerevisiae*, *F. oxysporum* and *C. carbonum*, in the basidiomycete *U. maydis* the *snf1* gene appears dispensable for the utilization of alternative sugars.

Deletion of the *U. maydis snf1* gene slightly reduces virulence

U. maydis is a plant pathogenic fungus and much of its life cycle must occur within its maize host plant. Therefore, the ability of *U. maydis* to sustain a pathogenic life style is crucial for the survival of the species. In order to test the pathogenic potential of Δ*snf1* strains, we co-inoculated 7 day old maize seedlings with pair-wise combinations of compatible wild-type and Δ*snf1* strains and monitored disease progression based on severity of symptoms. Table 4.2 summarizes the pathogenicity test corresponding to three independent biological replicates conducted with mutant strains 18/9 and 18/11. When plants were co-inoculated with compatible Δ*snf1* strains (treatment 4), the average disease index at either 7, 10 or 14 days after inoculation (dpi) was lower than when they were infected with any other strain combinations. However, dikaryons formed by compatible Δ*snf1* strains induced all the typical disease symptoms (chlorosis, anthocyanin production, gall formation) observed in wild type infections. These results indicate that in *U. maydis*, *snf1* is a relatively minor virulence determinant.

Discussion

The capacity of an organism to grow on a particular carbon source depends primarily upon the utilization of the appropriate enzyme set. Species that grow on a wide variety of carbon sources are able to do so because their genomes are equipped with the necessary genes encoding the enzymes that process each individual type of molecule. Nonetheless, it would be unnecessary and certainly wasteful, to constitutively produce all those enzymes, when the corresponding substrates are not available. Fortunately, cells have evolved strategies to avoid the energetically detrimental effect that such behavior would produce.

A common strategy among organisms is to adapt to their surroundings; that implies producing the required enzymes only when a particular carbon source becomes available. When multiple carbon sources are available, a particular carbon source is usually preferred over others. This is exemplified in *S. cerevisiae*, where glucose is the preferred carbon source and the production of enzymes involved in the degradation of other carbon sources is repressed at the transcriptional level when this sugar is accessible. This phenomon, known as "catabolite or glucose repression" has been extensively studied in this yeast where the *SNF1* gene, encoding a catalytic subunit of a protein-serine/threonine kinase (Celenza and Carlson 1984, Celenza and Carlson 1986), is a central regulator (Carlson 1999, Gancedo 1998, Hedbacker and Carlson 2008). The *S. cerevisiae SNF1* gene is required for the cell to utilize alternative carbon sources when glucose becomes limiting.

In many plant pathogenic fungi, the enzymes involved in the depolymerization of the plant cell wall constituents (CWDE) are under glucose repression. Deletion of *SNF1* orthologs in *C. carbonum* and *F. oxysporum* impaired the ability of these fungi to release glucose repression of CWDE genes, affecting their virulence as well. In this work, we explored the potential functions

of the *U. maydis SNF1* ortholog, *snf1*, as a determinant of CWDE expression and virulence in this fungus.

The *U. maydis snf1* um11293 gene was identified as the homolog of the *SNF1* α-catalytic subunit based on protein sequence similarity. Further examination of *U. maydis* genome indicated the presence of a homolog of activating γ-subunit Snf4p (um01350). Additionally, *U. maydis* appears to possess a single β-scaffolding subunit gene (um0668) instead of three paralogs as in *S. cerevisiea*. The fact that *U. maydis snf1* can functionally complement the phenotype of a yeast Δ*SNF1* mutant strain serves as strong evidence that this gene is indeed the *SNF1* ortholog. Therefore, in this work we were able to show that *U. maydis* seems to conserve all the genetic elements that make up the *SNF1* complex involved in the regulation of glucose repression.

With the notion that the identified *snf1* gene can function as a *SNF1* ortholog, we then sought to investigate if the gene was also involved in glucose repression of CWDE genes in *U. maydis*. We compared the expression patterns of several potential CWDE genes by northern blot analysis but only detected transcripts corresponding to one of six CWDE genes tested. When wild type cells were grown on glucose, no transcripts corresponding to xylanase gene, um03411, were detected. However, when cells were shifted to MM-xylan, a high accumulation of this transcript was observed, indicating that um03411 is as hypothesized, transcriptionally regulated by a mechanism of glucose repression. Unexpectedly, our results also revealed that after the shift to MM-xylan, the Δ*snf1* cells also accumulated high levels of um03411 transcript, strongly suggesting that *snf1* is irrelevant for releasing the transcriptional repression imposed by glucose on xylanase um03411. Therefore, our results clearly demonstrate that in *U. maydis* at least one CWDE enzyme gene, xylanase um03411, is under glucose repression. However, desrepression of um03411 when glucose is depleted from the growing medium occurs independently of *snf1*.

This comes as a contradiction to the well documented cases of *C. carbonum* and *F. oxysporumin* were the corresponding *S. cerevisiae SNF1* orthologs are required for the expression of CWDE in the absence of glucose. Because we failed to detect most of the CWDE transcripts by northern blot, we used qRT-PCR, a more sensitive approach to evaluate gene expression. Our results revealed two different patterns depending on the nature of the CWDE tested. We first expanded our analysis of xylan degrading enzyme genes by analyzing the expression of the four potential xylanase genes present in *U. maydis* genome. Expression of xylanases um04422 and um04897 was independent of glucose availability, indicating that they are not under glucose repression. On the other hand, for xylanases um06350 and um03411 transcripts increased on average 85- and 16-fold, respectively, when wild type cells were shifted to MM-xylan, indicating that these genes are subject to glucose repression. Unexpectedly, and in accordance to the pattern revealed by the northen results, the induction of um06350 and um03411 after the shift to MM-xylan was higher in Δ*snf1* than in the wild type cells. This result clearly argues against *snf1* acting as a "positive" regulator of um06350 and um03411 induction during growth in alternative carbon sources. It rather suggests that *snf1* somehow negatively regulates these genes when glucose is depleted.

We also investigated the pattern of expression of two CWDE involved in the degradation of other plant cell wall polymers. The fold increase of polygalacturonase um02510 and endoglucanase um02523 was 20 and 19, respectively, when wild type cells were shifted to MM-pectin, pointing to transcriptional repression by glucose of these genes. The relative transcript accumulation of these genes in the Δ*snf1* cells after the switch to MM-pectin was much lower than in wild type strain, indicating that in *U. maydis snf1* is required for full derepression of um02510 and um02523.

Therefore, in *U. maydis* although many CWDE enzymes are glucose repressed, *snf1* does not seem to be a universal element controlling this mechanism. In the case of xylanase genes, *snf1* appears to negatively regulate glucose repression while in the case of the other CWDE analyzed (um02510 and um02523), it acts as a positive regulator much in the same way that has been described for *SNF1* orthologs in other systems. Regardless of its role in the regulation of CWDE gene expression, its deletion did not affect the capacity of the mutant strains to metabolize compounds other than glucose, indicating that *U. maydis snf1* is not critical for survival in the absence of glucose. This is in marked contrast to *S. cerevisea, C. carbonum* and *F. oxysporumin* where *snf1* deletion impairs the ability of these fungi to thrive on alternative carbon sources. Additionally, preliminary microarray data (data not shown) indicate that in *U. maydis, snf1* is not required for derepression of glucose repressed genes. Moreover, many of the genes that were induced in the absence of glucose exhibited a higher degree of induction in the Δ*snf1* strain, reinforcing the notion that in *U. maydis snf1* may negatively regulate the expression of some genes.

U. maydis spends a great deal of its life living as a pathogen, prospering at the expense of its host, maize. It is only during this phase that sexual reproduction is possible in *U. maydis* and therefore pathogenic development is crucial for completing its life cycle. Because previous studies have demonstrated that *snf1* deletion affected virulence in other systems, we tested the capacity of Δ*snf1* strains to cause disease in maize seedlings. Our pathogenicity results showed that disease caused by Δ*snf1* strains were slightly less severe than those caused by wild type, suggesting that *U. maydis snf1* is required for full disease development.

In this work we have presented data showing that *U. maydis* does not strictly follow the *S. cerevisea, C. carbonum* and *F. oxysporumin snf1* paradigm. We showed rather that it may act

either as a positive or negative regulator of CWDE expression in relation to glucose repression. We also demonstrated that *snf1* is dispensable for *U. maydis* haploid growth on alternative carbon sources.

The *U. maydis* Δ*snf1* strains did however exhibit a minor reduction in virulence. This suggests that during pathogenic development, *snf1* is required for some processes associated with the ability of the fungus to cause disease. One explanation could be that *snf1* positively regulates additional CWDE genes that were not included in our analysis, which together with um02510 and um02523 are important for degradation of cell wall polymers during pathogenic growth. Microarray data showed that um02523 along with two other endoglucanase genes and endopolygalacturonase um02510 are highly induced during *U.maydis* infection (Doehlemann, *et al.* 2008). Our data indicates that induction of these genes is severely reduced in Δ*snf1* cells which may be one of the causes of the observed reduction in virulence.

References

Ahuatzi, D., Riera, A., Pelaez, R., Herrero, P. and Moreno, F. (2007) Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. *J Biol Chem*, **282**, 4485-93

Belien, T., Van Campenhout, S., Robben, J. and Volckaert, G. (2006) Microbial endoxylanases: effective weapons to breach the plant cell-wall barrier or, rather, triggers of plant defense systems? *Mol Plant Microbe Interact*, **19**, 1072-81

Carlson, M. (1999) Glucose repression in yeast. *Curr Opin Microbiol*, **2**, 202-7

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

Celenza, J. L. and Carlson, M. (1986) A yeast gene that is essential for release from glucose repression encodes a protein kinase. *Science*, **233**, 1175-80

de Jong, J. C., McCormack, B. J., Smirnoff, N. and Talbot, N. J. (1997) Glycerol generates turgor in rice blast. *Nature*, **389**, 244-244

De Vit, M. J., Waddle, J. A. and Johnston, M. (1997) Regulated nuclear translocation of the Mig1 glucose repressor. *Mol Biol Cell*, **8**, 1603-18

Deising, H. B., Werner, S. and Wernitz, M. (2000) The role of fungal appressoria in plant infection. *Microbes Infect*, **2**, 1631-41

Doehlemann, G., Wahl, R., Vranes, M., de Vries, R. P., Kämper, J. and Kahmann, R. (2008) Establishment of compatibility in the Ustilago maydis/maize pathosystem. *Journal of Plant Physiology*, **165**, 29-40

Emmett, R. W. and Parbery, D. G. (1975) Appressoria. *Annual Review of Phytopathology*, **13**, 147-165

Gancedo, J. M. (1998) Yeast carbon catabolite repression. *Microbiol Mol Biol Rev*, **62**, 334-61

Garcia-Maceira, F. I., Di Pietro, A. and Roncero, M. I. (2000) Cloning and disruption of pgx4 encoding an in planta expressed exopolygalacturonase from Fusarium oxysporum. *Mol Plant Microbe Interact*, **13**, 359-65

Garcia-Pedrajas, M. D., Nadal, M., Kapa, L. B., Perlin, M. H., Andrews, D. L. and Gold, S. E. (2008) DelsGate, a robust and rapid gene deletion construction method. *Fungal Genet Biol*, **45**, 379-88

Hedbacker, K. and Carlson, M. (2008) SNF1/AMPK pathways in yeast. *Front Biosci*, **13**, 2408-20

Holliday, R. (1965) Induced Mitotic Crossing-over in Relation to Genetic Replication in Synchronously Dividing Cells of Ustilago Maydis. *Genet Res*, **10**, 104-20

Holliday, R. (1974) *Ustilago maydis*. *In Handbook of Genetics (ed. R. C. King)*, 575–95

Juge, N. (2006) Plant protein inhibitors of cell wall degrading enzymes. *Trends Plant Sci*, **11**, 359-67

McCartney, R. R. and Schmidt, M. C. (2001) Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. *J Biol Chem*, **276**, 36460-6

Ospina-Giraldo, M. D., Mullins, E. and Kang, S. (2003) Loss of function of the Fusarium oxysporum SNF1 gene reduces virulence on cabbage and Arabidopsis. *Curr Genet*, **44**, 49-57

Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*, **29**, e45

Ronne, H. (1995) Glucose repression in fungi. *Trends Genet*, **11**, 12-7

Ruijter, G. J. and Visser, J. (1997) Carbon repression in Aspergilli. *FEMS Microbiol Lett*, **151**, 103-14

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**, 1191-200

Shah, D. A. and Madden, L. V. (2004) Nonparametric analysis of ordinal data in designed factorial experiments. *Phytopathology*, **94**, 33-43

Smith, F. C., Davies, S. P., Wilson, W. A., Carling, D. and Hardie, D. G. (1999) The SNF1 kinase complex from Saccharomyces cerevisiae phosphorylates the transcriptional repressor protein Mig1p in vitro at four sites within or near regulatory domain 1. *FEBS Lett*, **453**, 219-23

Snetselaar, K. M. and Mims, C. W. (1993) Infection of maize stigma by Usitlago maydis: light and electron microscopy. *Phytopathology*, **83**, 843-203

Snetselaar, K. M. and Mims, C. W. (1994) Light and electron microscopy of Ustilago maydis hyphae in maize. *Mycological Research*, **98**, 347-355

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**, 237-48

Vincent, O. and Carlson, M. (1999) Gal83 mediates the interaction of the Snf1 kinase complex with the transcription activator Sip4. *EMBO J*, **18**, 6672-81

Walton, J. D. (1994) Deconstructing the Cell Wall. *Plant Physiology*, **104**, 1113-1118

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

Tables

Table 4.1. *U. maydis* CWDE gene tested by qRT-PCR

Gene	enzyme	Forward primer	Reverse primer
um04422	endo-β-1,4-xylanase	GACCATCACCGATTTCGTCACCA	AGTAGCCGTTCTTCTTGAAGCCGT
um04897	endo-β-1,4-xylanase	AGAAGAATGTGCGTCCAGAGGGTT	TGATGCATGTCATCCTCGTGCTCT
um06350	endo-β-1,4-xylanase	AGTTTGCCACTGTCCTTGCTTTCG	TGGCAGCGTTACCGTTGTAGTTCT
um03411	endo-β-1,4-xylanase	AAAGCTGATCCGAGGTCATACGCT	ACCTTGCCCTTGTATCTTCCCACA
um02510	PGU1 - Endo-polygalacturonase	CCAACGTTCGCAACCAAGATGACT	ACACTACCAATCGACAAGCCGTGA
um02523	Endoglucanase 1 precursor	GCCTGGTTGCAAGTGGAGAATGAA	TGACAACCAGTTCGGTCGATGTGA
um03726	Cyclophilin (reference)	ACGCCGATTCACTTCGTC	AACGACGATCCCTCGTAACCGAA

Table 4.2. Effect of Δ*snf1* on pathogenicity

Treatment*	Dikaryon	Nro. of plants	Disease index[§]		
			7 dpi	10 dpi	14 dpi
1	+/+	3 X 20	2.7 ± 0.04 (a)[ψ]	2.9 ± 0.23 (a)	3.52 ± 0.4 (a)
2	+/-	3 X 20	1.9 ± 0.12 (b)	2.3 ± 0.08 (ab)	3.15 ± 0.1 (a)
3	-/+	3 X 20	2.2± 0.12 (a)	2.6 ± 0.22 (a)	3.68 ± .04 (a)
4	-/-	3 X 20	1.6 ± 0.05 (b)	1.9 ± 0.10 (b)	2.56 ± 0.1 (b)

* Treatment: paired strains are as follows 1 = (1/2 x 2/9); 2 = (1/2 x 18/11); 3 = (18/9 x 2/9); 4 = (18/9 x 18/11). Inoculation was of 10^6 cells/ml for all strains

§ Mean ± standard error were calculated for each treatment based on independent biological replicates.

ψ Statistical analysis was performed using a non parametric test of ordinal data in designed factorial experiments (Shah and Madden 2004).

Figures

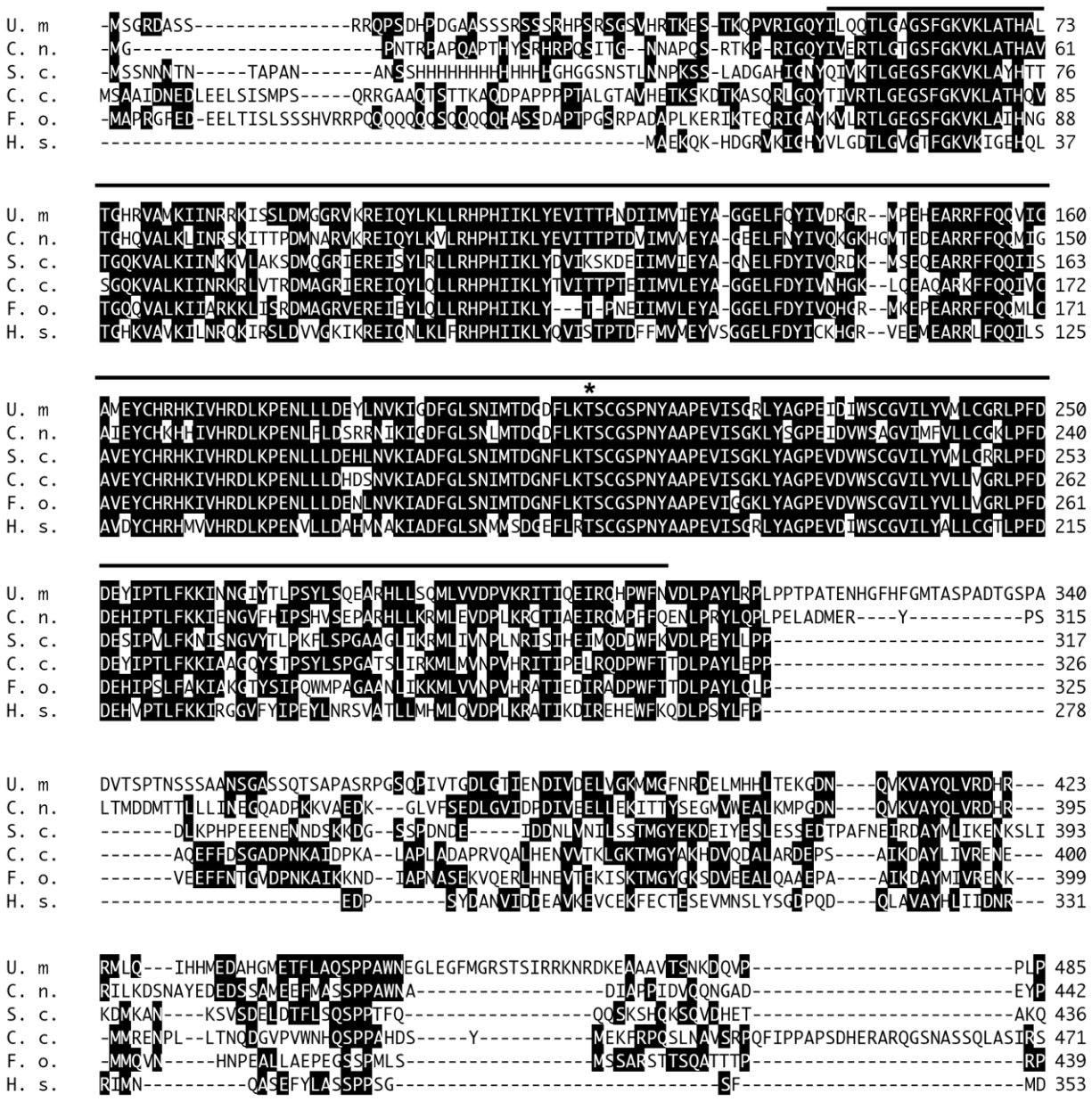

Figure 4.1. Alignment showing homology among different Snf1 orthologs. Snf1 orthologs are shown in the following order from top to bottom: *U. maydis, C. neoformans, S. cerevisiae, C. carbonum, Fusarium oxysporum and H. sapiens.* The black bar above the sequence indicates the S/T kinase domain. The asterisk indicates the threonine 207 of *S. cerevisiae* Snf1 critical for activation by upstream kinases.

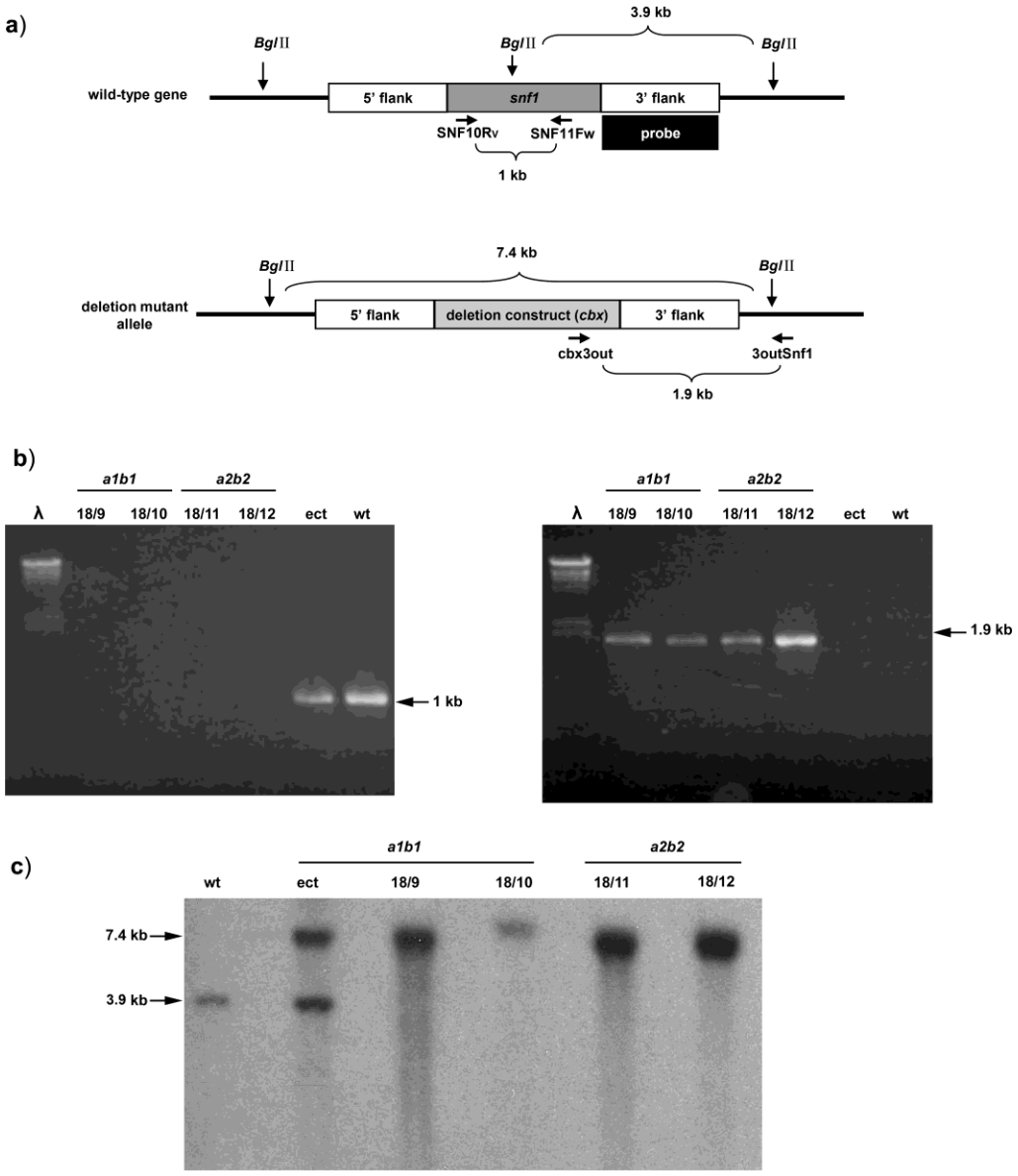

Figure 4.2. Deletion of *snf1* in *Ustilago maydis*. a) Schematic representation of wild type and the mutant allele generated by gene replacement. The position of PCR primers used to screen transformants are indicated (arrows). The Southern probe and position of the recognition sites of restriction enzymes used to digest fungal genomic DNA are indicated. The lengths of predicted Southern blot hybridizing bands are indicated. **b)** Transformants of both mating types, *a1b1* and *a2b2*, were initially screened for *snf1* gene replacement by PCR with the indicated primer sets. The length of PCR products is indicated on the sides of images (arrows). **c)** Gene deletion was

confirmed by Southern blot hybridization. Genomic DNA from wild type and *snf1* transformants were digested with *Bgl*II. The predicted size of hybridizing bands is indicated in kb. Strains are indicated above.

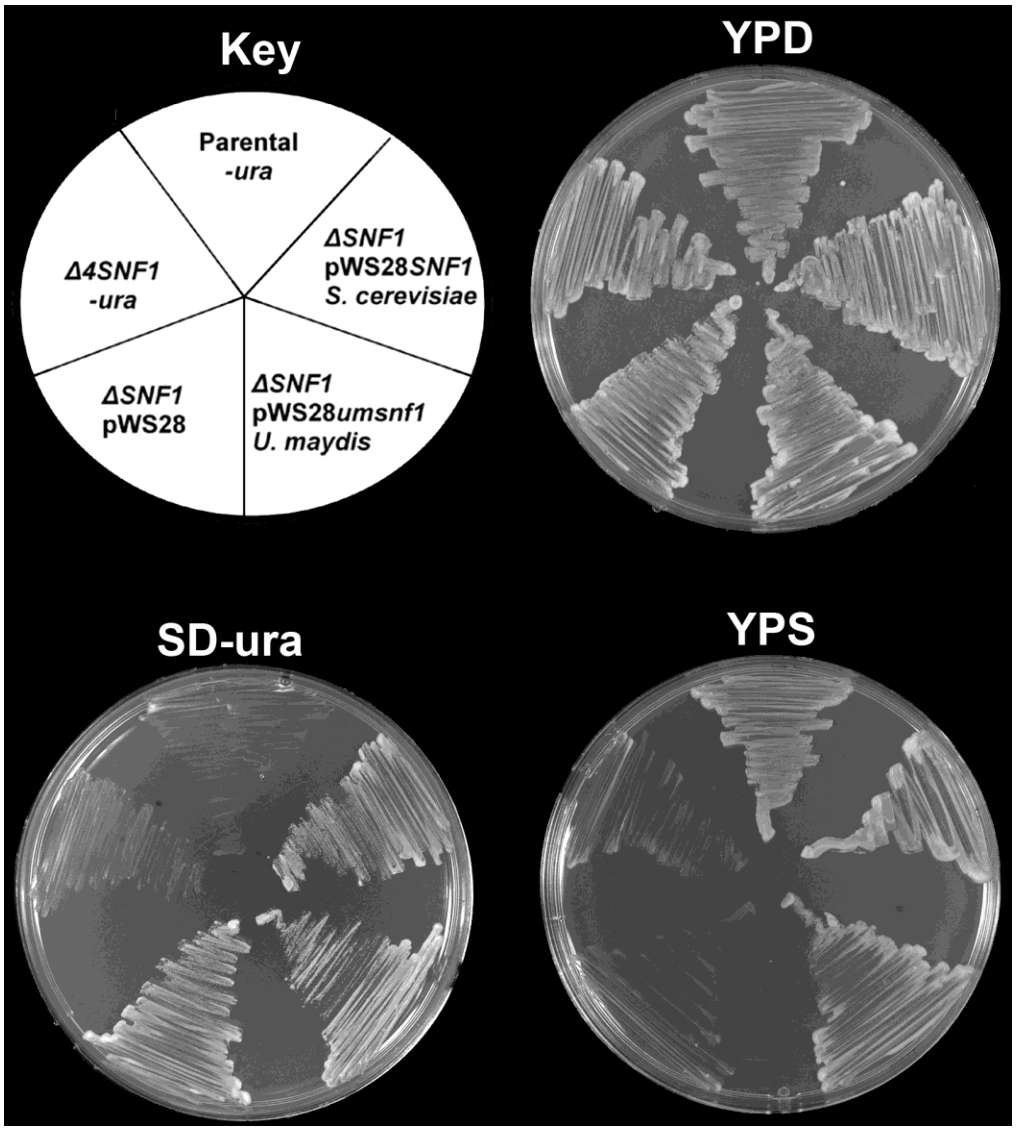

Figure 4.3. Complementation of the Δ*SNF1 S. cerevisiae* strain with the *U. maydis snf1* gene. Yeast reference strain W303 and derived Δ*SNF1 S. cerevisiae* strain MCY4908 were transformed with empty vector pWS28, pWS28*SNF1* (pWS28 carrying *S. cerevisiae SNF1*) and

pWS*um*28*snf1* (pWS28 carrying *U. maydis snf1*). YPD medium (yeast extract glucose); SD-ura (glucose medium lacking uracil); YPS medium (yeast extract sucorese) amended with antimycin (1µl/ml) and YPD (yeast extract glucose).

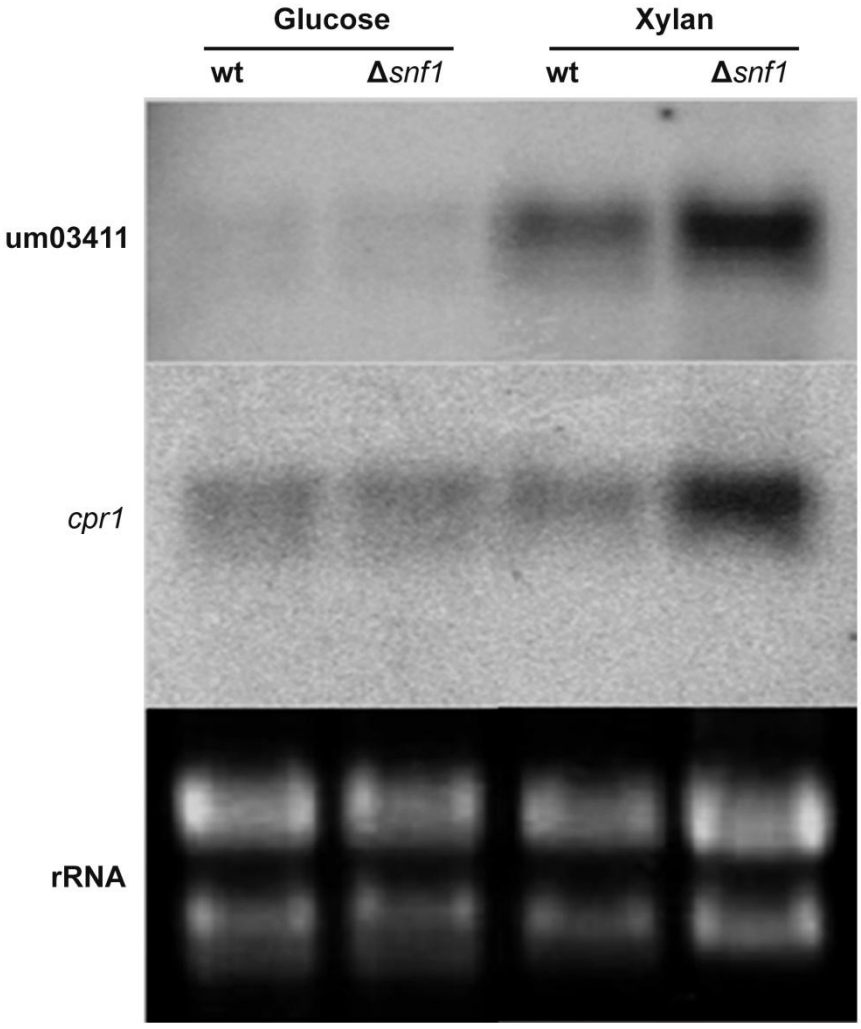

Figure 4.4. Relative expression of xylanase gene in wild-type and Δ*snf1* strains. Northen blot using total RNA probed with xylanase um03411 and loading control *cpr1* (um03726) sequences.

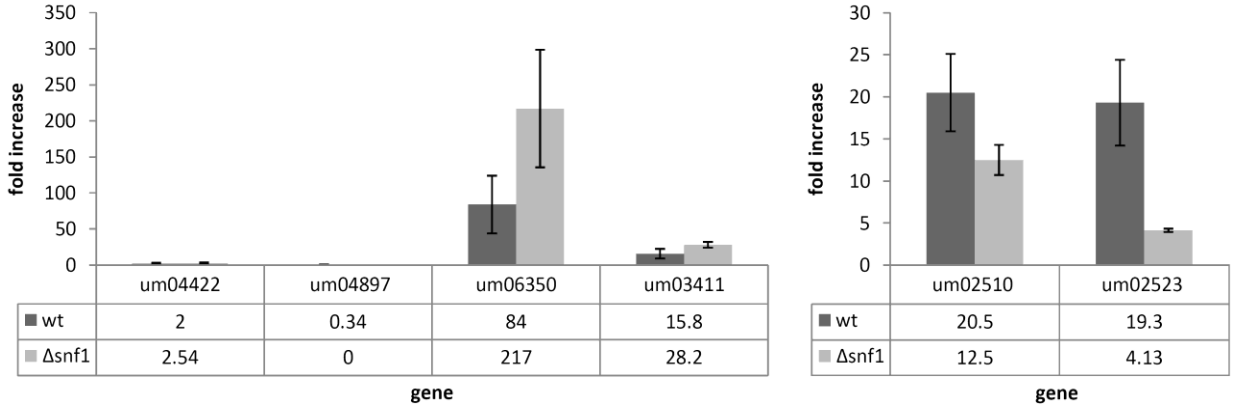

Figure 4.5. qRT-PCR analysis of relative expression of CWDE genes in *U. maydis* wild-type and Δ*snf1* mutant. Relative levels of xylanase (um04422, um04897, um06350 and um03411), endo-polygalacturonase (um02510) and endoglucanase (um02523) were calculated by qRT-PCR methodology. For each gene, relative gene expression was estimated based on transcript level in the non-repressive condition (MM-xylan or MM-pectin) relative to the level in the repressive condition (MM-glucose). For each condition, transcript levels were normalized to control gene *cpr1* (Doehlemann, et al. 2009). Indicated values correspond to means of three biological replicates; bars represent standard error of biological variation.

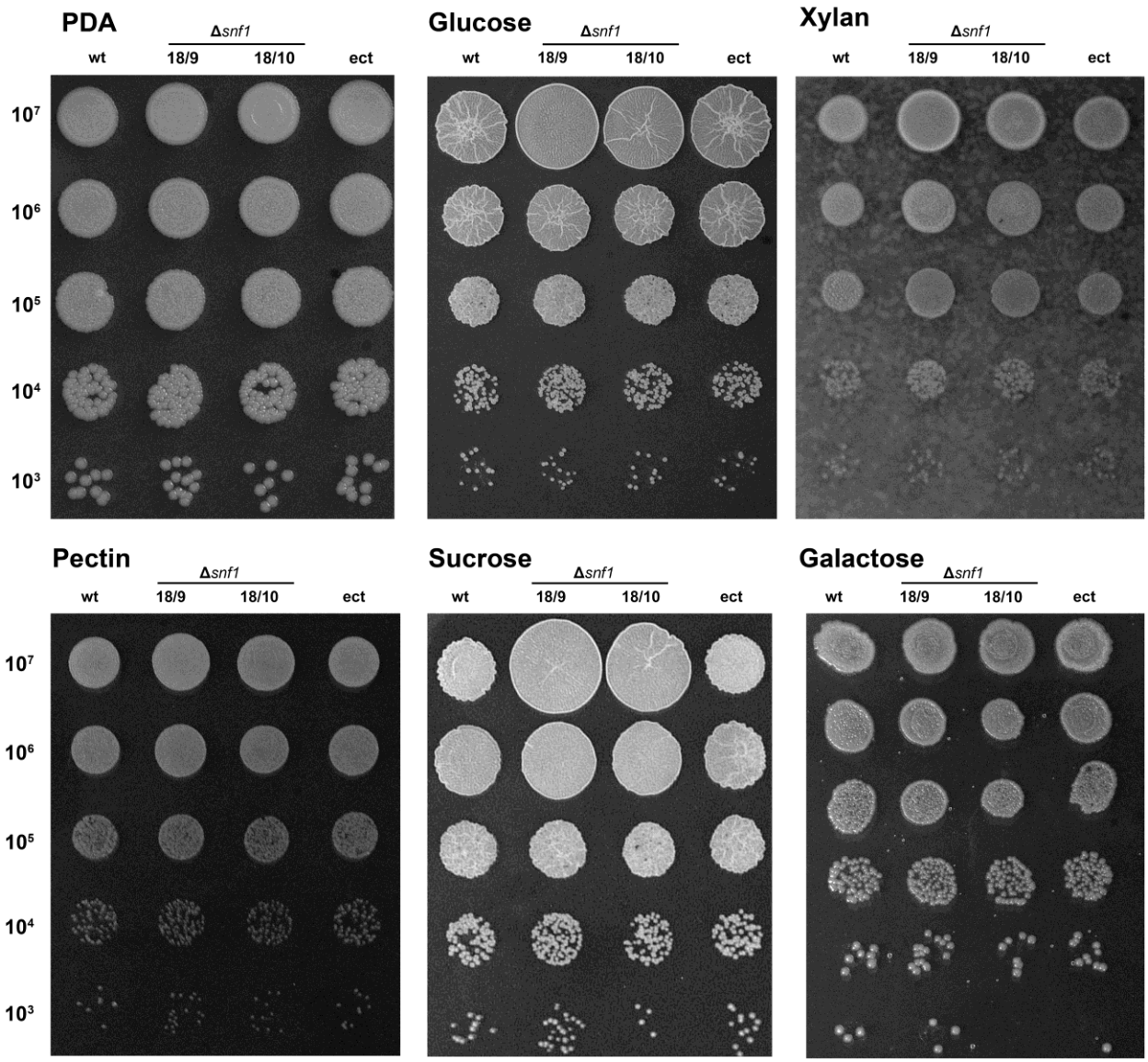

Figure 4.6. Comparative growth of *U. maydis* wild type and Δ*snf1* mutant strains on various carbon sources. Serial 10-fold dilutions of indicated strain cultures were plated on PDA and minimal medium containing indicated carbon sources and incubated at 28º C for 3 days.

CHAPTER 5

CONCLUSION

The plant pathogenic fungus *Ustilago maydis*, causal agent of corn smut disease, constitutes an ideal system for investigation of the basis of host-pathogen interactions, dimorphism, mating and pathogenicity. From a practical point of view, its status as a model system is even more remarkable as it is one of the few basidiomycete fungi of which the genetic and molecular determinants of their biological outcomes are studied in deep detail. Therefore, much of what is learned from its study can be used to pose hypotheses concerning other basidiomycete systems, most importantly regarding other plant pathogens such as smut and rust fungi.

During my Ph.D. research, I've adopted a reverse genetic approach to address the relevance of two well characterized pathways associated with controlling nutrient stress as determinants of *U. maydis* morphogenesis and pathogenic development.

One of the main focuses of this work was to investigate the role of autophagy in the context of *U. maydis* morphogenesis and pathogenic development. Autophagy has been shown to be crucial to many organisms to endure periods of nutritional stress as well as being important for many developmental changes. Our main hypotheses were that the process of autophagy would be important for *U. maydis*' ability to withstand a low nutrient environment and also undergo the developmental changes faced in its lifecycle. To test these hypotheses, we deleted the *U. maydis atg1* and *atg8* genes, homologs of two key *S. cerevisiae* autophagy genes, and then examined the phenotype of single as well as double mutant strains. The main conclusions drawn were:

1- The autophagy pathway plays a crucial role in some aspects of *U. maydis* morphogenesis. Disruption of key autophagy genes *atg1* and *atg8* severely affected the site of bud emergence as well as the mother-daughter cell separation process during budding of *U. maydis* haploid sporidia.

2- The process of autophagy is necessary for full pathogenic and morphological developments that take place in the host. Deletion of the autophagy genes *atg8* and *atg1* has serious consequences for *U. maydis* pathogenic capacity as well as affecting the sporulation process, an event in its lifecycle that occurs solely in the host.

3- *atg1* and *atg8* act additively , as the abnormal phenotypes exhibited by the double Δ*atg1*Δ*atg8* mutant strains were the same as those of the single *Δatg1* and *Δatg8* mutant strains but with a higher magnitude.

The main lesson learned from these conclusions is that in *U. maydis,* autophagy plays a crucial role in several phases of its life cycle. During the saprobic phase, autophagy is necessary for proper budding of haploid *U. maydis* sporidia. Failure to undergo autophagy results in defects in the site of the bud's emergence as well as cell separation. Strictly speaking, no clear notion exists of what the exact role of the saprobic phase is in nature, therefore no strong conclusion can be inferred on whether this abnormal budding pattern could be detrimental for the overall performance and existence of *U. maydis* as a species. Nevertheless, because *U. maydis* is a well established model system, these results are very valuable from a comparative point of view as they could potentially be extrapolated to other systems where appropriate.

Of supreme importance to *U. maydis'* ability to complete and reinitiate its lifecycle is the extremely detrimental effect that a lack of autophagy capacity has on virulence as well as

sporulation. Most importantly for progeny, blocking autophagy substantially reduces the amount of teliospores produced, therefore lowering fecundity.

Overall, our results indicate that autophagy in *U. maydis* in tightly linked to several essential processes of the life cycle of this plant pathogen fungus. Most precisely, losing the ability to undergo autophagy might lead to a reduction in *U. maydis* fitness and competitiveness, both of which could potentially displace it from its natural niche and drive it to species collapse.

A second focus of my research efforts was invested in the study of the role of *U. maydis* cell wall degrading enzymes (CWDE) have during pathogenic development. We established that *U. maydis snf1* was indeed the *Saccharomyces cerevisiae SNF1* ortholog by successful functional complemention of a *ΔSNF1* yeast strain with a copy of *U. maydis snf1* ORF. The core objective of our research was to investigate the role of the *snf1* gene as a potential regulator of glucose repression, particularly in regard to its role as a determinant of CWDE gene expression in concert with carbon source availability. We hypothesized that *U. maydis snf1* performed a similar role as a key genetic element controlling derepression of glucose repressed genes as was previously described for the ascomycete fungi *S. cerevisiae, Fusarium oxysporum* and *Cochliobolus carbonum*. More importantly, we thought that, based on previous reports, deletion of *snf1* in *U. maydis* would have a major effect in its potential as a pathogen. To test these hypotheses, *U. maydis* Δ*snf1* was compared to wild type in its ability to grow in alternative carbon sources as well as to express several CWDE genes depending on the presence or absence of glucose. Results of analyzing the phenotype of Δ*snf1* mutants in comparison to the wild type strains, produced the following main conclusions:

1- Certain CWDE enzymes in *U. maydis* are under glucose repression, in the sense that the corresponding genes are transcriptionally repressed when this sugar is available in the growing medium.

2- Contrary to the well characterized case of plant pathogens, *C. carbonum* and *F. oxysporum*, the *U. maydis snf1* gene does not act as a central positive regulator of CWDE in *U. maydis.* Instead, it performs a dual role, acting as a positive or a negative regulator depending on the particular CWDE gene. Additionally, the *snf1* gene is not required for *U. maydis* to metabolize alternative carbon sources.

3- Aligned with the previous conclusion, the *snf1* gene does not play a crucial role during *U. maydis* pathogenic development and no abnormal phenotypes were observed during its saprobic haploid sporidial growth.

One of the initial hypotheses implies that *U. maydis snf1* would behave similarly to its orthologs in other plant pathogenic fungi in regard to glucose repression and CWDE positive transcriptional regulation. Through my experimental work I was able to generate results to prove this hypothesis false. Therefore one of my main accomplishments has been to demonstrate that *U. maydis* does not strictly follow the well characterized and sometimes universally assumed paradigm of *snf1* as a specific positive regulator of CWDE enzymes and glucose repression. Taking into consideration several aspects of *U. maydis* biology, these conclusions are not completley surprising. A first consideration should be that the previously reported results regarding *snf1* orthologs as positive regulators of CWDE genes in plant pathogenic fungi are all derived from studies in ascomycete fungi and importantly necrotrophs. *U. maydis* is a basidiomycete, biotrophic organism, therefore the regulation of carbon utilization may differ significantly from *S. cerevisiae, C. carbonum* and *F. oxysporum*. A caveat to my conclusion is

that *snf1* might act as a crucial regulator of some other processes in *U. maydis* that were not investigated in this work. However, it is inferred from the phenotype of Δ*snf1* mutants during infection that such hypothetical processes are not major determinants of *U. maydis* pathogenic development.

As a corollary to these conclusions, it should be reinforced that appropriate consideration regarding taxonomy and ecological niche of the organism under study should be given when applying model systems as universal paradigms.

In summary, in this work we addressed the role of two important processes related to the *U. maydis'* ability to cope with changes, whether these changes come from the environment or they are predetermined by intrinsic imposed developmental programs. We provided evidence to show that *snf1* gene in *U. maydis* does not control the hypothesized process in *U. maydis* as is the case of its orthologs in ascomycete systems. Further analysis is required, for a specific role for this gene in *U. maydis* is to be ascribed. We also showed that the process of autophagy is crucial for *U. madyis* morphogenesis and pathogenic development. The precise role of autophagy during budding, in plant growth and spore formation will also require more in depth examination.